Mechanism of How Plants Survive Freezing Temperatures in Winter;

The Effects of Freezing and Light on Cold Acclimated Plants

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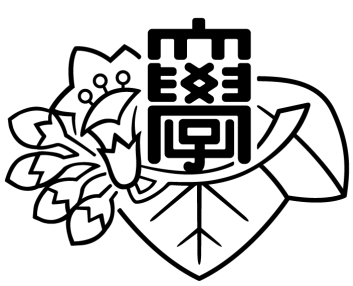


Contents

Chapter 6

Abbreviation

NA・・・Non-acclimation

CA・・・Cold acclimation

2PH・・・Second phase cold hardening

SZA・・・Sub-zero acclimation

2PFA・・・Second phase freezing acclimation

2PSA・・・Second phase supercooling acclimation

LT_{50}・・・Lethal temperature for 50% percent of the population

DCMU・・・3-(3,4-dichlorophenyl)-1,1-dimethylurea

RNA-seq ・・・RNA sequencing

DEGs・・・Differentially expressed genes

Chapter1

General introduction

Plants that grow in colder-than-temperate zones during overwintering

Plants must adapt and survive in a variety of environmental changes as they cannot move to an optional location. Plants are exposed to biotic stresses such as pathogens, insects, and environmental stresses such as temperature, drought, and light. Especially, freezing is an unavoidable stress for plants that grow in colder-than-temperate zones during overwintering.

Morioka City where I live, is one of the examples that are located near the boundary between the temperate zone (Cfa) and the subarctic zone (Dfa and Dfb) in the Köppen climate classification (Miyamoto, 2009). In mid-winter, temperatures drop to sub-zero even during the daytime, and consequently, plants in this area are exposed to freezing by frost. Snowfall begins gradually in late November, and snow begins to settle on the ground in late December. In general, temperatures do not drop as much under the snow because of its thermal insulating effect, and herbaceous plants covered by snow are less exposed to very low sub-zero temperatures than those exposed to the open air. In late fall and early winter, before the period of snow cover begins, radiative cooling causes frost to settle on the ground and plant surfaces. If temperatures fall below zero in the morning, plants undergo freezing in the presence of light (**Fig.1.1a-c)**. In addition, even during the heavy snowfall season, snow does not settle under large trees, so some herbaceous plants exposed to snow are also subjected to freezing in the presence of light (**Fig. 1.1d-f**). Thus, overwintering plants in colder-than-temperate zones force to cope not only with sub-zero temperature stress but also with freezing.

Low-temperature stress and freezing are particularly serious stresses that affect agricultural production. Furthermore, global warming has increased the damage caused by spring freezes. Generally, spring freezes are not surprising events, but it is believed that the recent global warming has resulted in unusually warm spring weather, causing plants to escape early dormancy and cold acclimation, which is the process that I will mention later, and that the subsequent freeze has caused significant damage. Thus, it is extremely important to have a comprehensive understanding of how overwintering plants respond to not only low temperatures but also freezing stress.

Mechanism of how plants deal with ice crystallization

When freezing occurs, plants die without exception when ice forms in the cytoplasm. Therefore, plants prevent the disorder by allowing ice to form outside the cell, called extracellular freezing. However, when ice is allowed to form outside the cell, the water inside the cell is absorbed by the ice due to the difference in chemical potential, so the inside of the cell is subjected to

osmotic stress, dehydration stress, and drought stress due to dehydration. In addition, cell walls adjacent to ice crystals are in addition to physical stresses. While cold damage is a temperature effect, freezing is multiple stresses including not only temperature effect but also osmotic dehydration and mechanical stress.

Cold acclimation (CA)

As an initial response toward overwintering, plants sense the drop in temperature and shorter daylengths starting from about fall. This triggers the process of acclimation to cold temperatures, which enhances their tolerance to freezing for successful overwintering. This process, known as cold acclimation (CA), proceeds at low temperatures (roughly from 11 to 0 °C) without freezing, and acclimation at lower temperatures leads to higher freezing tolerance (Tominaga et al., 2021).

Mechanism of cold acclimation at the molecular level

Several studies have revealed details of the CA process at the molecular level including gene expression (Guy et al., 1985), metabolisms (Guy, 1990), and proteins (Kawamura and Uemura, 2003). The roles of C-repeat binding factors (CBFs, a type of transcription factor) in the CA pathway of Arabidopsis are the best understood. Rapid transient expression of genes encoding CBF1, CBF2, and CBF3 occurs after plants are transferred to low temperatures, followed by the expression of *cold-regulated* (*COR*) genes to acquire freezing tolerance (Thomashow, 1999, 2001).

As mentioned above, when extracellular freezing occurs, cells are exposed to the osmotic stress associated with dehydration. Therefore, to avoid dehydration and for direct protection of the membrane, sugar is accumulated in response to low temperatures (Steponkus, 1984; Wanner and Junttila, 1999). Thus, during the CA process, freezing tolerance is acquired through changes at the molecular level, such as at the genetic, metabolic, and protein level.

Plant hormone signal transduction also involves in the CA process. It is reported that abscisic acid (ABA) can substitute for low-temperature stimulation (Chen and Gusta, 1983). It has been suggested that activation of the C-repeat element (CRT; dehydration-responsive) via CBF1- CBF3 may be induced by ABA (Knight et al., 2004). When plants grown under long-day conditions were given Dormin (later named ABA), it increased freezing tolerance in *Acer negundo* (Irving and Lanpear, 1968). Furthermore, in Arabidopsis, ethylene biosynthesis and signaling decrease freezing tolerance required by CA by suppressing the expression of *CBF1*-

CBF3 and type-A *Arabidopsis response regulators* (*ARR*) genes, which, are negative regulators of cytokinin signaling (Shi et al., 2012).

Mechanism of cold acclimation at the cellular level

It has been shown that at the cellular level, a low-temperature-responsive calcium signal is induced. The calcium signal, as a second messenger, is involved in various signaling pathways and stress responses including cold shock (Hepler and Wayne, 1985; Knight et al., 1993; Lecourieux et al., 2006; Plieth et al., 1999). Calcium influx has been shown to regulate the expression of genes involved in CA (Knight et al., 1996; Monroy et al., 1993). It has been reported that the response of cold-induced calcium signals is different before and after CA (Hiraki et al., 2019). In roots of non-acclimated (NA) Arabidopsis, the calcium signals are acutely responsive to cooling from 20°C, but less responsive to cooling from 2°C. Conversely, in 7-days CA Arabidopsis, the calcium signals are acutely responsive to cooling from 2°C, but show little responsiveness to cooling from 20°C. Furthermore, in Arabidopsis, freezing-induced mechanical stress triggers intracellular calcium signals because of plasma membrane damage, resulting in increased mechanical stress tolerance (Yamazaki et al., 2008).

Mechanism of cold acclimation at the tissue level

As changes at the plant tissue level, during the CA process, the lipid composition of the plasma membrane changes, which links to increasing freezing tolerance. For example, it has been reported that the proportion of plasma membrane lipid such as glucocerebrosides (CER) and free sterols (FS) in rye, oat, and Arabidopsis were altered before and after cold acclimation (Uemura et al., 1995; Uemura and Steponkus, 1994).

As another name for CA is cold hardening, cell wall modification occurs during the CA process (Chen et al., 2018, Takahashi et al., 2024). In leaves of broadleaf evergreen species such as *Buxus sempervirens* L., CA induces stiffer cell walls and increased tension compared to non-acclimated plants (Rajashekar and Lafta, 1996). In addition, various cell wall monosaccharides such as L-arabinose, D-galactose, and D-glucose are altered during CA and deacclimation (Kutsuno et al., 2023).

Second phase cold hardening (2PH) or sub-zero acclimation (SZA)

A second response in mid-winter (below 0°C) helps to ensure successful overwintering. Freezing tolerance is further enhanced if cold-acclimated plants are exposed to mild sub-zero temperatures, such as −2°C. This phenomenon, which has been observed in Arabidopsis, wheat,

and oat, is known as second-phase cold hardening (2PH) or sub-zero acclimation (Herman et al., 2006; Livingston and Henson, 1998; Livingston et al., 2007; Le et al., 2008, 2014; Takahashi et al., 2019, 2021). Although the number of papers is limited, there have been several reports on the elucidation of the mechanism of 2PH and SZA.

At the plant tissue level, cell structural changes occurred in cell organelles, including the endoplasmic reticulum (ER) and the Golgi apparatus in wheat (Herman et al., 2006). Furthermore, *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 19* (*xth19*) mutants showed decreased freezing tolerance after CA and SZA treatment due to differences in cell wall composition and structure, suggesting that cell wall remodeling is involved in the SZA process (Takahashi et al., 2021).

As changes at the plant cell and molecular level, compared to CA, the same level of sugar accumulation and the same pattern of gene expression changes in *CBF* and *COR* were observed, suggesting that the SZA process requires a unique pathway that differs from CA to dramatically enhance freezing tolerance (Le et al., 2007). Another change is that more intracellular water moved to apoplastic space during SZA compared with non-acclimated plants and cold-acclimated plants in Wheat (Herman et al., 2006).

2PH or SZA research challenges

While many studies have focused on CA, fewer have explored 2PH, and there is limited information on the physiological conditions and molecular mechanism of this phenomenon (Le et al., 2008, 2014; Takahashi et al., 2019, 2021). For example, no previous studies have examined the effect of light and photoperiod on the 2PH process although these are necessary for inducing the CA process to increase freezing tolerance. In addition, a recent paper studying on 2PH, SZA, detached leaves as samples were used (Le et al., 2008, 2015; Takahashi et al., 2019, 2021). The molecular mechanism may be different in detached leaves than in intact plants. Thus, studies using intact plants are also needed to develop molecular models of 2PH and SZA.

Construction of a freezing chamber that allows to maintain sub-zero temperatures under light conditions

One of the reasons for the slow progress in research on 2PH is the difficulty in constructing an experimental system. For this study, a previous lab member constructed a chamber capable of maintaining negative temperatures even under light conditions by adding fans and fluorescent lights (**Fig. 1.2a**). In addition, the plants were grown on agar plates so that they could be

acclimated to freezing conditions with ease and in their intact form (**Fig. 1.2b,c**). At sub-zero temperatures, water turns from liquid to ice, but without a certain degree of shock, the liquid is in a so-called supercooled state, in which no ice forms even at the freezing temperature. In this study, the agar plates, placed at -2°C, were in a supercooled state and no ice formed if I did not shock them. As described later (Methods in Chapter 2), I induced 2PH by two methods; one was induced the 2PH with ice crystallization by momentarily touching the agar with liquid nitrogen (second phase freezing acclimation: 2PFA) (**Fig. 1.2b**), one was induced 2PH without ice crystallization (second phase supercooling acclimation: 2PSA) (**Fig. 1.2c**). In previous studies in our laboratory, plants with ice crystals in agar plates i.e., 2PFA during the 2PH showed higher freezing tolerance than those without ice crystals in agar plates i.e., 2PSA (**Fig. 1.3**) (Sugita et al., 2024).

Outline of this PhD. thesis

It is expected that the plants are exposed to light during the 2PH process in an outdoor environment at sub-zero temperatures as shown in **Fig. 1.1c**. Thus, in this doctoral dissertation, in addition to 2PSA and 2PFA treatment during 2PH, two more 2PH treatment conditions were tested i.e., light conditions during the 2PH. In other words, four patterns of treatment conditions during 2PH process (without ice nucleation (2PSA) in the (1) dark and (2) light conditions, and with ice crystal (2PFA) in the (3) dark and (4) light conditions) were tested (**Fig. 1.4**). In this doctoral dissertation, to understand the overwintering mechanisms of plants, I focused on elucidating the physiological conditions and molecular mechanisms of 2PH. Specifically, in the first half of the chapter (Chapter 2), I focused on elucidating 1) the physiological conditions in plants with ice crystals that showed higher freezing tolerance than the plants without ice crystals during 2PH. Furthermore, in the latter chapters (Chapter 3-5), I focused on 2PH plants with ice crystals under light conditions which have remarkable freezing tolerance among all 2PH plants, and elucidating 2) the molecular mechanisms. To achieve these two goals, specifically, I verified the following points.

1) Physiological conditions to induce 2PH with ice crystallization.

[1] The effect of duration of 2PH with ice crystallization on freezing tolerance (Chapter 2).

[2] The effect of light and changes in photoperiod during 2PH with ice crystallization on freezing tolerance (Chapter 2).

2) Molecular mechanism of 2PH with ice crystallization under light conditions.

[3] The expression of the *CBF* regulon in 2PH (Chapter 3).

[4] Role of the electron transport system in the chloroplast in the process of 2PH with ice crystallization under light conditions (Chapter 3).

[5] Role of photoreceptors in the process of 2PH with ice crystallization under light conditions (Chapter 3).

[6] Sugar content during the 2PH process and regulation of transcript levels of genes (Chapter 4).

[7] Large-scale expression analysis for a comprehensive understanding of 2PH mechanisms (Chapter 4,5).

Through a multifaceted approach such as screening analysis, pharmacological analysis, sugar analysis, and RNA-seq analysis, I investigated the basic physiological conditions and molecular mechanism of 2PH focusing on various factors for increasing freezing tolerance including CBF, light, sugar, cell wall, and plant hormones. Elucidating the mechanistic basis of 2PH will help reduce frost damage and expand the range and timing of crop growth.

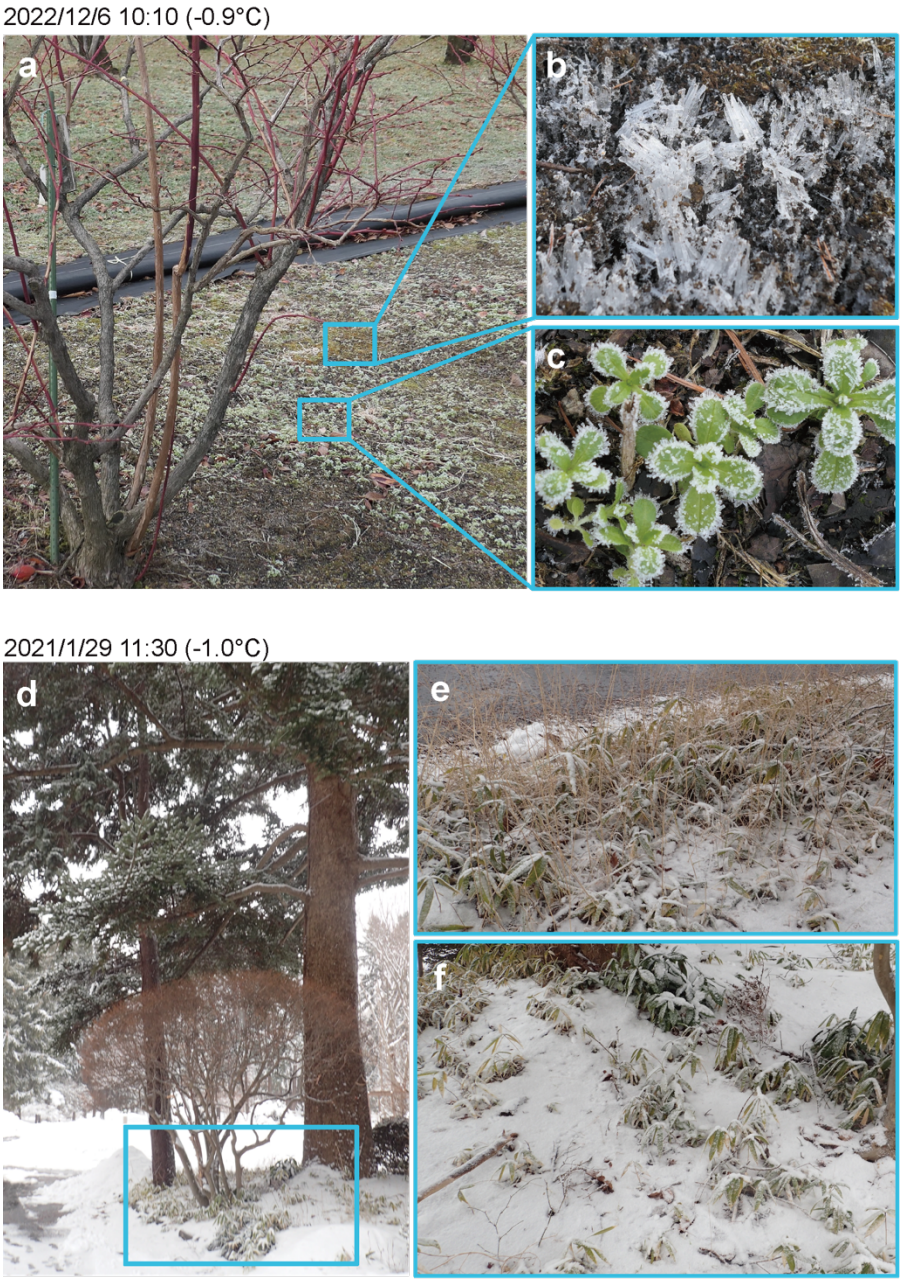


Fig. 1.1. (a) Outdoor site at the Field Science Center, Iwate University, Iwate on December 6, 2022. (b) Enlarged picture of A, showing frost on the ground. (c) Enlarged picture of A showing plants subjected to freezing under light conditions. (d) Outdoor site at the Faculty of Agriculture, Iwate University, Iwate on January 29, 2021. (e) and (f) Enlargements of (d). Trees protect plants growing below from snowfall, so plants are frozen without being covered by snow.

Fig. 1.2. (a) Freezing chamber used for 2PFA and 2PSA treatments. Temperature fluctuations were negligible and stable (±0.2°C) during the experiments because air was circulated by two fans. Fluorescent lights allowed control of light conditions during the experiments. The pictured was quoted from 2022 Takahashi master's thesis. (b) Images of 2PFA plants and (c) 2PSA plants. Pictures were taken after freezing acclimation or supercooling acclimation at −2°C for 3 days under 12-h light/12-h dark photoperiod after cold acclimation treatment. Scale bar = 10 mm.

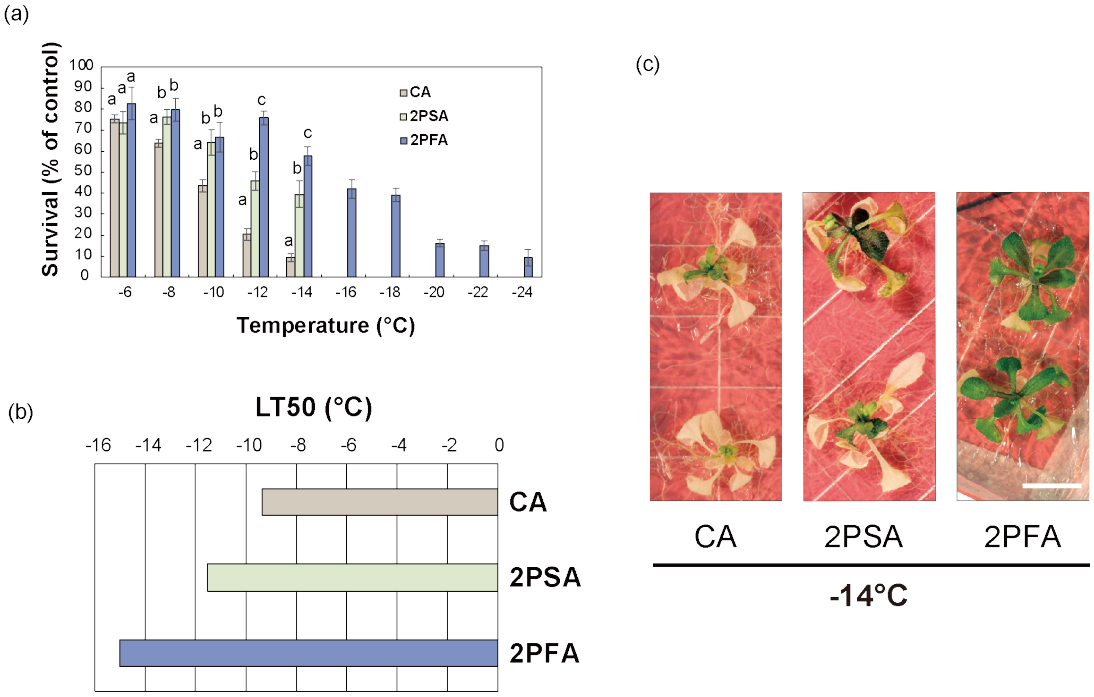


Fig. 1.3 Freezing treatment after cold acclimation further enhances freezing tolerance. (a) Survival rates of CA, 2PSA, and 2PFA plants. Error bars indicate SE (n=27–36). All data were evaluated by one-way ANOVA with Tukey-Kramer test ($p < 0.01$) and different letters at each temperature show significant difference. These data were partially modified Takahashi master's thesis, 2022. (b) LT_{50} values for CA, 2PSA, and 2PFA plants calculated from data shown in (a). (c) Representative images of CA, 2PSA, and 2PFA plants after freezing at −14°C and then thawing for 3 days at 23°C. Scale bar = 10 mm.

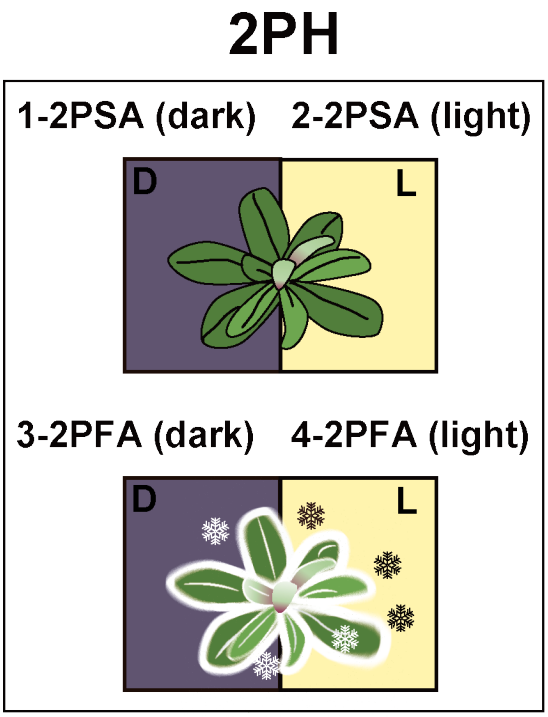


Fig. 1.4 Experimental design of the treatment during 2PH.
As 2PH treatments, plants were kept at −2°C without ice crystals (2PSA) in the (1) dark, and (2) light conditions, and with ice crystals (2PFA) in the (3) dark, and (4) light conditions in the dark in the freezing chamber.

Chapter2

Understanding the physiological conditions to achieve the second phase cold hardening

Abstract

Overwintering plants survive sub-zero temperatures by cold acclimation (CA), wherein they acquire freezing tolerance through short-term exposure to low temperatures above 0°C. The freezing tolerance of CA plants increases when they are subsequently exposed to mild sub-zero temperatures, a phenomenon known as second-phase cold hardening (2PH). A previous study showed that, during 2PH, a freezing treatment further enhanced the freezing tolerance in Arabidopsis compared with supercooling treatment. This required CA as a pre-treatment to enhance of the freezing tolerance. Here, I designated the phenomenon as second-phase freezing acclimation (2PFA), and the objective of this chapter was to elucidate the physiological conditions of 2PFA. In addition to the results showing that the 2PFA plants in dark conditions enhanced freezing tolerance, I revealed that the 2PFA plants under light conditions get further enhanced freezing tolerance. However, in the 2PFA plants under light conditions, changes in photoperiod did not affect the freezing tolerance. These results show that freezing and light play important roles in the enhancement of freezing tolerance in cold-acclimated Arabidopsis. After midwinter, some plants overwinter under light conditions with severe cold may be adapted to the natural conditions through the 2PFA process.

Introduction

Numerous studies have been conducted to elucidate the physiological conditions of cold acclimation (CA). For example, in Arabidopsis, approximately 7 days of CA treatment is required for the enhancement of the freezing tolerance (Tominaga et al., 2021; Uemura et al., 1995). Moreover, light is required for photosynthesis to enhance the freezing tolerance during CA process (Wanner and Junttila. 1999). Light during CA process is also required as a signal for pathways triggered by photoreceptors including cryptochromes and phytochrome (Imai et al. 2021, Jiang et al. 2020). Furthermore, light intensity and spectral quality play important role for enhancing the freezing tolerance during CA process (Kameniarová et al. 2022). Daylength (photoperiod) also affects freezing tolerance though CA process. Generally, in the case of herbaceous plants, short days CA treatment increases freezing tolerance (Levitt et al., 1980), but long days CA treatment in Arabidopsis and some barley varieties enhances it (Limin et al., 2007; Wanner and Junttila. 1999). Interestingly, the regulation of one of the COR genes, *COR15A*, by daylength shows different patterns depending on the temperature range (Tominaga et al., 2021).

Previous experiments conducted in our laboratory have shown that the effect of 2PH treatment were enhanced by ice crystals on the agar compared to supercooling at sub-zero temperature (**Fig. 1.3**) (Sugita et al., 2024). These results suggest that not only sub-zero temperatures but also ice crystals play important roles in enhancing freezing tolerance during the 2PH process. To distinguish between these 2P treatments with or without freezing on the agar, I called as freezing acclimation during 2P: 2PFA (at −2°C with ice crystallization) and supercooling acclimation during 2P: 2PSA (at −2°C without ice crystallization). Previous studies in our laboratory also showed that the CA process was necessary as a pre-treatment for achievement of the 2PFA in the dark conditions and the maximum freezing tolerance of the plants was reached within 7 days CA (Sugita et al., 2024). While some information about the role of CA process to achieve 2PFA in the dark conditions, there is limited information on the physiological conditions of 2PFA such as the effect of the duration and light conditions on the freezing tolerance.

For this study, I focused on the physiological conditions including duration of 2P, light and photoperiod during the 2P treatment. The results show that 1 day of 2PFA treatment in the dark is required to enhance the freezing tolerance. In addition, compared with 2PFA in the dark conditions, 2PFA in the light conditions further increased the effect to improve freezing tolerance, showing an LT50 value below −20°C even in Arabidopsis, and 3 days of 2PFA treatment in the light is required to achieve maximum freezing tolerance. Furthermore, in the

2PSA treatment, light plays a role in furthermore increasing the freezing tolerance compared with that of the treatment in the dark conditions as well as the 2PFA treatment.

Materials and Methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were surface-sterilized with 20% (v/v) bleach and 1% (v/v) sodium dodecyl sulfate and placed on Hoagland's medium containing 1% (w/v) sucrose and 1% (w/v) agar in square (96 mm ×96 mm) Petri plates. Two days after stratification at 4°C in the dark, plants were grown in a controlled-environment chamber at 23°C for 2 weeks under a 16-h light/8-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps. These plants were designated as non-acclimated (NA) plants. The NA plants were transferred into a growth chamber at 2°C under a 12-h light/12-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps and grown for 7 days as the CA treatment. The CA plants were transferred into the freezing chamber at −2°C. After keeping the plates at −2°C for 10 min in the freezing chamber, ice was induced to form by momentarily touching the agar with liquid nitrogen, and then the plants were incubated for 1 day, 3 days, or 7 days at −2°C either in the dark or under an 8-h light/16-h dark, 12-h light/12-h dark, or 24-h light photoperiod with light supplied at 80 μmol m^{-2} s^{-1} as the second-phase freezing acclimation (2PFA) treatment (**Fig. 1.2b**). For the second-phase supercooling (2PSA) treatment, CA plants were transferred into the freezing chamber at −2°C in the same way as in the 2PFA treatment, but ice was not induced to form on the agar (**Fig. 1.2c**). The experimental design was shown in **Fig. 2.1**.

All experiments were repeated at least three times, and mean values were calculated.

Freezing tolerance test

For the NA, CA, 2PSA, and 2PFA treatments, seedlings grown on agar plates were placed vertically in the dark in an SU-641 controlled-temperature chamber (Espec, Osaka, Japan), and were rotated in the chamber at 1 rpm to equalize all plates temperatures through air circulation by the fan. After 10 min at −2°C, ice was induced to form on the agar by touching a corner of the plate with liquid nitrogen for a few seconds. This treatment was not applied if ice crystals had already been formed in the 2PFA treatment. The plates were kept for 2 h at −2°C to freeze the agar and plants completely, and the temperature was gradually lowered to −22°C at −2°C h^{-1} in the dark. Plates were taken out at the specified temperature and transferred to a growth

chamber at 23°C with a 16-h light/8-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps. The plants were kept under these conditions for 1 day to allow thawing, and then the survival rate was measured.

Measurement of survival rate

The survival rate of plants was calculated from the maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m) measured on whole Arabidopsis leaves (Ehlert and Hincha, 2008). A recent study established a chlorophyll fluorescence imaging method based on F_v/F_m, which can easily be used to evaluate the freezing tolerance of intact plants, and correlates well with results obtained using the electrolyte leakage method (Hiraki et al., 2020). The chlorophyll fluorescence of dark-adapted Arabidopsis plants (15 min) was measured using a Fluor Cam instrument (800 MF, Photon Systems Instruments, Brno, Czech Republic). I used the value of F_v/F_m multiplied by the area of using the whole plant and calculated the survival rate the following equation:

$$\text{Survival rate at the specified temperature} = \frac{(F_v/F_m \times \text{Area})\ \text{after 1 day of freezing test}}{(F_v/F_m \times \text{Area})\ \text{before CA treatment}}$$

$$\text{Fixed survival rate} = \frac{\text{Survival rate at the specified temperature}}{\text{Survival rate of plants that were not freezing tested (control)}}$$

LT_{50} was calculated by proportional formula using data from two temperatures between 50% survival.

Statistical analysis

Statistical significance of differences was determined by one-way ANOVA with Tukey-Kramer test for multiple comparisons ($p < 0.01$) with R (version 4.3.1) using RStudio (version 2023.09.1+494).

Results

One day of freezing treatment in the dark is required to achieve the 2PFA.

After CA at 2°C for 7 days, for the second-phase treatment (2P), plants were kept at −2°C with ice crystal (freezing acclimation during 2P: 2PFA) or without ice crystallization (supercooling acclimation during 2P: 2PSA) for 3 days in the dark in the freezing chamber. The freezing tolerance of plants was estimated using a chlorophyll fluorescence imaging method based on F_v/F_m.

Firstly, the effect of the duration of the 2PFA treatment in the dark on freezing tolerance was evaluated. CA plants were subjected to 1, 3, and 7 days of 2PFA treatment at -2°C under the dark conditions. The results show that the survival rates of 1 day 2PFA plants at −14°C and −18°C were approximately 50% and 40% of that of 0 day 2PFA plants respectively. However, the 3-day and 7-day 2PFA treatments under dark conditions did not increase freezing tolerance thereafter the 1-day 2PFA treatments. From these results, the maximum freezing tolerance of 2PFA plants in the dark was obtained within 1 day (**Fig. 2.2**).

Light during 2PFA treatment further enhances freezing tolerance

As shown in **Fig. 1a-c**, there are some cases that plants are subjected to freezing under light conditions. Thus, the effects of light exposure and photoperiod during the 2PFA treatment were studied by varying the photoperiod from 8-h light/16-h dark to 24-h light. Compared with the 2PFA plants in the dark, 2PFA plants under the light conditions showed significantly higher survival rates at temperatures below −16°C (**Fig. 2.3a,b**).

Three days of freezing treatment in the light conditions is required to achieve the 2PFA.

Next, I examined the effect of the duration of the 2PFA treatment with a 12-h light/12-h dark photoperiod on freezing tolerance. CA plants for 7 days were subjected to 1, 3, and 7 days of 2PFA treatment at −2°C under the light conditions (**Fig. 2.3c**). Unlike the 2PFA treatment in the dark, the maximum freezing tolerance of 2PFA plants in the light conditions was not obtained within 1 day. For example, at −18°C and −22°C, the survival rate of 1 day 2PFA plants in the light conditions were approximately 35% and 20% of that of 0-day 2PFA plants respectively. While, at −18°C and −22°C, the survival rate of 3 days 2PFA plants in the light conditions were approximately 65% and 50% of that of 0 day 2PFA plants respectively. However, there was no difference of freezing tolerance between plants subjected to 3 and 7 days of the 2PFA treatment in the light conditions. This result indicated that 3 days of 2PFA treatment under light conditions was required to induce the maximum freezing tolerance in 2PFA plants in the light conditions (**Fig. 2.3c**).

Light during 2PSA treatment also enhances freezing tolerance

The second phase supercooling acclimation (2PSA) treatment, which represents supercooling conditions, was also tested under light conditions. The freezing tolerance of plants after 2PSA treatment in the dark or under a 12-h light/12-h dark photoperiod was measured and their LT_{50} was calculated. The freezing tolerance acquired after 2PSA treatment in the light was more than that acquired after 2PSA treatment in the dark (**Fig. 2.4**). These results indicate that light during the 2P treatment further enhances the effects of freezing and sub-zero temperatures to establish freezing tolerance.

Discussion

Freezing and light during 2P treatments significantly increases the freezing tolerance of plants.

The results show that the freezing tolerance of CA plants was further enhanced by exposure to mild sub-zero temperatures that did not cause injury (**Figs. 2.2-3**). At this time, it was greatly enhanced by the addition of a freezing treatment on the agar (i.e., 2PFA), while freezing tolerance could be enhanced by a supercooling treatment (i.e., 2PSA) (Sugita et al., 2024, **Fig. 2.4**). Maximum freezing tolerance was achieved after 1 day of the 2PFA treatment in the dark conditions. (**Fig. 2.2**). Interestingly, the effect of 2PFA to increase the freezing tolerance were further enhanced by exposure to light and the maximum freezing tolerance of 2PFA plants in the light conditions was obtained within 3 days (**Fig. 2.3**). The difference of photoperiod during 2PFA process in the light conditions has no effect on the freezing tolerance (**Fig. 2.3a**).

One day of 2PFA treated plants in both dark and light conditions showed that freezing tolerance was the same (**Fig. 2.2, 2.3c**). This indicates that the freezing tolerance mechanism acquired on day 1, i.e., the acquisition of tolerance by freezing treatment, is common to light and dark conditions. In addition, the 2PFA plants under light conditions continued to increase their freezing tolerance after 1 day of treatment, and maximum freezing tolerance was achieved after 3 days. This indicates that light plays a role in increasing the freezing tolerance of the 2PFA plants under light conditions between 1 day and 3 days.

As shown in **Figure 2.4**, the effect of light during the second phase was observed in both the supercooling (2PSA) and in the freezing (2PFA) treatments. The LT_{50} of 2PSA plants was −11.5°C and −13.5°C under dark and light conditions, respectively, and the LT_{50} of 2PFA plants was −15.0°C and −20.4°C under dark and light conditions, respectively (**Fig. 2.4**). The difference in the LT_{50} of plants between 2PFA and 2PSA was 3.5°C under dark conditions and 6.9°C under light conditions, which is not the same value. These results suggest that a new

freezing tolerance mechanism is responsible for the substantial enhancement of freezing tolerance when 2PFA treatments are applied under light conditions.

In previous studies where 2P treatments were applied under laboratory conditions, the freezing or sub-zero temperature treatments were applied in the dark (Livingston et al., 2007; Le et al., 2008, 2015; Takahashi et al., 2019, 2021). In the context of those experiments, it was assumed that herbaceous plants are often buried under snow in winter, and consequently live in low-light or dark conditions during overwintering (Le et al., 2008). However, snow is not always present in sub-zero environments, for example, herbaceous plants growing under trees are not covered by snow during winter (**Fig. 1.1d-f**). Plants in these environments are likely to be exposed to scattered and sometimes direct sunlight even at sub-zero temperatures. Therefore, I examined the effects of light on the second phase treatment.

Another study showed contrasting results, i.e., when soil-grown Arabidopsis plants were subjected to second-phase treatments in the dark at −3°C after CA, the degree of freezing tolerance was not affected by whether the leaves were frozen or not (Le et al., 2008). Although we do not know the exact reason for this, the main difference between our study and theirs is that the plants were grown in soil before the freezing treatment in their study, but on agar plates in ours. Compared with plants grown on agar, potted plants grown in soil are surrounded by a larger volume of soil. Soil holds more water than does agar. Because the heat capacity of water is very large and water also generates heat when it freezes, it takes more time to freeze when the substrate has a high water content. Thus, the difference in freezing speed may explain differences in results between their study and mine.

In this and previous studies, ice was induced to form on the agar by touching a corner of the agar plate with liquid nitrogen for a few seconds as 2PFA treatment (**Fig. 1.2b**, Sugita et al.,2024). Since the agar is definitely frozen, the plants is probably also frozen from the roots, most likely due to propagation of agar ice. In previous studies, plants have been reported to freeze from their roots and lower tissues at sub-zero temperatures (Livingston et al., 2018; Stier et al., 2003). When liquid water freezes, it releases energy in the form of heat. Therefore, the use of infrared thermography cameras allows the observation of freezing plant tissues. In recent years, progress has been made from analog systems to digital systems, allowing for higher resolution observations. In this study, I did not verify whether plants were actually frozen. Thus, infrared thermography analysis will be revealed that plants are frozen definitely.

CA plants further enhance their freezing tolerance when exposure to mild sub-zero temperatures as 2P treatment. In this study, the temperature was kept constant at −2°C during the 2P process. The increasing in freezing tolerance by the 2P treatment may be changed by

sub-zero temperatures during 2P process. Thus, during 2P process, it would be interesting to examine the freezing tolerance at various temperatures such as −3 or −4 °C.

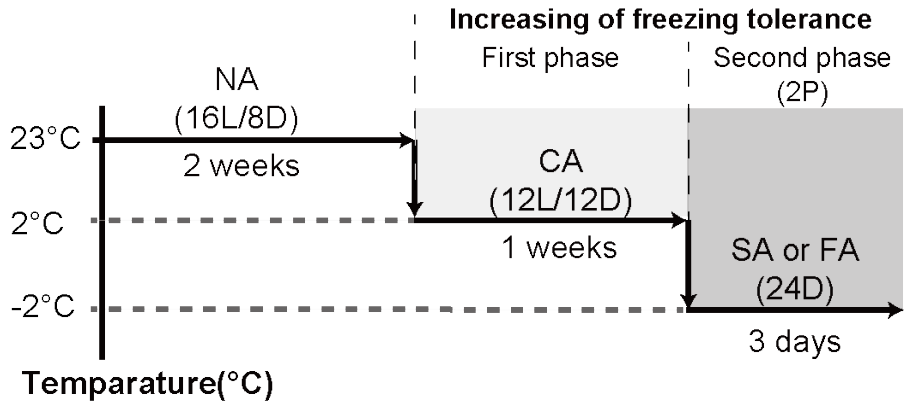


Fig. 2.1 Freezing treatment after cold acclimation further enhances freezing tolerance. (a) Overview of experimental design. Plants were cold-acclimated at 2°C (CA) for 7 days under a 12-h light/12-h dark photoperiod. For second-phase (2P) treatments, CA plants were kept at −2°C with ice crystal (second-phase freezing acclimation: 2PFA) or without ice crystallization (second-phase supercooling acclimation: 2PSA) for 3 days in the dark.

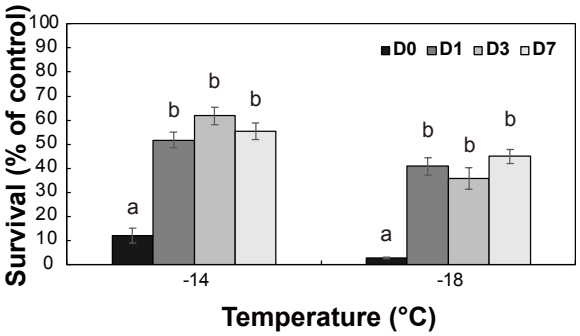


Fig. 2.2 To achieve the 2PFA effect, 1 day of freezing treatment in the dark are required. (a) Survival rates at −14°C and −18°C of plants freezing-acclimated for 0, 1, 3, and 7 days (D0, D1, D3, D7) at −2°C in the dark after 7 days of CA. Error bars indicate SE (n=11–47). Different letters at each temperature show significant difference at $p < 0.01$ (Tukey-Kramer test).

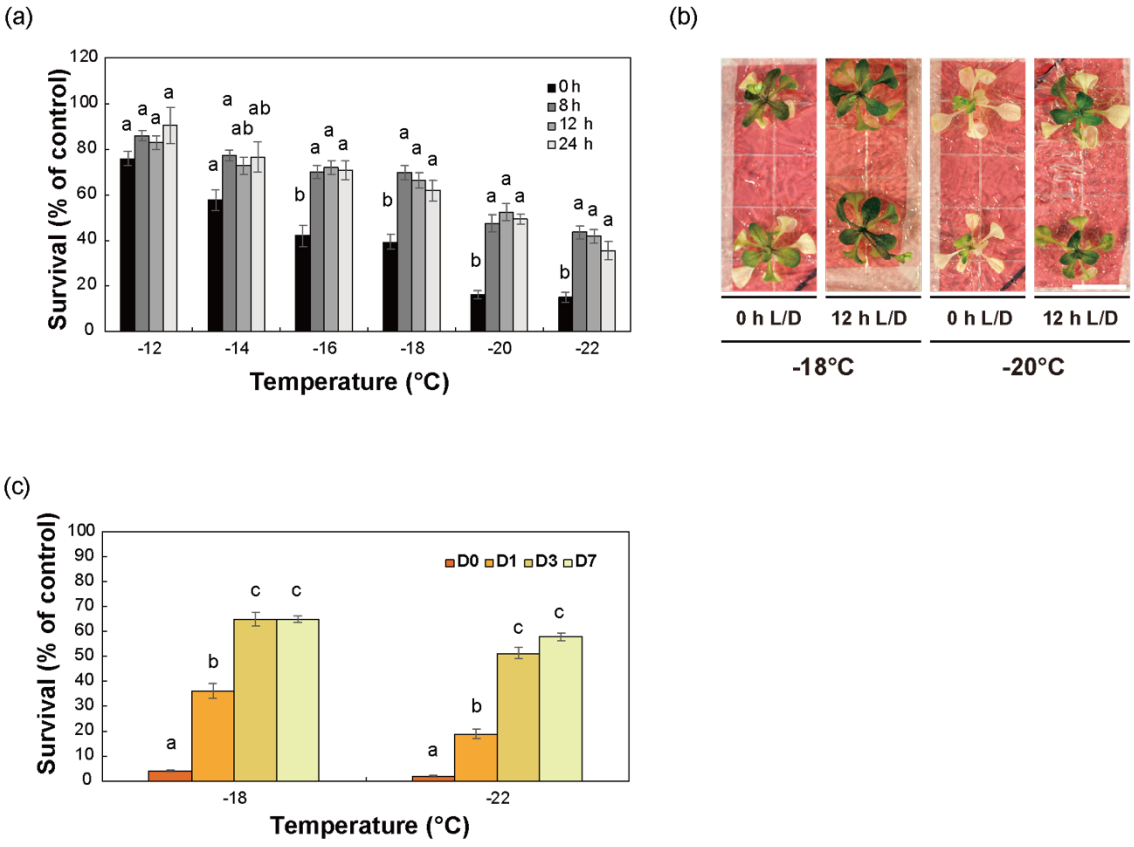


Fig. 2.3 Light during freezing treatment after cold acclimation further enhances freezing tolerance.

(a) Survival rates of CA7 plants subsequently freezing-acclimated at −2°C for 3 days under 0-h photoperiod (0 h), 8-h light/16-h dark photoperiod (8 h), 12-h light/12-h dark photoperiod (12 h), or 24-h light photoperiod (24 h). Error bars indicate SE (n=11–50). Different letters at each temperature indicate significant difference at $p < 0.01$ (Tukey-Kramer test). (b) Representative pictures of plants frozen at −18°C and −20°C after freezing-acclimation for 3 days under 0-h photoperiod and 12-h photoperiod. Pictures were taken 3 days after thawing at 23°C. Scale bar = 10 mm. (c) Survival rate after freezing at −18°C and −22°C of CA7 plants freezing-acclimated for 0 day (D0), 1 day (D1), 3 days (D3), and 7 days (D7) at −2°C under 12-h photoperiod. Error bars indicate SE (n=11–46). Different letters at each temperature show significant difference at $p < 0.01$ (Tukey-Kramer test).

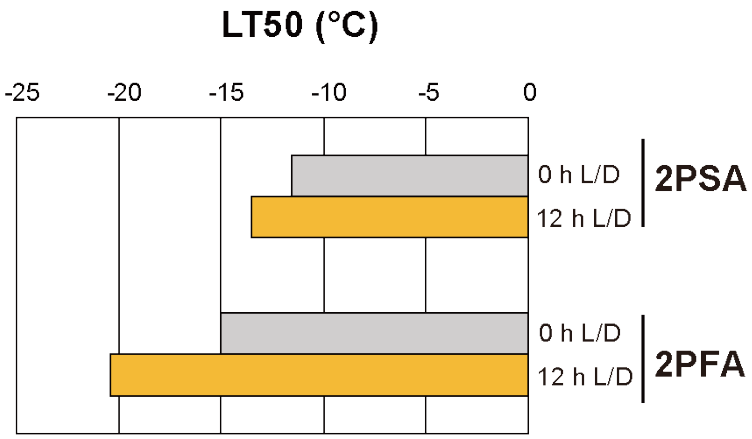


Fig. 2.4 Light during 2P process after cold acclimation further enhances freezing tolerance.
LT_{50} of plants freezing-acclimated at −2°C for 3 days with/without ice crystallization under a 0-h photoperiod or 12-h photoperiod.

Chapter3

Elucidating the molecular mechanism of how second phase freezing acclimation under light conditions further enhance freezing tolerance

Abstract

Plants that grow in colder-than-temperate zones are exposed to freezing under light conditions even in winter in some cases. Many overwintering plants enhance freezing tolerance by cold acclimation (CA). In addition, CA plants increase further freezing tolerance by exposing to mild freezing temperatures such as −2℃ or −3℃, which is a phenomenon known as the second-phase cold hardening (2PH). Previous chapter revealed that the effect of 2PH were increased by ice during the process, and I designed this phenomenon as second phase freezing acclimation (2PFA). In addition, light play important role in increasing freezing tolerance during 2PFA process. Here, I explored the molecular mechanism of how 2PFA plants under light conditions enhance freezing tolerance dramatically. C-repeat binding factor (*CBF*) and *cold-regulated* (*COR*) genes were down-regulated by light during the 2PFA treatment, different expression profiles from that during CA. The freezing tolerance of 2PFA plants was decreased by the presence of the photosynthetic electron transfer inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) during the 2PFA treatment. Consistently, chlorophyll fluorescence observation in leaves under freezing conditions showed that electron transport system was active. Furthermore, compared with wild-type plants, *phototropin1,2* and *phyb* mutants showed lower freezing tolerance after 2PFA treatment. These results show that freezing under light conditions further increases freezing tolerance *via* pathways involving some photoreceptors and photosynthetic electron transfer.

Introduction

Several studies have revealed details of the CA process at the molecular level. The CBFs (C-repeat binding factors) pathway of Arabidopsis is one of the best-understood gene regulatory networks during CA processes. Rapid transient expression of *CBF1*, *CBF2*, and *CBF3* genes is induced after transferring plants to low temperatures, followed by the expression of cold-regulated (*COR)* genes to acquire freezing tolerance (Thomashow, 1999, 2001).

In addition to research on the CBF-COR pathway in response to low temperature, studies about the role of light effect on the CA process have progressed at the molecular level. In response to low temperatures, light is used as not only an energy source for photosynthesis but also an environmental signal. Experiments using DCMU have suggested that the photosynthetic electron transfer pathway may contribute to increasing of freezing tolerance during the CA process (Tominaga et al., 2021; Wanner and Junttila, 1999). For example, the plants cold-acclimated under light conditions in the presence of DCMU exhibited lower freezing tolerance compared with plants cold-acclimated under light conditions in the absence of DCMU. Furthermore, DCMU inhibits sugar accumulation, which is induced in CA treatment under light conditions (Wanner and Junttila, 1999). These results suggested that during the CA process, photosynthesis may be required to accumulate sugars as compatible solutes, which improve the freezing tolerance.

Furthermore, light is used as a signal for pathways involved in photoreceptors during the CA process. Photoreceptors depend on the wavelength of light received. For example, while blue light is perceived via cryptochromes (CRY1 and CRY2) and phototropins (PHOT1 and PHOT2), phytochromes (phyA to phyE) known as the red-far-red light-absorbing. CRY-CBF pathway is involved in the early process of CA by perceiving blue light (Imai et al., 2021). Blue light-induced accumulation of COR15b in barley at low temperature (Crosatti et al., 1999). In addition, phototropins act as a temperature sensor, sensing low temperatures and positioning chloroplasts in an optimal position for photosynthesis through movement along actin filaments (Fujii et al., 2017). It has been reported that the phytochromeB pathway via CBFs- PIF3 plays an important role in enhancing freezing tolerance in response to low temperatures (Jiang et al., 2020). Thus, photoreceptors are involved in the CA process or process of sensing low temperatures.

Thus, although molecular mechanisms centered on CBFs and light in response to low temperatures are being elucidated, molecular mechanisms related to second phase cold hardening have been limited study. Especially, previous chapter (see Chapter 2) showed that second phase freezing acclimation (2PFA) under light conditions leads to significant freezing

tolerance. Still, it is unclear whether 2PFA follows a similar molecular pathway or independent pathway to CA to enhance freezing tolerance. In this study, I aimed to understand the molecular mechanism of how 2PFA plants under light conditions increases freezing tolerance, and whether 2PFA follows the same molecular pathway as CA or not. The results in this chapter show that the expression of *CBFs* and *CORs* was down-regulated by freezing and light conditions at -2 °C. Furthermore, 2PFA plants under light conditions further increase freezing tolerance *via* pathways involving photosynthetic electron transfer and photoreceptors including phototropins and phytochrome.

Materials and Methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were surface-sterilized with 20% (v/v) bleach and 1% (v/v) sodium dodecyl sulfate and placed on Hoagland's medium containing 1% (w/v) sucrose and 1% (w/v) agar in square (96 mm ×96 mm) Petri plates. After two days of stratification at 4°C in the dark, plants were grown in a controlled-environment chamber at 23°C for 2 weeks under a 16-h light/8-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps. These plants were designated as non-acclimated (NA) plants. The NA plants were transferred into a growth chamber at 2°C under a 12-h light/12-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps and grown for 7 days as the CA treatment. The CA plants were then transferred into a freezing chamber at −2°C. After keeping the plates at −2°C for 10 min in the freezing chamber, ice was induced to form by momentarily touching the agar with liquid nitrogen, and then the plants were incubated for 1 day, 3 days, or 7 days at −2°C either in the dark or under an 8-h light/16-h dark, 12-h light/12-h dark, or 24-h light photoperiod with light supplied at 80 μmol m^{-2} s^{-1} as the second-phase freezing acclimation (2PFA) treatment. As the second-phase supercooling (2PSA) treatment, CA plants were transferred into the freezing chamber at −2°C in the same way as in the 2PFA treatment, but the ice was not induced to form on the agar.

Several Arabidopsis mutants were used in this study. *csk1* was a kind gift from Professor Mitsumasa Hanaoka (Chiba University). *Hy4-2.23N/fha* (designated as *crycry2*) derived from the Landsberg erecta ecotype (Ahmad et al., 2002) and *phot1-5phot2-1* (designated as *phot1phot2*) derived from the *GLABRA1* mutant (*gl1*) (Kinoshita et al., 2001) were kind gifts from Professor Akira Nagatani (Kyoto University). *phyB* (SALK022035) was obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu). All seeds used in this study were obtained from plants backcrossed with wild-type plants at least two times.

All experiments were repeated at least three times, and mean values were calculated.

RNA extraction and qRT-PCR analyses

Plants were grown in a controlled-environment chamber for 2 weeks at 23°C under a 16-h light/8-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps. The plants were then transferred to a growth chamber at 2°C with a 12-h light/12-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps, and samples were collected at 0, 3, 6, 12, 24, 36, 48, 60, and 72-h (designated as CA in Figure 5a). After 7 days, the CA plants were then subjected to CA+ treatment (2°C), 2PFA treatment (−2°C with ice), or 2PSA treatment (−2°C without ice). Samples were collected at 0, 3, 6, 12, 24, 36, 48, 60, and 72-h of these treatments. Then, RNA was extracted from the aerial parts of plants using an ISOSPIN Plant RNA kit (Nippon Gene, Tokyo, Japan). The extracted RNA was treated with DNase, and then 500 ng total RNA was used to synthesize cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan), according to the manufacturer's instructions. Quantitative real-time RT-PCR analyses were performed using the Thermal Cycler Dice™ Real-Time System (Takara, Otsu, Japan) with SYBR Premix Ex Taq II (Takara), according to the manufacturer's protocol. The relative transcript levels of genes were calculated from real-time PCR data using the $2^{-\Delta\Delta Ct}$ method with Thermal Cycler Dice™ Real-Time System Single Software (Livak and Schmittgen, 2001). The reference genes were *PP2A* and *GAPDH*. All primers used for qRT-PCR were blasted using Primer-BLAST at the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov). Primer sequences are listed in **Table 3.1**.

Freezing tolerance test

For the NA, CA, 2PSA, and 2PFA treatment plants, seedlings growing on agar plates were placed vertically in the dark in an SU-641 controlled-temperature chamber (Espec, Osaka, Japan), which were rotated at 1 rpm to equalize all plates temperatures through air circulation by the fan. After 10 min at −2°C, ice was induced to form on the agar by touching a corner of the plate with liquid nitrogen for a few seconds. This treatment was not applied if ice crystals had already been formed in the 2PFA treatment. The plates were kept for 2 h at −2°C to freeze the agar and plants completely, and the temperature was gradually lowered to −22°C at −2°C h^{-1} in the dark. Then plates were taken out at the specified temperature. Plants were transferred to a growth chamber at 23°C with a 16-h light/8-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps. The plants were kept under these conditions for 1 day to allow thawing, and then the survival rate was measured.

Measurement of survival rate

The survival rate of plants was calculated from the maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m) as described previous chapter (Chapter 2).

The leaf survival rate was calculated as the proportion of dead leaves out of total leaves 3 days after the freezing test. Leaves that were completely yellow were regarded as dead leaves as described by Zhang et al. (2013).

Treatment with a photosynthetic electron transport inhibitor

The CA seedlings were transferred from the growth chamber at 2°C to room temperature and misted twice with 0.5% v/v ethanol with or without 50 μM 3–(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). After 1 h at room temperature, the chlorophyll fluorescence of the plants was measured. Then, the plants were transferred into the growth chamber at −2°C with ice crystal for 3 days under dark conditions or a 12-h light/12-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps. The experimental design is shown in **Fig. 3.1**. After the freezing tolerance test, the leaf survival rate was calculated. The effect of ethanol used as a solvent for DCMU on freezing tolerance was also examined, and it was concluded that it did not affect survival (**Fig. 3.2**).

Statistical analysis

Statistical significance of differences was determined by Student's t-test ($p < 0.05$) between two group in Excel (version 16.78.3).

Results

***CBFs* and *CORs* were down-regulated in the 2PFA plants under light conditions.**

CBF genes and their targets, *COR* genes, encode components of one of the major pathways involved in the CA process (Thomashow, 1999, 2001). Thus, the transcriptional profiles of these genes within 72-h after the treatments were compared between the 2P and CA processes. In this experiment, the 2P treatments were freezing (2PFA) or supercooling (2PSA), and were applied in the dark or under a 12-h light/12-h dark photoperiod after CA treatment (**Fig. 3.3a**). Because 2P treatments effects other than temperature and freezing could affect the given results, one of the controls was then subjected to a further CA process (CA+) under the same time conditions as the 2P treatment at 2°C.

During the CA process at 2°C under a 12-h light/12-h dark photoperiod, the three *CBF* genes showed rapid and transient increases in their transcript levels, as previously reported in

CA Arabidopsis plants (**Fig. 3.3b-d and 3.5a-c**, Gilmour et al., 1998). The transcript levels of the three *CBF* genes peaked at 3 h, then decreased within 24 h and did not increase until 72 h. Conversely, their transcript levels did not change in the CA+ treatment, suggesting that their transcription responded to low temperature.

In the 2PSA treatments in the dark and light, the three *CBF* genes showed transient increases in their transcript levels between 3 h and 6 h, similar to that observed in the CA treatment (**Fig. 3.3b-d and Fig. 3.4, 3.5a-c**). When comparing the effects of the 2PSA treatment between light and dark conditions, interestingly, the *CBF* transcript levels were higher in the dark than under light conditions, showing an inverse relationship with the magnitude of freezing tolerance. The increased transcript levels of *CBF1* and *CBF2* in the dark were similar to those in CA plants, while the transcript level of *CBF3* was relatively low. In the 2PFA treatments in the dark and light, the *CBF1* and *CBF2* genes showed transient increases in their transcript levels between 3 h and 24 h (**Fig. 3.3b-c and Fig. 3.5a-b**). The *CBF3* genes showed that in the light condition, the transcript level was an increased between 3 h and 6 h followed by a gradual decrease, while in the dark condition, the transcript level was a gradual increase up to 24 h, then followed by a decrease (**Fig. 3.3d and Fig. 3.5c**). The three *CBF* transcript levels were higher in the dark than under light conditions. Analyses of *CBF* genes revealed clear differences in their transcript profiles between the 2PFA and 2PSA treatments. Two peaks in the transcript levels of *CBF1* and *CBF2* occurred at 3- and 24-h in both light and dark conditions (**Fig. 3.3b-c and 3.5a-b**). In all the 2PFA treatments, the transcript levels of *CBF* were lower under light conditions than under dark conditions.

Next, the transcriptional profiles of *COR* genes were determined. First, in the CA treatment, the transcript level of *COR15A* continued to increase up to 72 h, whereas those of *COR47* and *COR78* peaked between 24 h and 36 h and then decreased (**Fig. 3.3e-g and 3.5d-f**). After 7 days of CA, namely CA+ in the figure, the *COR15A* transcript levels remained high, whereas those of *COR47* and *COR78* remained lower than their respective levels in CA. In the 2PSA treatment in the dark, all the *COR* genes showed increased transcript levels. In the 2PSA treatment under light conditions, only the transcript level of *COR47* was increased relative to that in CA+. In the 2PFA treatment in the dark, all the *COR* genes showed increased transcript levels. In the 2PFA treatment under light conditions, all the *COR* genes showed lower transcript levels compared with those in the dark, and none of them showed a clear increase in its transcript levels between 3 h and 72h.

These analyses of gene transcriptional profiles show that the significant increase in freezing tolerance caused by the 2PFA treatment under light conditions cannot be explained by

the expression of the CBF regulon. Therefore, next, I focused on the possibility that photosynthesis and photoreceptors are involved in the effect of light to strengthen freezing tolerance induced by the 2PFA treatment.

The electron transport pathway in chloroplasts is involved in the 2PFA process under light conditions.

In the CA process, light is essential for the expression of cold-inducible genes and the enhancement of freezing tolerance. Experiments using DCMU have suggested that the photosynthetic electron transfer pathway may contribute to this process (Tominaga et al., 2021; Wanner and Junttila, 1999). To confirm the contribution of photosynthesis to the effects of light to strengthen freezing tolerance after the 2P treatments, I conducted experiments using DCMU. The plants were sprayed with DCMU at the timing after CA and before the 2P treatments. However, because DCMU treatment not only increases F_0 values but also decreases F_m values depending on the treatment conditions (Tóth et al., 2005), our freezing tolerance test based on F_v/F_m could not be used in this experiment. Therefore, I measured the survival rate by counting the number of living leaves after the freezing test. There was a strong correlation between the survival rate determined by measuring F_v/F_m and that determined by counting the number of living leaves (**Fig. 3.6**). The plants subjected to 2P treatments under light conditions in the presence of DCMU exhibited lower freezing tolerance at −14°C, −16°C, −18°C, and −22°C compared with plants subjected to the same 2P treatments in the absence of DCMU (**Fig. 3.7**). To confirm whether DCMU inhibits light-enhanced freezing tolerance during 2PFA treatment, I also tested the survival rate of plants subjected to 2P treatments in the dark in the presence of DCMU. However, for plants subjected to 2P treatments in the dark, the presence of DCMU did not significantly affect their survival rate at −14°C, −16°C, −18°C, and −22°C (**Fig. 3.7**), indicating that DCMU acts only through the light-mediated pathway.

Taken together, these results suggest that the photosynthetic electron transport pathway is involved in the effect of light to strengthen the freezing tolerance acquired after a 2PFA treatment.

Some photoreceptors, phytochrome, and phototropin are involved in the 2PFA process under light conditions.

A red light photoreceptor phytochrome (PHYB), and blue light photoreceptor cryptochromes, are involved in the CA process of *Arabidopsis thaliana* (Imai et al., 2021; Jiang et al., 2020). Phototropins are temperature-sensing photoreceptors that function in cold-induced chloroplast

positioning for photosynthesis (Fujii et al., 2017). To determine whether these photoreceptors are involved in the acquisition of freezing tolerance in 2PFA plants under light conditions, I screened the photoreceptor mutants, *cry1cry2*, *phot1phot2*, and *phyb*. Compared with wild-type plants, 2PFA *cry1cry2* mutants exhibited lower freezing tolerance, whereas *cry1cry2* plants subjected to 2P treatments showed the same freezing tolerance (**Fig. 3.8a,b**). Conversely, the freezing tolerance of CA *phot1phot2* mutants was not different from that of the control, but the freezing tolerance of 2PFA *phot1phot2* plants was lower than that of the control (**Fig. 3.8c,d**). The CA *phyb* mutants exhibited decreased freezing tolerance, consistent with previous studies (Jiang et al., 2020). Conversely, 2PFA *phyb* mutants also showed a reduced survival rate (**Fig. 3.8e,f**). These results suggest that the two photoreceptors phototropin and PHYB are involved in the effect of light to further enhance the freezing tolerance of 2PFA plants.

Discussion

Freezing during 2P treatments significantly increases the freezing tolerance of plants. Comparisons of the expression profiles of several *CBF* and *COR* genes revealed a clearly different expression profile of *CBF* genes in the 2PFA treatment. These results indicate that freezing signals and low-temperature signals are different. An enhancement of freezing tolerance caused by sub-zero temperature treatments after CA was hardly observed in NA plants (Sugita et al., 2024), indicating that CA treatment is necessary as a pretreatment for the acquisition of freezing tolerance for 2PFA treatments. In fact, the effect of 2PFA was enhanced by the number of days of CA, and the intracellular changes during the CA process were necessary for the 2PFA treatment to enhance freezing tolerance (Sugita et al., 2024). These results suggest that the mechanisms of perception and processing of the freezing signals in cells may differ before and after CA.

The calcium signal, as a second messenger, is involved in various signaling pathways and stress responses including cold shock (Hepler and Wayne, 1985; Knight et al., 1993; Lecourieux et al., 2006; Plieth et al., 1999). Calcium influx has been shown to regulate the expression of genes involved in CA (Knight et al., 1996; Monroy et al., 1993). Interestingly, it has been reported that the response of cold-induced calcium signals is different before and after CA (Hiraki et al., 2019). In roots of NA Arabidopsis, the calcium signals are acutely responsive to cooling from 20°C, but less responsive to cooling from 2°C. Conversely, in 7-day CA Arabidopsis, the calcium signals are acutely responsive to cooling from 2°C, but show little responsiveness to cooling from 20°C. As a result of plants being more sensitive to temperature changes at low temperatures after CA, for example, during the 2PSA treatment, calcium signals

may result in significantly enhanced freezing tolerance. Compared with supercooling, freezing may cause an additional significant increase in calcium signaling that may further enhance freezing tolerance. In addition, freezing-induced mechanical stress triggers intracellular calcium signals because of plasma membrane damage, resulting in increased mechanical stress tolerance in Arabidopsis (Yamazaki et al., 2008). I believe that further studies should investigate calcium signaling to elucidate the mechanisms of enhanced freezing tolerance that occurs following 2P treatments after CA.

Light exposure during 2P treatments significantly enhances freezing tolerance

Real-time PCR results also showed that the regulation of gene expression during the second phase differed between light and dark conditions (**Fig. 3.3, 3.5**). These results suggest that a new freezing tolerance mechanism is responsible for the substantial enhancement of freezing tolerance when 2PFA treatments are applied under light conditions. Because these genes encode key factors involved in increasing freezing tolerance, these results were unexpected, but imply that other mechanisms are involved in the enhancement of freezing tolerance by 2P treatments. In our previous study on *cry1cry2* mutants subjected to CA for 1 day, the freezing tolerance of the mutants estimated by chlorophyll fluorescence imaging was lower than that of wild type, whereas the freezing tolerance of the mutants estimated by plant recovery or plasma membrane stability was higher than that of wild type (Imai et al., 2021). Interestingly, in the *cry1cry2* mutants, anthocyanin biosynthesis genes were down-regulated and *CBF* and *COR* were up-regulated during a 1-day CA treatment. These results indicate that in the early stages of CA, the regulation of gene expression by cryptochromes is more focused on protecting chloroplasts than on protecting the plasma membrane against freezing conditions. Thus, on the basis of the results of the present study, i.e., suppression of the increase in *CBF* and *COR* transcript levels during 2P treatments under light conditions, I hypothesize that plant cells shift gene expression to support stable photosynthesis at sub-zero temperatures, and this may lead to better processing of light energy and subsequent freezing tolerance.

Molecular mechanisms of 2PFA under light conditions

I conducted several experiments focusing on the molecular mechanisms of 2PFA plants under light conditions which showed the highest freezing tolerance. First, DCMU, which inhibits electron transfer from PSII to the plastoquinone (PQ) pool, was used to elucidate the relationship between 2PFA and the photosynthetic pathway. The results show that DCMU inhibited the enhancement of freezing tolerance after 2PFA treatment under light conditions,

whereas this did not occur after 2PFA treatment in the dark (**Fig. 3.6**). Therefore, this decrease in freezing tolerance caused by DCMU is light-dependent. Because DCMU has been reported to generate reactive oxygen species (ROS) at PSII under light conditions (Ozgur et al., 2015), it is possible that the decrease in freezing tolerance caused by DCMU could be due to ROS-induced damage. To estimate the effect of this side effect of DCMU, the F_v/F_m values were measured in plants treated with DCMU before 2PFA treatments under light and dark conditions. As shown in **Fig. 3.9** the F_v/F_m values were decreased by DCMU treatment before the 2PFA treatment, and the F_v/F_m values further decreased after the 2PFA treatment under both dark and light conditions. While there was a difference in the degree of reduction in the F_v/F_m values between dark and light conditions, the difference was negligible ($\Delta F_v/F_m = 0.06$). This implies that, in plants subjected to a 2PFA treatment under light conditions, PSII may be only slightly damaged by the ROS produced by the combination of light and DCMU. Taken together, these results suggest that electron transfer from PSII to the PQ pool is involved in the light-induced enhancement of freezing tolerance after 2PFA treatment.

Electron transfer from PSII to the PQ pool was shown to be involved in the up-regulation of *COR15A* expression during CA (Tominaga et al., 2021). Therefore, it is possible that this electron transfer may be similarly involved in the regulation of gene expression in 2PFA plants, although both *COR* and *CBF* were down-regulated during the 2PFA treatment under light conditions. It has been reported that the chloroplast sensor kinase CSK regulates the expression of chloroplast genes by sensing the redox state of the PQ pool (Puthiyaveetil et al., 2008). The role of CSK in 2PFA under light conditions was determined using a *CSK*-deficient mutant, *csk-1*, but there was no difference in freezing tolerance after 2PFA treatment between the wild-type and the mutant (**Fig. 3.10**). This result suggests that CSK is not involved in the regulation of freezing tolerance, at least in 2PFA plants.

The role of photoreceptors during the 2PFA process under light conditions

Next, the role of photoreceptors in 2PFA was studied using several photoreceptor mutants. The results obtained suggest that phototropin and phytochrome are involved in the enhancement of freezing tolerance. Phototropin was clearly involved in the enhancement of freezing tolerance by 2PFA treatments (F**ig. 3.8c,d**). It has been reported that phototropins act as a temperature sensor, sensing low temperatures and positioning chloroplasts in an optimal position for photosynthesis through movement along actin filaments (Fujii et al., 2017). Similarly in the 2PFA plants, it is possible that phototropins relocate chloroplasts to positions with moderate light levels under extracellular freezing conditions, thereby protecting them from excess light

energy and allowing for efficient photosynthesis (**Fig. 3.11**). In the *phyB* mutant, freezing tolerance was lower than that of wild type after both the 2PFA treatment and CA (**Fig. 3.e,f**). It is possible that the mutation had similar negative effects on CA and on freezing tolerance acquired under the 2PFA treatment. As mentioned above, I speculate that the mechanisms by which 2PFA enhances freezing tolerance differ from the CA mechanisms. It is possible that phytochrome PHYB also functions in 2PFA, and it enhances freezing tolerance *via* a different mechanism from that of CA. It has been reported that PHYB is involved in photoperiodic control (Jackson et al., 1996; Osugi et al., 2011). In addition, PHYB is involved in the enhancement of freezing tolerance by photoperiodic regulation of the CBF pathway (Lee and Thomashow, 2012). In this study, although the results of the experiments using *phyb* mutants were found to be involved in the 2PFA process under light conditions, the changes in photoperiod during the 2PFA process under light conditions had no effect on the freezing tolerance. These results indicate that the PHYB-mediated photoperiodic control is not functional at sub-zero temperatures, or not involved in the enhancement of freezing tolerance in 2PFA plants under light conditions. The mechanism of how PHYB is involved in the 2PFA process remains unknown.

Because *phot1phot2* and *phyB* plants subjected to 2PFA treatments showed decreased freezing tolerance (**Fig. 3.8**), it is expected that blue light and red-far-red light are involved in the 2PFA process. In fact, during the CA process, among various lights, blue light is the most effective wavelength for maintaining Fv/Fm (Imai et al., 2021). Thus, in further research, to determine which light qualities are involved in the 2PFA process, it will be interesting during the 2PFA treatment, plants are grown with different monochromatic lights including white, blue, red, far-red, and green.

In this chapter, as 2PFA treatment under light conditions, CA plants were incubated at -2℃ for 3 days under 12-h light/12-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1}. The light intensity of the experiment in this study was very low compared to the outdoors. Thus, differences in light intensity may affect freezing tolerance in 2PFA plants under light conditions. Furthermore, chloroplasts exhibit evasive movement when blue light intensity increases (Jarillo et al., 2001). If chloroplast stereotactic movement occurs under extracellular freezing conditions, it is likely that chloroplasts are being placed in optimal locations for photosynthesis depending on light levels. Thus, it will be interesting to investigate whether the difference of light intensity during 2PFA process under light conditions affects movement of chlorophyll and enhancement of freezing tolerance.

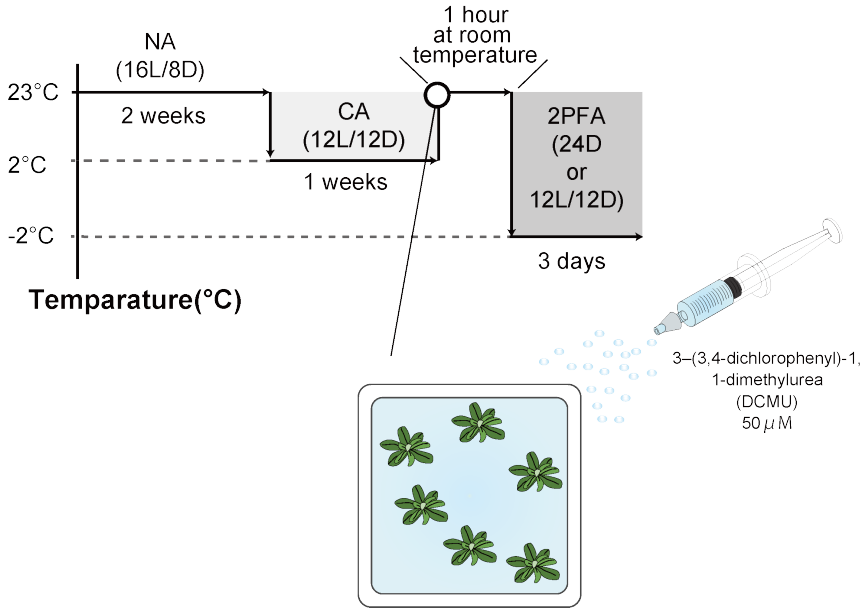


Fig. 3.1 Overview of experimental design using DCMU.
DCMU were sprayed to plants were cold-acclimated at 2°C (CA) for 7 days under a 12-h light/12-h dark photoperiod, and the plants were kept it at room temperature for 1 h. 2PFA treatment was conducted in the dark or under a 12-h light/12-h dark photoperiod.

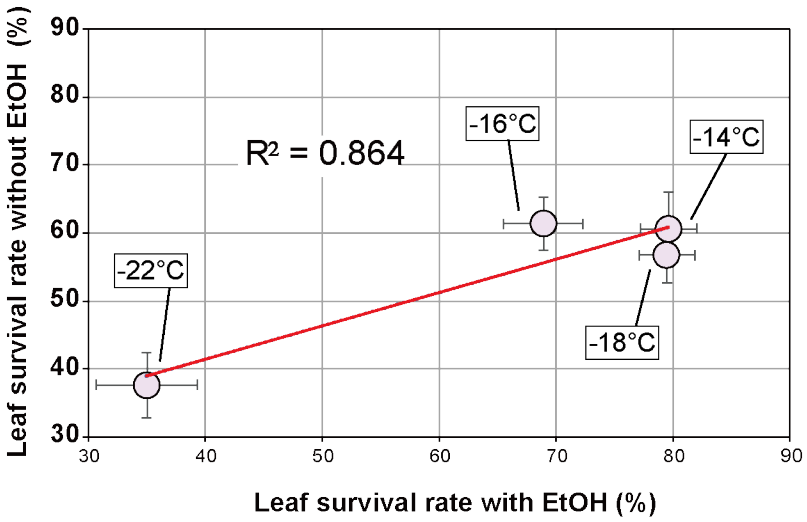


Fig. 3.2. Correlation between leaf survival rate with and without ethanol treatment. Plants cold-acclimated for 7 days were sprayed with ethanol, which is used for dissolving DCMU and then subjected to a 2PFA treatment (−2°C for 3 days). In addition, water was sprayed to plants cold-acclimated for 7 days instead of ethanol (without ethanol). Leaf survival rate was calculated by dividing the number of dead leaves by the number of total leaves at 3 days after the 2PFA treatment. Error bars indicate SE (n=22–36).

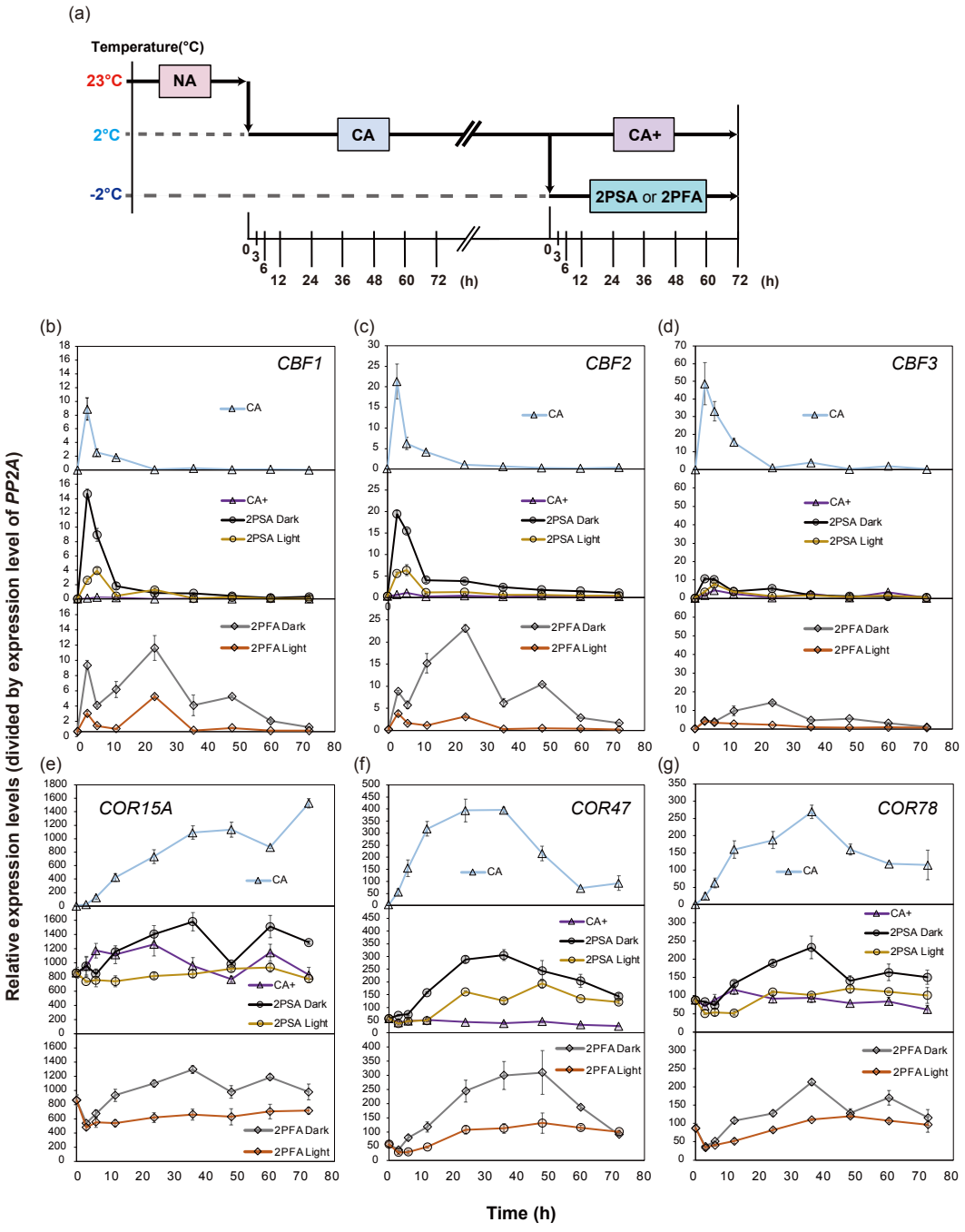


Fig. 3.3 Downregulation of *CBF* and *COR* genes in 2PFA plants under light conditions.
(a) Overview of experimental design. RNA was extracted from plants at 0, 3, 6, 12, 24, 36, 48, 60, and 72 h after transfer into a growth chamber at 2°C from 23°C (CA samples). In addition, RNA samples were extracted at 0, 3, 6, 12, 24, 36, 48, 60, and 72 h after transferring CA7 plants (cold-acclimated for 7 days at 2°C) into a freezing chamber at −2°C with or without ice crystal (2PFA and 2PSA, respectively), or after keeping CA plants for a further 7 days at 2°C (CA+). Relative transcript levels of (b) *CBF1*, (c) *CBF2*, (d) *CBF3*, (e) *COR15A*, (f) *COR47*, and (g) *COR78* (n=3) were determined after normalization against that of *PP2A*.

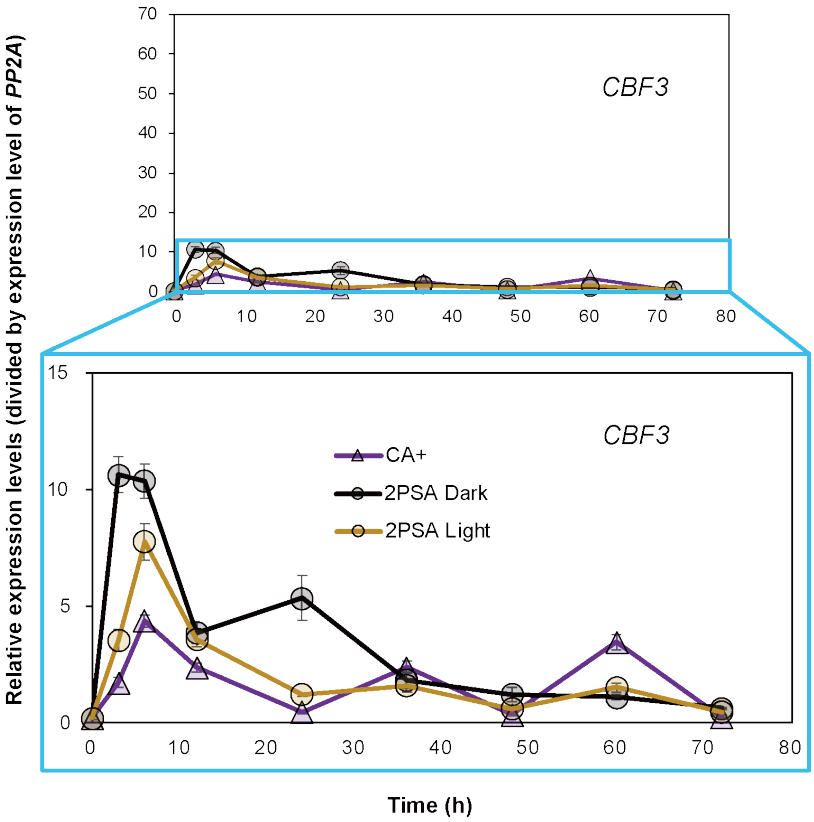


Fig. 3.4. Enlarged graph of Fig. 3.3d.
RNA was extracted from plants at 0, 3, 6, 12, 24, 36, 48, 60, and 72 h after transfer into a growth chamber at 2°C from 23°C (CA samples). In addition, RNA samples were extracted at 0, 3, 6, 12, 24, 36, 48, 60, and 72 h after transferring CA7 plants (cold-acclimated for 7 days at 2°C) into a freezing chamber at −2°C without ice crystal (2PSA), or after keeping CA plants for a further 7 days at 2°C (CA+). Relative transcript levels of *CBF3* (*n*=3) were determined after normalization against that of *PP2A*.

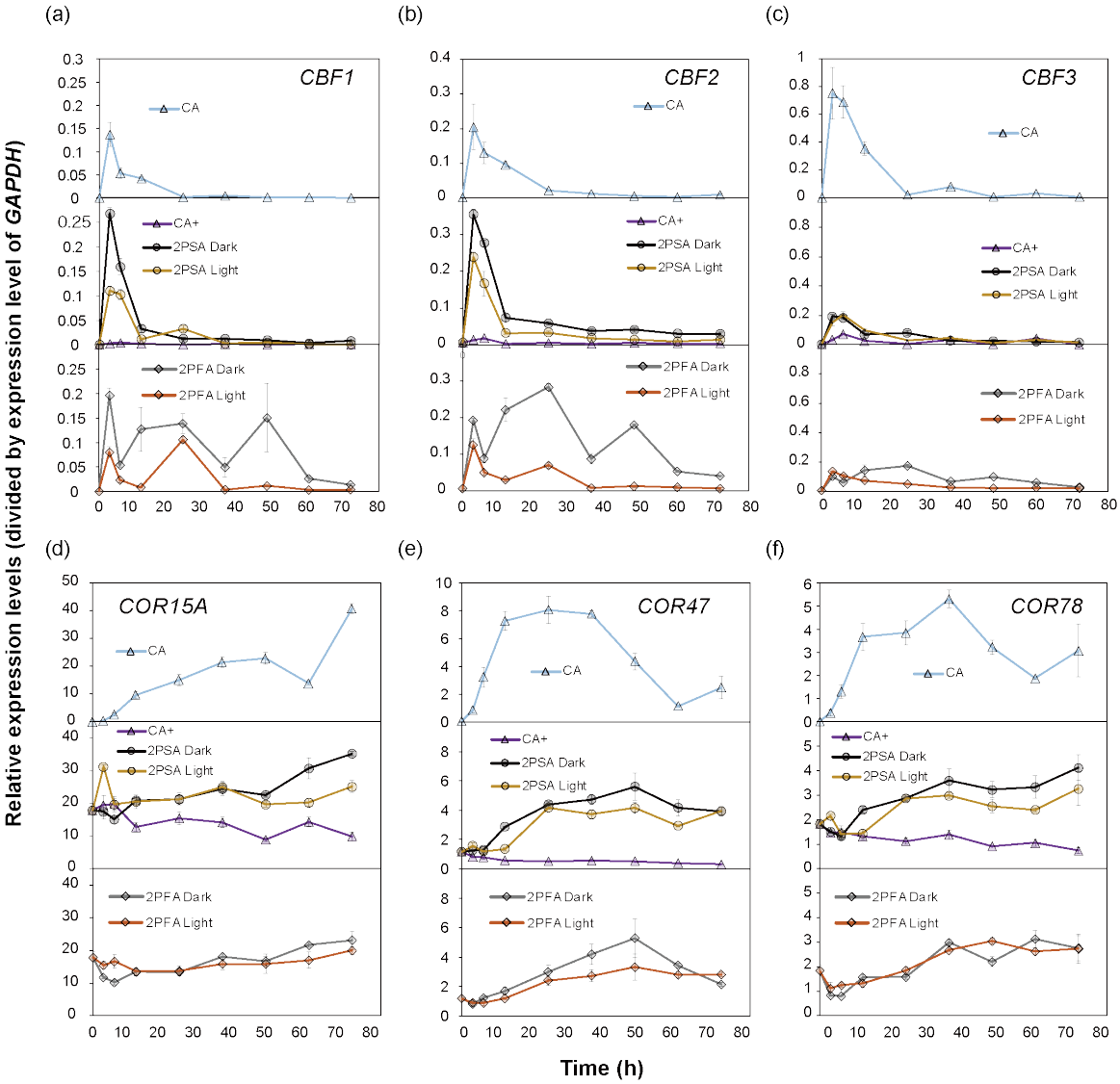


Fig. 3.5. Downregulation of *CBF* and *COR* genes in 2PFA plants under light conditions. Transcript levels of *CBF* and *COR* genes in plants at 0, 3, 6, 12, 24, 36, 48, 60, and 72 h after transferring to a growth chamber at 2°C from 23°C (CA samples), and in cold-, freezing- and supercooling-acclimated plants (CA+, 2PFA, and 2PSA, respectively) after 0, 3, 6, 12, 24, 36, 48, 60, and 72 h. Relative transcript levels of (a) *CBF1*, (b) *CBF2*, (c) *CBF3*, (d) *COR15A*, (e) *COR47*, (f) *COR78* (n=3). Gene transcript levels were determined after normalization against that of GAPDH.

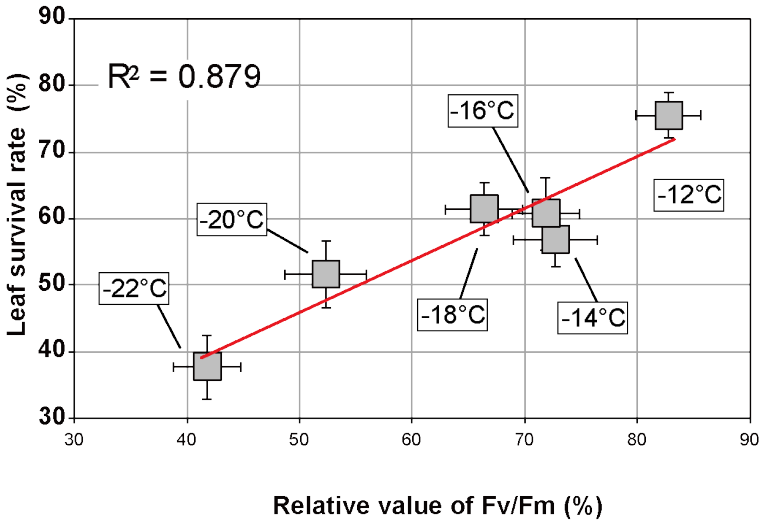


Fig. 3.6. Correlation between survival rate calculated from F_v/F_m and survival rate calculated from the number of surviving leaves.
Plants cold-acclimated for 7 days were subjected to a 2PFA treatment (−2°C for 3 days with ice crystal). To calculate survival rates, F_v/F_m was measured 1 day after the freezing tolerance test, and the number of surviving leaves was counted 3 days after the freezing tolerance test.

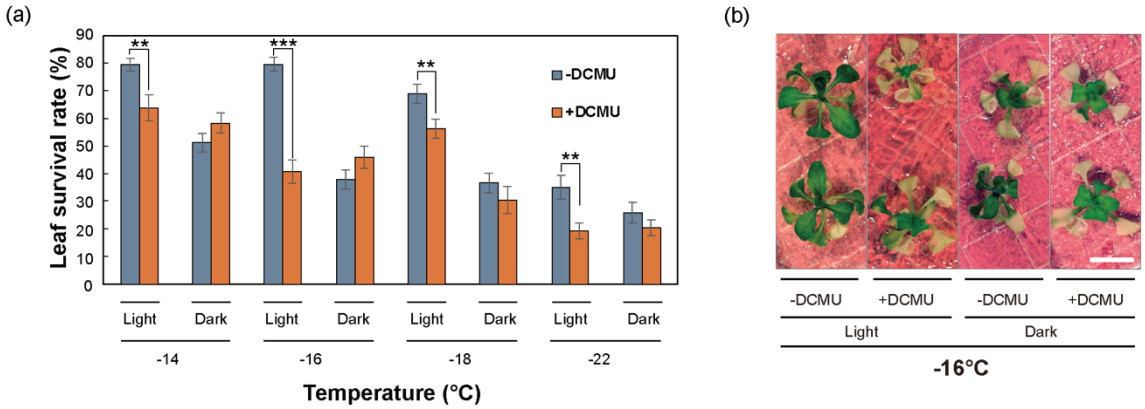


Fig. 3.7. DCMU treatment decreases the extent of the acquisition of freezing tolerance in 2PFA plants under light conditions.

(a) Leaf survival rate of 2PFA plants treated with DCMU. CA7 plants (cold-acclimated for 7 days at 2°C) were treated with 50 μM DCMU and kept at room temperature for 1 h before 2PFA treatment (−2°C with ice for 3 days under a 12-h light/12-h dark photoperiod or in the dark). Survival rate at −14°C, −16°C −18°C and −22°C was calculated by dividing the number of dead leaves by the total number of leaves after thawing for 3 days at 23°C. Error bars indicate SE (n=22–36). Asterisks at each temperature show significant difference (Student's t-test, ** p < 0.01, *** p < 0.001). (b) Representative pictures of plants frozen at −16°C after 2PFA for 3 days under 0-h photoperiod or 12-h light/12-h dark photoperiod. Pictures were taken 3 days after thawing at 23°C. Scale bar = 10 mm.

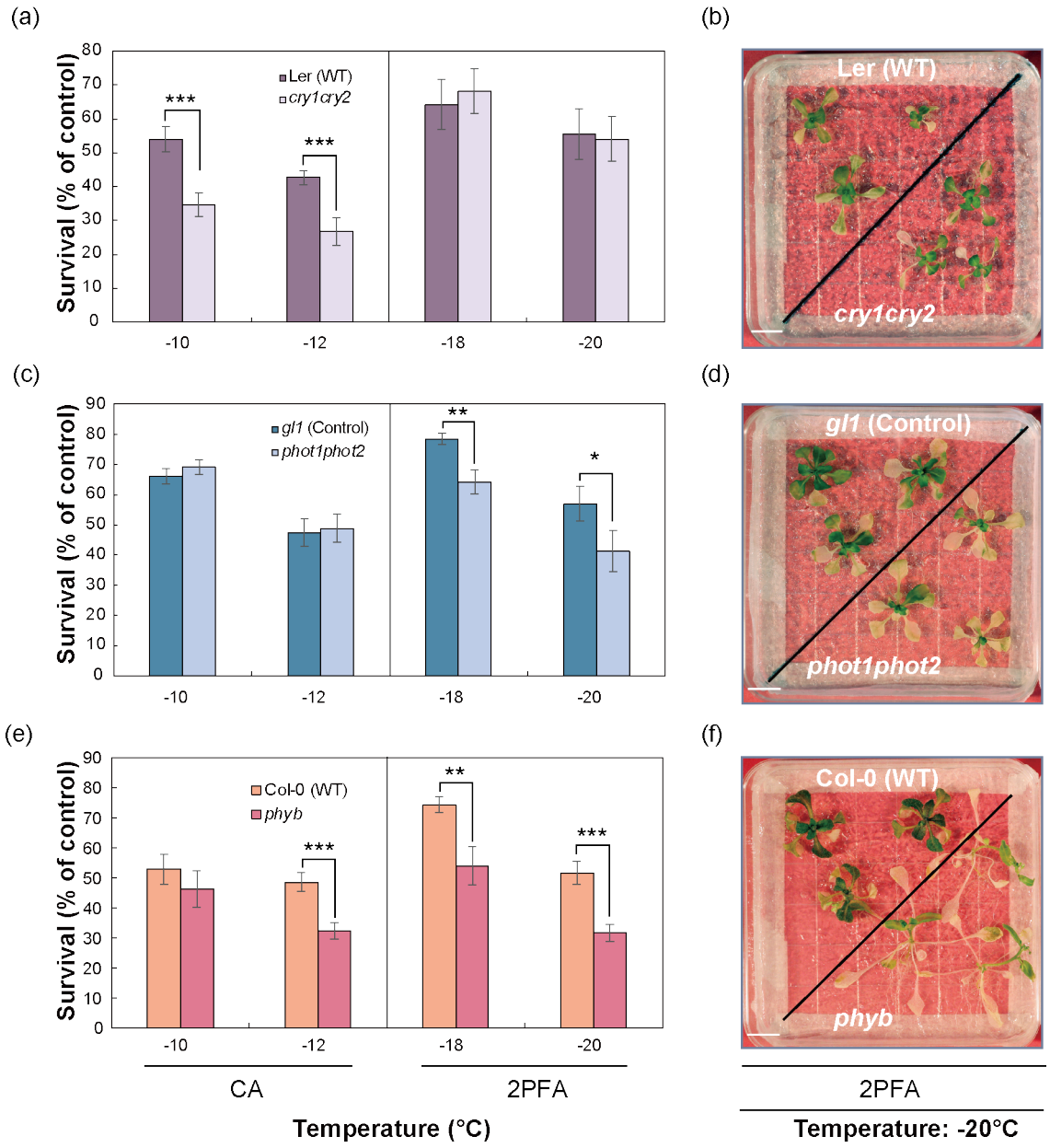


Fig. 3.8. Photoreceptors PHOTOTROPIN and PHYB are involved in the 2PFA process. Freezing tolerance of photoreceptor mutants (a) *cry1cry2*, (c) *phot1phot2* and (e) *phyb*. Each mutant was incubated at 2°C for 7 days under a 12-h light/12-h dark photoperiod to induce CA and then subjected to a 2PFA treatment (−2°C for 3 days with ice under a 12-h light/12-h dark photoperiod). Error bars indicate SE (n=8–18). Different letters at each temperature show significant difference (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Representative pictures of (b) *cry1cry2*, (d) *phot1phot2* and (f) *phyb* plants frozen at −20°C after freezing acclimation. Pictures were taken 3 days after the freezing test. Scale bars = 10 mm.

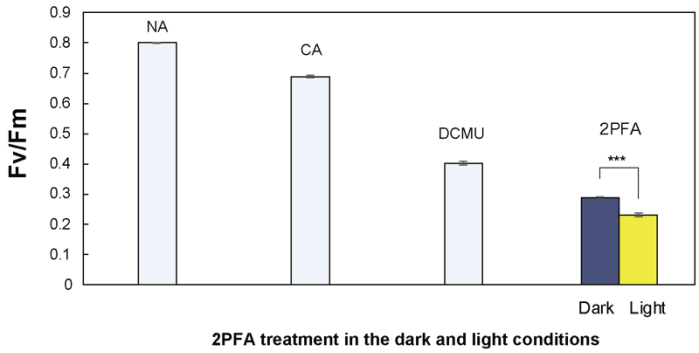


Fig. 3.9. F_V/F_m of plants during 2PFA in the dark and light conditions after CA.
F_V/F_m was measured in the plants after 2PFA at −2°C in the dark or light conditions for 3 days, and then the plants were thawed at 23°C for 3 days. F_V/F_m of the same samples was measured when the plants were grown at 23°C for 2 weeks (NA), at 2°C for 1 week (CA), and with DCMU after the CA treatment. Error bars indicate SE (n=12–36). Asterisks at each temperature show a significant difference (Student's t-test, *** $p < 0.001$).

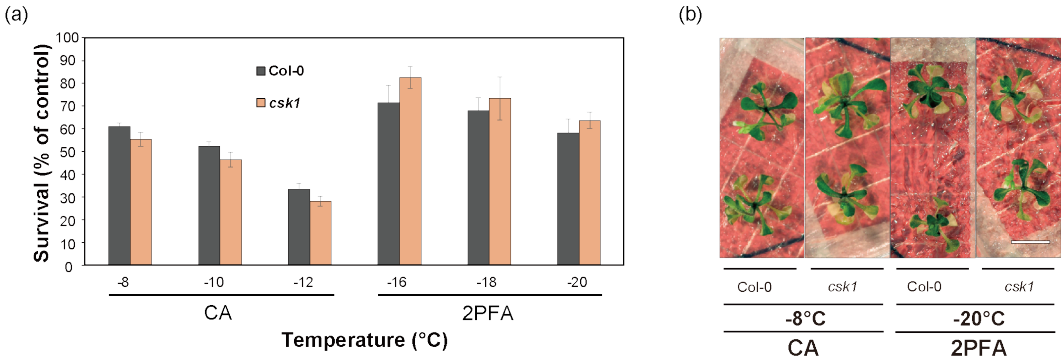


Fig. 3.10. Chloroplast sensor kinase CSK is not involved in the 2PFA process.
(a) Freezing tolerance of *csk1* mutant. Plants were incubated at 2°C for 7 days under a 12-h light/12-h dark photoperiod as the CA treatment and then subjected to a 2PFA treatment (−2°C for 3 days under the same photoperiod). Error bars indicate SE (n=15–18). (b) Representative pictures of wild-type (Col-0) and *csk1* plants after freezing at −8°C (CA plants) and −20°C (3d-2PFA plants). Pictures were taken 3 days after the freezing test. Scale bar = 10 mm.

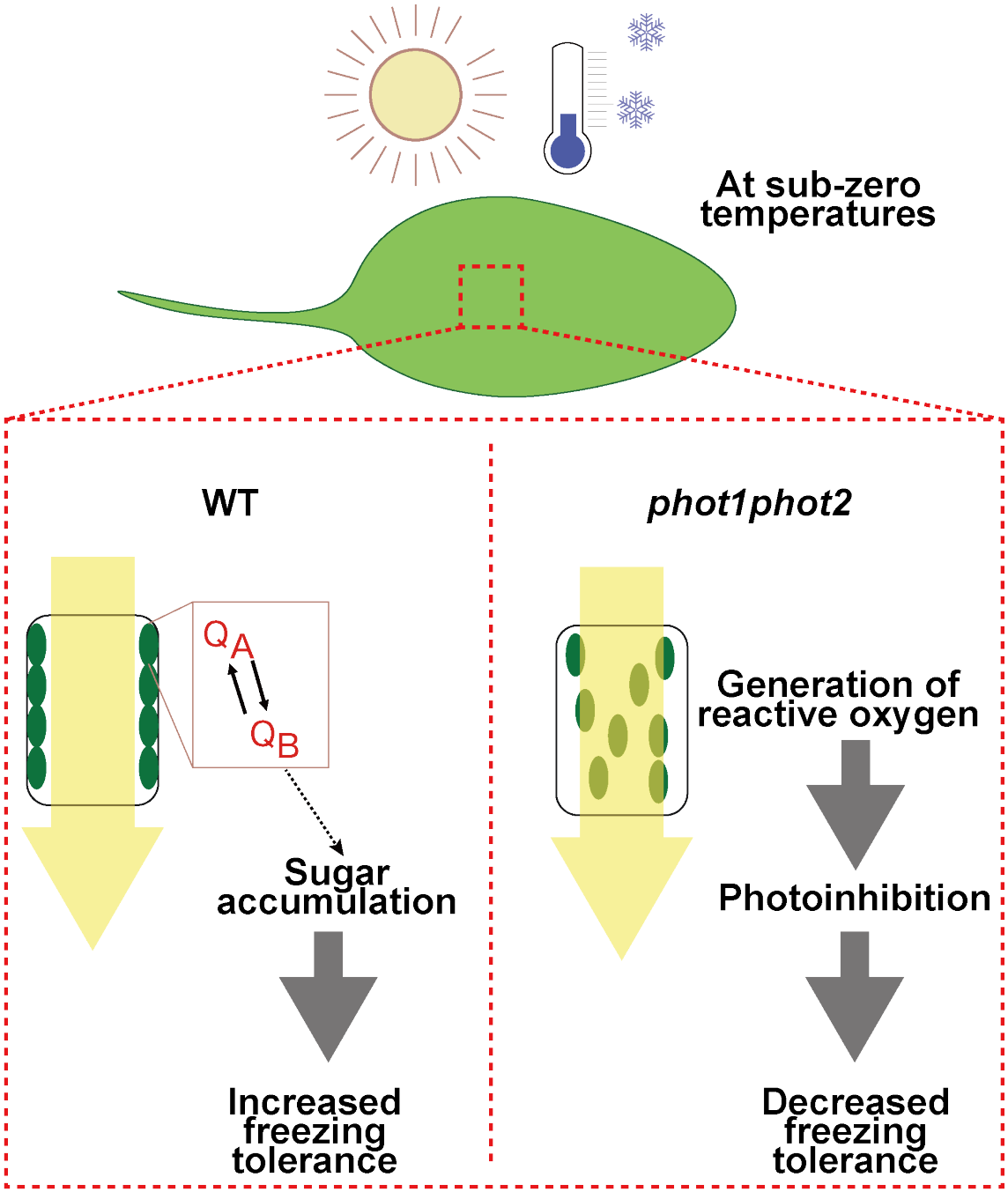


Fig. 3.11. Proposed mode of acquiring freezing tolerance during 2PFA under light conditions.

At sub-zero temperatures, chloroplasts are relocated via phototropins for accepting moderate light level. The signals electron transfer from Q_A to Q_B in photosystem II result in accumulating sugars and increasing freezing tolerance. In *phot1phot2*, chloroplasts are not relocated via phototropins, reactive oxygen was produced and causes photoinhibition.

Table 3.1. Primer list used in this study

PP2A_RT_FW	TAACGTGGCCAAAATGATGC
PP2A_RT_RV	GTTCTCCACAACCGCTTGGT
GAPDH_RT_FW	TTGGTGACAACAGGTCAAGCA
GAPDH_RT_RV	AAACTTGTCGCTCAATGCAATC
CBF1_RT_FW	CCGCCGTCTGTTCAATGGAATCAT
CBF1_RT_RV	TCCAAAGCGACACGTCACCATCTC
CBF2_RT_FW	CGGAATCAACCTGTGCCAAGGAAA
CBF2_RT_RV	AGACCATGAGCATCCGTCGTCATA
CBF3_RT_FW	TTCCGTCCGTACAGTGGAAT
CBF3_RT_RV	AACTCCATAACGATACGTCGTC
COR15A_RT_FW	AACGAGGCCACAAAGAAAGC
COR15A_RT_RV	CAGCTTCTTTACCCAATGTATCTGC
COR47_RT_FW	ACAAGCCTAGTGTCATCGAAAAGC
COR47_RT_RV	TCTTCATCGCTCGAAGAGGAAG
COR78_RT_FW	GCACCAGGCGTAACAGCT
COR78_RT_RV	AAACACCTTTGTCCCTGGTGG

Chapter4

Molecular and genetic approaches to increasing soluble sugars during second phase freezing acclimation under light conditions.

Abstract

Overwintering plants survive at sub-zero temperatures through two phases of increasing freezing tolerance. The first phase process, which is known as cold acclimation (CA), works at low temperatures above zero and the second phase (2P) process works at sub-zero temperatures. There are four patterns in the 2P process, in which plants are exposed to sub-zero temperatures without ice 1) in the dark conditions and 2) in the light conditions, and in which plants are exposed to sub-zero temperatures with ice 3) in the dark conditions and 4) in the light conditions. Here, I focused on the CA plants treated with freezing under light conditions that acquire maximum freezing tolerance in the four patterns in the 2P process. Transcriptome analysis by RNA sequencing was used to examine the genetic control of plants that experienced freezing under light conditions. These results suggested that plants treated with ice during the 2P process under light conditions increased their freezing tolerance by coping with the osmotic stress associated with dehydration due to ice treatment rather than the sub-zero temperature stress. Consistently, the osmotic levels and soluble sugar contents including sucrose and galactinol were highly increased in the plants of 2P treatment with ice under light conditions compared with the other 2P treatment. These results show that accumulation of soluble sugars via regulation of gene expression involved in synthetic enzymes of sugars increases freezing tolerance in the plants of 2P treatment with ice under light conditions

Introduction

Plants that are grown in temperate and frigid zones are exposed to sub-zero temperatures in winter. At sub-zero temperatures, ice crystals are formed in the intercellular spaces of many plants. Water moves toward the ice crystals, which have a lower chemical potential, from inside the cells, which have a higher chemical potential. Thus, at sub-zero temperatures, plants are subjected to multiple stresses including not only temperature but also cellular dehydration stress (Hincha and Zuther. 2014).

As a first phase responds toward overwintering, plants increase their freezing tolerance by sensing the drop in temperatures in the fall (cold acclimation, CA). This phenomenon further proceeds by exposure to lower temperatures, but not freezing temperatures (Tominaga et al., 2021). At mid-winter, if cold-acclimated plants experience lower temperatures below 0°C, these plants enhance furthermore freezing tolerance through a second phase response. This phenomenon is known as second-phase cold hardening (2PH) or sub-zero acclimation, and, is reported in Arabidopsis, wheat, and oat (Herman et al., 2006; Livingston and Henson, 1998; Livingston et al., 2007; Le et al., 2008, 2015; Takahashi et al., 2019, 2021). A recent study showed that there are four patterns in the 2PH process, in which plants are exposed to sub-zero temperatures with (Second Phase Freezing Acclimation; 2PFA) or without ice (Second Phase Supercooling Acclimation; 2PSA) under dark or light conditions (Sugita et al., 2024). Surprisingly, 2PFA plants under light conditions exhibited the highest freezing tolerance in the 2PH patterns, showing an LT_{50} value roughly below −20°C in Arabidopsis (Sugita et al., 2024).

During the CA process, plants increase the expression of genes encoding C-repeat binding factors/dehydration responsive element binding factors (CBFs/DREBs, a type of transcription factor) transiently. Subsequently, their downstream target genes, cold-regulated (COR) genes enhance freezing tolerance (Thomashow, 1999, 2001). However, in a previous chapter (Chapter 3), I revealed that the expression of the CBF regulon is not regulated in the 2PFA plants under light conditions. It has also been reported that during the CA process, sugars were accumulated as compatible solutes to prevent dehydration inside the cells and the resultant accumulation of toxic substances (Steponkus, 1984; Wanner and Junttila, 1999). Furthermore, in protoplasts isolated from rye leaves, freeze-induced dehydration affects lamellar-to-hexagonalII phase transitions in biological membranes, which is the cause of injury (Gordon-Kamm and Steponkus, 1984). Sugars also may act as a direct buffer to prevent the fusion of cellular membranes induced by dehydration (Steponkus, 1984). There is a positively correlation between accumulation of sugar and freezing tolerance (Yano et al., 2005). Thus, the accumulation of sugar during cold acclimation important role in enhancing their freezing

tolerance. In Arabidopsis, Sucrose, Glucose, Fructose, and Raffinose were accumulated during the CA process (Gilmour et al., 2000), while Sucrose, Glucose, and Fructose were accumulated in Cabbage seeds (Sasaki et al., 1996).

The changes in sugar content during the CA process were regulated by the expression of the transcriptome that encodes proteins involved in sugar metabolism. Transcripts that encode Sucrose-phosphate-synthase (SPS), and Sucrose synthase (SuSy) were up-regulated in response to low temperatures (Fowler and Thomashow, 2002, Gilmour et al., 2000). Transcripts for genes that encode galactinol synthases such as *galactinol synthase 1* (*GolS1*), *3* (*GolS3*), and *4* (*GolS4*), which are known as enzymes that catalyze the first committed step in the synthesis of raffinose, were shown to be accumulated in response to low temperatures (Fowler and Thomashow, 2002). Especially, *GolS3* is a member of the CBF regulon, and the expression is induced by low temperatures and overexpression of CBF3/DREB1a (Fowler and Thomashow 2002, Taji et al., 2002). Sugar is accumulated during not only cold acclimation but also various stresses including drought and salt stresses. In response to salt and drought stress, raffinose, and galactinol were accumulated through regulation of *Gosl1* and *Gols2* expression in Arabidopsis (Taji et al., 2002). Thus, sugar accumulation is one of the important intracellular changes in the stress response.

A previous study showed that freezing tolerance is dramatically enhanced in 2PFA plants under light conditions via some photoreceptors and chloroplast electron transfer signals (Sugita et al., 2024). Conversely, the expression of *CBFs* and *CORs*, which are the executioner units of freezing tolerance acquisition, were not upregulated (Sugita et al., 2024), suggesting that freezing tolerance is enhanced in 2PFA plants under light conditions by another pathway rather than CBF regulon, but the specific molecular mechanism is still unclear.

For this study, RNA-seq analysis was conducted to investigate how the 2PFA plants under light conditions enhance dramatically freezing tolerance. Furthermore, I investigated the accumulation of sugar and its effect on freezing tolerance in 2PFA plants under light conditions. These results suggested that the levels of Sucrose and Galactinol were increased in 2PFA plants under light conditions through regulation of gene expression of the coding sugar synthetase for enhancing their freezing tolerance.

Materials and Methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were surface-sterilized with 20% (v/v) bleach and 1% (v/v) sodium dodecyl sulfate and placed on Hoagland's medium containing 1%

(w/v) sucrose and 1% (w/v) agar in square (96 mm ×96 mm) Petri plates. Two days after stratification at 4°C in the dark, plants were grown in a controlled-environment chamber at 23°C for 2 weeks under a 16-h light/8-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps. These plants were designated as non-acclimated (NA) plants. The NA plants were transferred into a growth chamber at 2°C under a 12-h light/12-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps and grown for 7 days as the CA treatment. The CA plants were transferred into a freezing chamber that could maintain stable sub-zero temperatures even under light conditions by adding circulation fans and LED lights to a ready-made freezing chamber (IN602N, Yamato Scientific, Tokyo, Japan) as described in a previous study (Sugita et al., 2024). Using this chamber, after keeping the plates at −2°C for 10 min in the freezing chamber, ice was induced to form by momentarily touching the agar with liquid nitrogen, and then the plants were incubated for 3 days at −2°C either in the dark or under a 12-h light/12-h dark with light supplied at 80 μmol m^{-2} s^{-1} as the second-phase freezing acclimation (2PFA) treatment. As the second-phase supercooling (2PSA) treatment, CA plants were transferred into the freezing chamber at −2°C in the same way as in the 2PFA treatment, but the ice was not induced to form on the agar. To compare the different temperature treatments, plants cold-acclimated for 7 days at 2°C were subjected to 2°C (CA+) as one of the controls.

Chlorophyll fluorescence measurement

For measuring the chlorophyll fluorescence of plants under freezing conditions, Arabidopsis seedling was placed on Hoagland medium containing 1%(w/v) sucrose and 1%(w/v) agar in a Petri dish (diameter: 40 mm). After stratification, the plates were grown at 23℃ for 2℃ weeks and placed at 4℃ for inducing cold acclamation. Then, plants were placed in the growth chamber at −2℃, and ice crystals was inoculated to agar by touching the plates with liquid nitrogen after 10 minutes incubation. The plants were grown at −2℃ under dark conditions or 12-h photoperiod at 80 μmol m^{-2} s^{-1}.

To measure PAM fluorescence in the frozen state, I used a low-temperature water tank (Tokyo Rikakikai, Tokyo, Japan) to circulate antifreeze (EtOH) through a jacked beaker (**Fig.4.1**). Plates after incubation 0, 24, 48, and 72h at −2℃ subjected to CA treatment for 7 days was placed in the jacketed beaker and chlorophyll fluorescence was measured while the agar was maintained at freezing temperature. Insulating sheets were wrapped around the jacked beaker and hose connecting the low-temperature bath to stabilize the temperature.

RNA extraction

Plants were grown in a controlled-environment chamber for 2 weeks at 23°C under a 16-h light/8-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps. The plants were transferred to a growth chamber at 2°C with a 12-h light/12-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps, and samples were collected at 0, 3, 24, and 48 h (designated as CA). After 7 days, the CA plants were then subjected to CA+ treatment (2°C), 2PFA treatment (−2°C with ice), or 2PSA treatment (−2°C without ice), and these plants were collected at 0, 3, 24, and 48 h. Then, RNA was extracted from the aerial parts of each sample using an ISOSPIN Plant RNA kit (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. An overview of the experimental design of samples for RNA-Seq analysis was designed in **Fig.4.3a**. The plants under various treatments at 3 h were used for detection genes that are expressed in response to the treatments such as sub-zero temperature, ice crystals, and light. Conversely, the plants under various treatments at 24 and 48 h were used for investigation of the key genes for increasing freezing tolerance.

RNA-Seq analyses

As RNA sequencing (RNA-seq) library preparation method, lasy-Seq was used. Reverse transcription (RT) of total RNA was performed by oligo-dt primers including index sequencing. This method does not require mRNA as in poly-A selection, and a unique index sequence was added to each sample; see a paper for detailed requirements for RNA-Seq library preparation (Kamitani et al., 2019). A HiSeq 2500 was used for sequencing. To convert raw data obtained by sequencing into count data (data that can be used for analysis), dynacomkobe/biodocker_rnaseq_pipeline: ver.0.5.0 for quality trimming using Trimmomatic such as removing adapter sequences and low-quality bases for quantification by RSEM (version 1.3.3) and Bowtie (version 1.2.3) in docker were used. Through these steps, the data obtained from sequencing were used to trim reads, map them to genome sequences, and count the number of mapped reads, all in a Docker environment (version 4.13.0 (89412)). The Trimmed Mean of M values (TMM) method implemented in edgeR was used to normalize the RNA-seq data to perform the likelihood ratio test (LRT). Furthermore, the *k*-means method, Gene ontology (GO) analysis, and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway gene set enrichment analysis were performed with R (version 4.3.2) software using Fragments Per Kilobase of exon per Million mapped fragments (FPKM), which is the expression level normalization method.

qRT-PCR analyses

Quantitative real-time RT-PCR analyses were performed using the Thermal Cycler Dice™ Real-Time System (Takara, Otsu, Japan) with SYBR Premix Ex Taq II (Takara), according to the manufacturer's protocol. The relative transcript levels of genes were calculated from real-time PCR data using the $2^{-\Delta\Delta Ct}$ method with Thermal Cycler Dice™ Real-Time System Single Software (Livak and Schmittgen, 2001). The reference gene was *PP2A*. All primers used for qRT-PCR were blasted using Primer-BLAST at the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov). For each time treatment of plants, see "RNA extraction and qRT-PCR analyses" in Chapter 3.

Measurement of osmolality

Plants were grown in a controlled-environment chamber for 2 weeks at 23°C under a 16-h light/8-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps. The plants were then transferred to a growth chamber at 2°C with a 12-h light/12-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps, and samples were collected at 0, 24, 48, and 72 h (designated as CA). After 7 days, the CA plants were subjected to CA+ treatment (2°C), 2PFA treatment (−2°C with ice), or 2PSA treatment (−2°C without ice), and these plants were collected at 0, 24, 48 and 72 h. Each plant was frozen with liquid nitrogen and ground with a pestle. After centrifugation at 15,000 rpm at 4°C for 5 min, the supernatant was collected and the osmolality was measured with Vapro® Vapor Pressure Osmometer (Xylem, New York, United States).

Measurement of sugar content

The aerial parts of each sample (approximately 0.1 g) were frozen with liquid nitrogen. Plant materials were added to the internal standard (maltopentaose) and homogenized with 80% (v/v) ethanol using a pestle. The homogenate was boiled for 30 min on a heat block at 80°C and then centrifuged for 5 min. The residues were re-homogenized with 80% (v/v) ethanol and centrifuged as above. These extraction steps were repeated three times. The supernatants were dried on the heat block at 80°C. Leaf pigments were removed by chloroform, methanol, and water (chloroform:methanol: water, 1:1:0.9, v/v/v). The extracts were dried on the heat block at 80°C and dissolved in 0.5 mL of distilled water that was filtered through a 0.22 μm pore filter membrane. Sucrose, raffinose, galactinol, glucose, and fructose were analyzed by high-performance liquid chromatography (HPLC) using SUGAR SC1011 (Resonac, Tokyo, Japan) and SUGAR KS-801 (Resonac) columns at 30°C with a refraction index detector. Samples (5μl)

were injected and eluted with ultrapure water at a flow rate of 0.2 ml min^{-1}. Each sugar was quantified from the calibration curve using an external standard method and corrected as a ratio of the area relative to that of the internal standard.

Treatment with a photosynthetic electron transport inhibitor

CA seedlings were misted twice with 0.5% (v/v) ethanol with or without 50 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The experiment was performed at 2℃ under green-filtered low light. After spraying of DCMU, the plants were kept at 2℃ for 1 h under dark conditions and then were transferred into the growth chamber at −2°C with ice crystal for 3 days under dark conditions or a 12-h light/12-h dark photoperiod with light supplied at 80 μmol m^{-2} s^{-1} by fluorescent lamps. Then, the osmotic pressure and sugar contents of the aerial parts of the plants were measured.

Results

Choloroplasic electron transfer during the 2PFA process under light conditions

Previous studies in our group showed that the cold-acclimated plants that were treated with freezing (2PFA) on the agar at –2°C under light conditions dramatically enhanced the freezing tolerance (Sugita et al., 2024). In addition, the freezing tolerance of the 2PFA plants under light conditions was decreased by 3- (3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), suggesting that the photosynthetic electron transport pathway is involved in the increased freezing tolerance during the 2PFA process. Thus, I hypothesized that photosynthesis is involved in the process of 2PFA plants under light conditions to strengthen freezing tolerance. To gain insight into the hypothesis, I constructed an experimental system using a chlorophyll fluorescence spectrometer that could measure photosynthetic activity in intact (non-destructive) frozen plants (**Fig. 4.1**). Firstly, I analyzed the effect of cold or freezing temperature on PSII photochemical efficiency by the chlorophyll fluorescence *Fv/Fm* ratio (**Fig. 4.2.a**). CA Arabidopsis plants were subjected to 24, and 48 h of 2PFA treatment at –2°C under the dark conditions or light conditions. While *Fv/Fm* value of NA plants and 0 h 2PFA plants showed approximately 0.8 and 0.7, respectively, *Fv/Fm* value of 24 and 48 h 2PFA plants showed decreased value, which decreased, to ~0.5. There was no significant difference in the value between light and dark conditions in 2PFA plants. The results showing the decreased *Fv/Fm* values in 2PFA plants suggested that the photosynthetic activity in electron transport system in chloroplast is reduced. Next, I observed photochemical quenching (*qP*) which is a parameter indicating the percentage of opening

photosystem II (**Fig. 4.2.b**). Compared to NA plants and 0 h 2PFA plants (2℃), *qP* values of 24 and 48 h 2PFA plants were decreased.

These results suggested that the electron transport system in chloroplast is still active during the 2PFA process, even though it was suppressed.

Plants respond to dehydration stress at a late stage during the 2PFA process under light conditions.

Next, large-scale gene expression analysis using RNA sequencing (RNA-seq) was performed to understand the molecular mechanism at the transcription levels how 2PFA plants under light conditions enhance their freezing tolerance. In this study, cold-acclimated plants were treated with freezing (2PFA) or without freezing (2PSA) on the agar at −2°C either in the dark or under a 12-h light/12-h dark photoperiod (**Fig. 4.3.a**). Because 2P treatments effects other than temperature and freezing could affect the given results, one of the controls was then subjected to a further CA process (CA+) under same time conditions as the 2P treatment at 2°C.

Firstly, a cluster analysis was performed to analyze gene expression trends in the plants under various treatments (CA, CA+, 2PSA, and 2PFA). The genes were classified into 12 clusters (A-L) by the *k*-means method (**Fig. 4.3.b**). In clusters, E and F, the gene expression was up-regulated only in the CA plants. In clusters, B and C, the transcription levels were up-regulated in the CA plants and CA+ plants, CA plants, and 2PFA plants in the dark conditions, respectively. In clusters, A, H, and K, the transcription levels were up-regulated only in the CA+ plants, 2PSA plants under light conditions, and 2PFA plants in the dark conditions. In the cluster G, the gene expression of CA, 2PSA plants under light conditions, or 2PFA plants either in the dark conditions and light conditions was up-regulated, and that of CA+ and 2PSA in the dark conditions was down-regulated. In the gene belonging to the cluster L, while the expression of 2PSA plants under light conditions, 2PFA plants either in the dark conditions and light conditions were up-regulated, the gene expression of CA and CA+ were down-regulated. In the cluster J, gene expression of the 2PFA or 2PSA plants in the dark conditions was up-regulated, and that of the CA, CA+, 2PFA, and 2PSA plants under light conditions, was down-regulated. Interestingly, genes classified in cluster I (N=197) showed elevated expression in the 2PFA under light conditions compared with other groups (**Fig. 4.3.c**).

To examine the specific expression variations of genes belonging to cluster I in detail, a heat map for the time series analysis from 3 to 48 h in CA, CA+, 2PSA, and 2PFA groups was conducted (**Fig. 4.4.a**). The results showed that in the 2PFA plants under light conditions, gene expression was up-regulated at 3 h, 24 h, and 48 h. Furthermore, their expression patterns varied

at each time. Therefore, I again performed the *k*-means method to classify the expression patterns by time. The 197 genes of 2PFA plants under light conditions at 3, 24, and 48 h were further classified into 4 clusters (1-4) (**Fig. 4.4.b**). The transcript levels of genes in the cluster I-1 (n=53) rapidly increased within 3 h and then decreased between 3 h and 48 h. In the cluster I-2 (*n*=35), the genes showed increases in their transcript levels between 0 h and 24 h, and decreases at 48 h. The genes in the cluster I-3 (n=47) were slowly up-regulated within 3 h, then rapidly up-regulated between 3 h and 24 h, then slowly down-regulated between 24 h and 48 h. In the cluster I-4 (*n*=62), the genes were up-regulated at 48 h.

To provide biological attributes of the 197 genes, gene ontology (GO) enrichment analysis was conducted (**Fig. 4.4.c**). The GO enrichment analysis classified the genes into three GO categories: 1) biological process (BP), 2) molecular functions (MF), and 3) cellular component (CC). The results showed that, in the BP category, GO terms associated with defense such as “Defense response”, “Response to wounding” and “Defense response to bacterium” were enriched in all the clusters. In addition, “Response to water deprivation” was the top enriched GO term in the cluster I-4. In the MF category, “Molecular_function” and “Protein binding” were the top enriched GO terms in the cluster I-1, I-2, and I-3, but “DNA-binding transcription factor activity” was enriched in the cluster I-4 instead of “Molecular_function”. In the CC category, “Nucleus”, and “Chloroplast” were highly enriched GO terms in all clusters. These analyses of the genes that were upregulated in the 2PFA plants under light conditions showed that the function and location where the genes products carry out a molecular function differ depending on the time series variation. In particular, to detect the genes that their gene products carry out a molecular function associated with photosynthesis, the genes enriched “Chloroplast” were represented in **Table 4.1**. In the genes enriched “Chloroplast”, *Glutathione S-transferase family protein* (AT1G19550) in the cluster I-2, *Cysteine-rich RLK RECEPTOR-like protein kinase 38* (AT4G04510) and *WRKY family transcription factor* (AT4G23810) in the cluster I-3, were highly upregulated. However, I did not detect genes directly involved in the function of photosynthesis such as encoding chromoprotein and light-harvesting complex. Furthermore, since it was expected that 2PFA plants under light conditions were exposed to water deprivation induced by extracellular freezing and drought stress, the genes enriched “Response to water deprivation” in the BP category of the cluster I-4 were listed in **Table 4.2**. The result showed that *LEA domain-containing protein 13* (AT2G18340) related to drought stress (López-Cordova et al., 2021) was upregulated at a late stage during the 2PFA process. These results suggested that the 2PFA

plants under light conditions regulated the gene involved in water stress rather than regulating the genes related to the function of photosynthesis.

KEGG pathway analysis showed that genes related to sugar metabolisms were regulated in the 2PFA plants under light conditions.

Next, the false-discovery rate (FDR) approach was then implemented to identify differentially expressed genes (DEGs) that were regulated by 2PFA treatment under light conditions. A volcano plot between the 2PFA plants under light conditions and CA+ plants displayed that differentially expressed genes (FDR < 0.05, log fold-change > 1) (**Fig. 4.5**). The analysis by likelihood ratio test (LRT) revealed that 762 genes were up-regulated, and 59 genes were down-regulated in the 2PFA treatment under a 12-h light/12-h dark photoperiod.

Next, I performed the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis to identify the key metabolic pathways during the 2PFA process under light conditions. The 821 DEGs (762 up-regulated and 59 down-regulated genes) derived from the 2PFA under light conditions, relative to CA+ were mapped into the KEGG database (**Fig. 4.6**). KEGG pathway analysis indicated that the DEGs were enriched in numerous metabolic pathways. "Starch and sucrose metabolism (ath00500)", "Galactose metabolism (ath00052)", "Glutathione metabolism (ath00480)", "Glycerophospholipid metabolism (ath00564)", and "Amino sugar and nucleotide sugar metabolism (ath00520)" were highly enriched KEGG pathways (**Fig. 4.6a**). Since 2PFA plants were expected to accumulate soluble sugars as compatible solute through regulating these metabolisms, DEGs were mapped into "Starch and sucrose metabolism" (**Fig. 4.7**). The results showed that 2PFA plants regulated the genes encoding enzyme that catalyzes the synthesis of trehalose, D-glucose, maltose, and sucrose, i.e. *trehalose-6-phosphate synthase (TPS)*, *trehalose-6-phosphate phosphatase (TPP)*, and *β-amylase (BAM)*, and *sucrose phosphate synthase 2F (SPS2F)*. The heatmap analysis of time-series experiments revealed that the transcript levels of genes encoding sugar metabolism enzymes were increased between 24 and 48 h in 2PFA plants under light conditions. In the 2PFA treatment, the transcript levels of *TPS11*, *TPPF*, and *BAM1* were increased relative to those in CA, CA+, and 2PSA (**Fig. 4.8**).

Next, the DEGs were mapped into "Galactose metabolism". A heatmap showing the time-series expression levels revealed that 2PFA plants regulated the genes encoding enzyme that catalyzes the synthesis of raffinose, i.e. *UDP-glucose 4-epimerase (UGE)*, *Galactinol synthase (GolS)*, and *raffinose synthase or seed imbibition protein (SIP1)* (**Fig. 4.9, 10**). Interestingly, the transcript level of *GolS2* was increased only in the 2PFA treatments at 24 and 48 h, relative to that in CA, CA+, and 2PSA (**Fig. 4.10**).

These results suggest that changes in sugar metabolism are the key metabolic pathways in the 2PFA plants under light conditions. Therefore, I speculated that sugars play a role in strengthening freezing tolerance induced by the 2PFA treatment.

2PFA plants under light conditions increased the osmolality

It has been reported that to increase freezing tolerance during the CA process, sugars were accumulated as a compatible solute for preventing cellular dehydration and the resultant accumulation of toxic substances (Steponkus 1984; Wanner and Junttila, 1999). Furthermore, RNA-seq analysis in this study revealed that gene expression related to sugar metabolism is regulated in the 2PFA plants under light conditions. Thus, I measured the osmolality of 2PFA and 2PSA plants either in the dark or under a 12-h light/12-h dark photoperiod (**Fig. 4.11.a**). During the CA process at 2°C under a 12-h light/12-h dark photoperiod, osmolality was increased within 3 h and did not increase thereafter until 72 h (**Fig. 4.11.b**). In the 2PSA treatments, the osmolality was higher under light conditions than in the dark conditions at 48 and 72 h. Similarly, in the 2PFA plants, the osmolality was higher under light conditions than in the dark conditions. In addition, 2PFA plants under light conditions exhibited the highest osmolality compared with CA, CA+, and the other 2P treatments at all times.

These results suggested that the plants during the 2P process under light conditions increase the osmolality, and if freezing occurs, the osmolality is further increased.

Sugar was accumulated in the 2PFA plants under light conditions

The results of measuring the osmolality shown in **Figure 4.11** suggested that soluble substances were accumulated in 2PFA plants under light conditions. Thus, I measured the content of soluble sugars including raffinose (Raf), sucrose (Suc), galactinol (GalOH), glucose (Glc), and fructose (Fru). In the CA treatment, consistent with the results of measuring the osmolality, total leaf sugars accumulated gradually within 72 h (**Fig. 4.12.a**). In the 2PSA treatments, the plants did not accumulate sugars during 2PSA in the dark conditions, similar to that observed in the CA+ treatment. In contrast, significant sugar accumulation was observed from 24 h to 72 h under light conditions. In the 2PFA treatments in the dark conditions, sugar contents exhibited no changes until 72 h. Conversely, the 2PFA treatments under light conditions showed a clear increase in sugars. When comparing the sugar accumulation between the 2PSA and 2PFA under light conditions, 2PFA plants further accumulated sugars, which is consistent with the results of measuring the osmolality. These results suggested that the accumulation of soluble sugar content could be related to enhanced freezing tolerance in 2PFA plants under light conditions.

For each sugar, in the CA treatment, Suc, Glc, and Fru were gradually accumulated during 72 h (**Fig. 4.12.b**). For Raf, a slight increase was observed at 72 h in CA plants. In the plants after 7 days of CA (CA+), Raf accumulated slightly over time, while other sugars did not accumulate. In the 2PSA treatment in the dark conditions, the content of the investigated sugars did not change over time. Conversely, in the light conditions, Suc, Glc, and Fru levels were increased, similar to that observed in the CA treatment. During the 2PFA process, in the dark conditions, the content of all sugars did not change over time. In contrast, the 2PFA plants under light conditions showed increased contents of Suc, Glc, and Fru over time, Raf at 48 h slightly, GalOH at 48 and 72 h. A comparison of data between 2PSA and 2PFA under light conditions revealed that the content of Suc, GalOH, and Fru was specifically increased in the 2PFA plants under light conditions.

Taken together, these results suggested that increasing Suc, Glc, and Fru contents is important for the enhancement of freezing tolerance during the 2P process under light conditions. Furthermore, the plants accumulated the content of Suc and GalOH specifically during the 2PFA process under light conditions to enhance their freezing tolerance.

Chloroplast redox state is involved in the 2PFA-induced osmolality increase and sugar content under light conditions

In a previous study, experiments using DCMU have suggested that the photosynthetic electron transport pathway is involved in the enhancement of freezing tolerance in 2PFA under light conditions (Sugita et al., 2024). This led me to hypothesize that the accumulation of soluble sugars, which are the product of the photosynthesis and the resultant increase in the osmolality was responsible for the enhancement of freezing tolerance in 2PFA under light conditions via the photosynthetic electron transfer pathway. Thus, plants before the treatment of 2PFA were sprayed with the DCMU. The plants treated with the DCMU and subsequently subjected to 2PFA treatment for 72 h under light conditions showed lower osmolality compared with plants subjected to the 2PFA treatment in the absence of the DCMU pretreatment (**Fig. 4.13.a**). Interestingly, the osmolality in the DCMU-treated plants subjected to 2PFA treatments under the dark conditions did not change, suggesting that the chloroplast redox state is involved in the 2PFA-induced osmolality increase under light conditions. Next, I measured the content of soluble sugars in the 2PFA plants in the presence of DCMU. **Fig. 4.13.b** showed that the accumulation of soluble sugars is reduced in the presence of DCMU in the 2PFA plants under light conditions at 72 h. Conversely, in the dark conditions, sugar accumulation was not changed between with or without the DCMU pretreatment.

Taken together, these results suggested that the photosynthetic electron transport pathway is involved in the processes of 2PFA under light conditions for increasing osmotic pressure and sugar accumulation.

Discussion

2PFA plants under light conditions increased soluble sugars and enhanced their freezing tolerance via chloroplast redox state.

When light is absorbed by chlorophyll, the energy is mainly used for 1) photochemical reactions in photosystem II, 2) dissipated as heat, and 3) dissipated as fluorescence (Murchie and Lawson, 2013). In this study, I constructed a new experimental system that could measure photosynthetic activity in intact frozen plants (**Fig. 4.1**). Using this new experimental system, I measured the *Fv/Fm*, *qP* values under freezing conditions in *Arabidopsis* (**Fig. 4.2**). The results showed that *Fv/Fm* and *qP* values in the 2PFA plants at 24 and 48 h either in the dark or under light conditions were lower than those in CA and 2PFA plants at 0 h. Because each of these values was non-zero, light energy is still used for photochemical reactions. However, the percentage of light energy going to photochemical reactions is low during the 2PFA. These results led me to hypothesize that light energy is dissipated as heat during the 2PFA. Thus, I measured non-photochemical quenching (NPQ), which is a parameter indicating the magnitude of heat dissipation (**Fig. 4.14**). Compared to NA plants, the NPQ value of 2PFA plants at 0, 24, and 48 h either in the dark or under light conditions was low. Furthermore, NPQ values of 2PFA plants under light conditions at 24 and 48 h were lower than those of 2PFA plants in the dark conditions (**Fig. 4.14**). These results suggested that light energy is more dissipated as heat in the light condition than in the dark condition during 2PFA, but both values are lower than those of NA. Thus, much light energy is likely to be dissipated as fluorescence when the 2PFA plants are exposed to light.

The experiments using DCMU showed that the photosynthetic electron transfer pathway is involved in the CA and 2PFA process to enhance the freezing tolerance (Sugita et al., 2024; Tominaga et al., 2021; Wanner and Junttila, 1999). RNA-seq analysis showed that the main changes in metabolic pathways are sugar metabolism related to sucrose and galactose metabolism in the 2PFA plants under light conditions (**Fig. 4.5-10**). Interestingly, I revealed that products of photosynthesis (i.e. sugars) including sucrose and galactinol were dramatically increased in the 2PFA plants under light conditions (**Fig. 4.12**). DCMU treatment inhibited the increase of soluble sugars in the 2PFA plants under light conditions (**Fig. 4.13**). Thus, since freezing tolerance was decreased in the presence of DCMU during the 2PFA process under light

conditions (Sugita et al., 2024), sugar accumulation via the electron transfer pathway was involved in the enhancement of freezing tolerance in 2PFA plants under light conditions. Previous studies showed that during CA process, carbon dioxide was absorbed, and sucrose was accumulated by regulation of the sucrose phosphate synthase (SPS) activity (Nägele et al., 2012; Strand et al., 2003). Thus, the accumulation of sugars through photosynthesis has been advocated at low temperatures (Wanner and Junttila, 1999). Considering these results, it is possible that, during the processes of 2PFA under light conditions, the plants accumulate soluble sugars via photosynthesis and enhance their freezing tolerance. Alternatively, if photosynthesis was not taking place during the 2PFA process under light conditions, it is conceivable that sucrose in the 2PFA plants under light conditions may be accumulated through the degradation of starch that was accumulated during the CA process. In fact, plants during CA accumulate starch to the same extent as non-acclimated plants (Nägele et al., 2012). In addition, the transcription level of *β-amylase* (*BAM*), which encodes the enzyme that converts starch into maltose, was increased in the 2PFA plants under light conditions (**Fig. 4.8.b**). Thus, it may be interesting to verify the origin of sucrose and galactinol that were accumulated in the 2PFA plants under light conditions. I considered three methods to investigate whether 2PFA plants under light conditions do photosynthesis or not.

1) Measuring starch content. In addition to the conventional method, i.e., iodo-starch reaction, a new starch visualization method using the fluorescent reagent fluorescein has been established (Ichikawa et al., 2024).

2) 2PFA treatment under light conditions without carbon dioxide. Soda lime is an effective carbon dioxide-absorbent material. The main components are inorganic compounds such as calcium hydroxide, sodium hydroxide, and silicates. When soda lime reacts with carbon dioxide, the following chemical reactions occur:

$CO_2 + Ca(OH)_2 \rightarrow CaCO_3 + H_2O$

$CO_2 + 2NaOH \rightarrow Na_2CO_3 + H_2O$

Thus, by placing soda lime in an enclosed space during 2PFA treatment under light conditions, and measuring freezing tolerance, it is possible to determine the effect of carbon dioxide needed for photosynthesis on freezing acclimation.

3) Performing metabolic flux analysis using $^{13}CO_2$. Photosynthesis requires carbon dioxide. Thus, the stable isotope $^{13}CO_2$ is taken up by *Arabidopsis thaliana* during 2PFA to analyze carbon dynamics during 2PFA.

In **Chapter 2**, I revealed that 2PFA treatment dramatically increases freezing tolerance in cold-acclimated *Arabidopsis* (LT50 = –20℃). In this chapter, the accumulation of sugar was shown to be associated with the enhancement of freezing tolerance in the 2PFA under light conditions (**Fig. 4.12**). However, in the 2PFA plants under dark conditions, sugar concentration was not increased, suggesting that in the 2PFA plants in the dark, freezing tolerance is enhanced not by the sugar accumulation. Other factors will be discussed in detail in the next chapter.

2PFA plants under light conditions deal with dehydration stress at the transcription levels. I conducted a large-scale gene expression analysis using RNA-seq to understand the molecular mechanism of how 2PFA plants under light conditions dramatically enhance freezing tolerance. The cluster analysis by *k*-means and GO analysis revealed that "Water deprivation" in the BP categories was enriched in the cluster I-4, which was the cluster that genes are up-regulated in the 2PFA plants at 48 h (**Table 4.2**). After incubation of ice in the agar, agar releases heat as coagulation heat. This is expected to temporarily warm the plants, making them less likely to freeze instantly, suggesting that the plant begins to freeze as the heat of freezing from the agar decreases. Thus, the 2PFA plants under light conditions were gradually exposed to dehydration stress in the later stages of the process.

RNA-seq analysis in this study revealed that *trehalose-6-phosphate synthase (TPS)*, *trehalose-6-phosphate phosphatase (TPP)*, and *galactinol synthase 2 (GolS2)* were up-regulated in the 2PFA plants under light conditions (**Fig. 4.10**). Previous studies showed that overexpression lines of *TPP* and *GolS2* were shown to be resistant to drought stress (Lin et al., 2019; Nishizawa et al., 2008). In this study, I revealed that galactinol is dramatically increased in the 2PFA plants under light conditions (**Fig. 4.12**). Thus, galactinol may prevent dehydration inside of cells from freezing-induced drought stress in the 2PFA plants under light conditions. GolSs are enzyme that catalyzes the first committed step in the synthesis of raffinose from galactinol (Fowler and Thomashow 2002, Taji et al., 2002). At low temperatures, *Gols1*, *3*, and *4* expression was induced, and raffinose in Arabidopsis was accumulated (Gilmour et al., 2000, Taji et al., 2002). However, in cold-acclimated Arabidopsis, freezing tolerance measured by electrolyte leakage method was not altered in mutants deficient in the gene encoding raffinose synthase and galactinol synthase overexpressing lines, indicates that increasing galactinol and raffinose during CA process were not involved in protection of the plasma membrane (Zuther et al., 2004). In another paper, it was demonstrated that raffinose played a role in protecting chloroplasts under freezing conditions in the cold-acclimated plant (Knaupp et al., 2011). There is an example that Arabidopsis protects chloroplasts rather than the plasma membrane via

photoreceptor cryptochromes in the early stages of CA (Imai et al., 2021). Thus, it is possible that raffinose functions as protection of the chloroplasts rather than the plasma membrane under freezing-induced drought stress. I will test whether galactinol and raffinose are involved in increasing freezing tolerance by protecting the chloroplast or membrane during the 2PFA process under light conditions using defective mutants that cannot synthesize raffinose or galactinol.

In this study, I measured the expression level of *GolS2* by real-time PCR approaches. The result showed that in the 2PFA plants either in the dark or light conditions, the expression level of *GolS2* gradually increased and peaked between 12 h and 36 h, and then decreased. These results are consistent with the results of galactinol accumulation over 24 h to 48 h in the 2PFA plants under light conditions. Conversely, sugar analysis revealed no accumulation of galactinol in 2PFA plants in the dark conditions. These results indicated that light energy is required for galactinol synthesis through the regulation of *GolS2* expression.

There is a correlation between increasing of the sugar content and freezing tolerance in 2PFA plants under light conditions.

During CA process, soluble sugars, proline, and glycine betaine accumulated as osmotic regulators in herbaceous plants including wheat and *Arabidopsis* (Kamata and Uemura. 2004; Xin and Browse, 1998). I showed that the 2PFA plants under light conditions dramatically increased osmotic pressure and sugar contents compared with CA+, the other 2P treated plants (**Fig. 4.11,12**). Correlation plots showed that there is a weak positive correlation between the osmotic pressure and sugar content in the CA plants ($R^2 = 0.6103$) (**Fig. 4.16**). This result suggests that, in addition to sugars, other compatible solutes such as proline and betaine may be accumulated, in accordance with previous studies (Kamata and Uemura, 2004; Xin and Browse. 1998). Conversely, a stronger positive correlation was observed between osmotic pressure and sugar content in 2PFA plants under light conditions ($R^2 = 0.7164$) compared with the CA plants, indicate that the accumulation of sugar is strongly involved in the increase in osmotic pressure rather than the accumulation of the proline and betaine. From the RNA-seq data, since the expression of genes related to proline biosynthesis was hardly regulated (**Fig. 4.17**), it is reasonable that the factor contributing to the enhancement of freezing tolerance in 2PFA plants under light conditions is not the accumulation of proline.

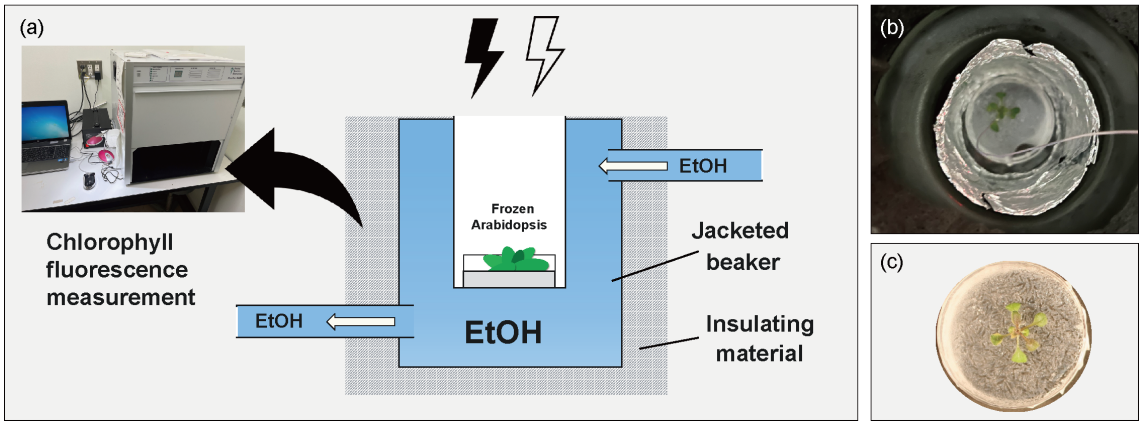


Fig. 4.1. An experimental system to measure photosynthetic activity in intact frozen plants.
(a) Schematic diagram of the experiment for measurement of chlorophyll fluorescence. Antifreeze solutions (EtOH) were circulated in a jacked beaker using a low-temperature water tank. Insulating sheets were wrapped around the beaker and hose connecting between the beaker and low-temperature bath. (b) Plants were placed in the beaker. The leaf temperature was kept at −2°C. (c) Ice was inoculated to agar by touching the plates with liquid nitrogen after 10 minutes of incubation.

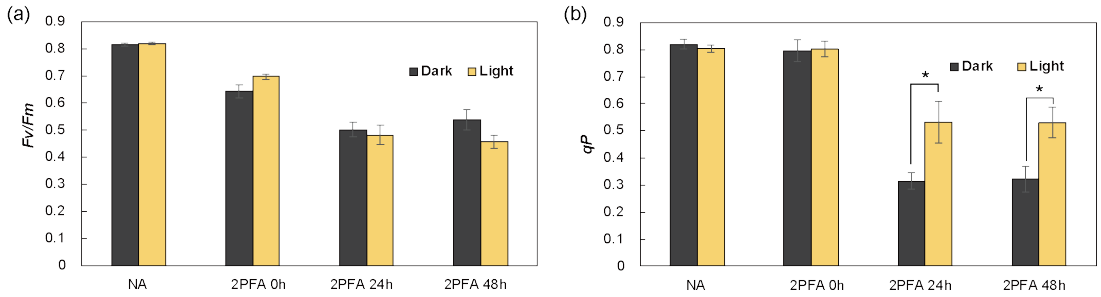


Fig. 4.2. *Fv/Fm* and *qP* values of plants during 2PFA in the dark and light conditions after CA.

(a) *Fv/Fm* and (b) *qP* were measured in the plants that were freezing acclimated at −2°C in the dark or light conditions at 0, 24, and 48 h. As a control, *Fv/Fm* and *qP* of the same samples used for the 2PFA in the dark and light conditions were measured when the plants were grown at 23°C for 2 weeks (NA). Error bars indicate SE (n=3-8). Asterisks at each temperature show a significant difference (Student's t-test, * $p < 0.05$).

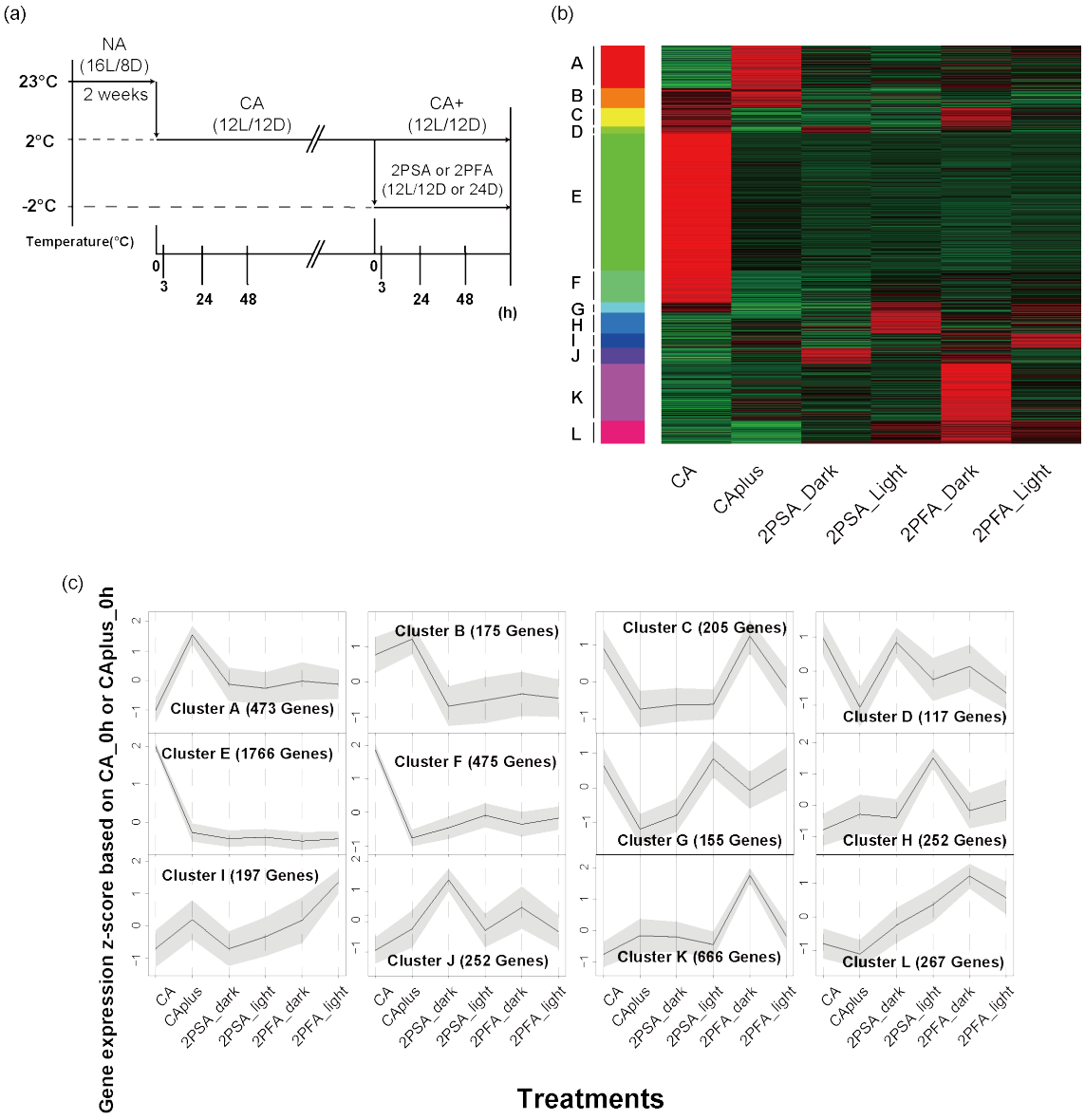


Fig. 4.3. Heatmap showing expression patterns among groups (CA, CA+, 2PSA, and 2PFA) by *k-means* analysis.

(a) Overview of experimental design. RNA was extracted from plants at 0, 3, 24, and 48 h after transfer into a growth chamber at 2°C from 23°C (CA samples). In addition, RNA samples were extracted at 0, 3, 24, and 48 h after transferring CA7 plants (cold-acclimated for 7 days at 2°C) into a freezing chamber at −2°C with or without ice crystallization (2PFA and 2PSA, respectively) in the dark or light conditions, or after keeping CA plants for a further 7 days at 2°C (CA+). (b) Heatmap analysis of genes responding to cold acclimation (CA), CA+, 2PSA, and 2PFA under dark or light conditions. The genes were classified into 12 clusters (Cluster A-L) using the *k*-means method, and random 1000 genes of them were visualized in the heatmap. The *k*-means analysis was performed with R (version 4.3.2) using RStudio (version

2023.09.1+494). (c) Line graphs showing the kinetics of changes in gene expression profile within all clusters shown in (b). The gray range represents SE (*n*=3).

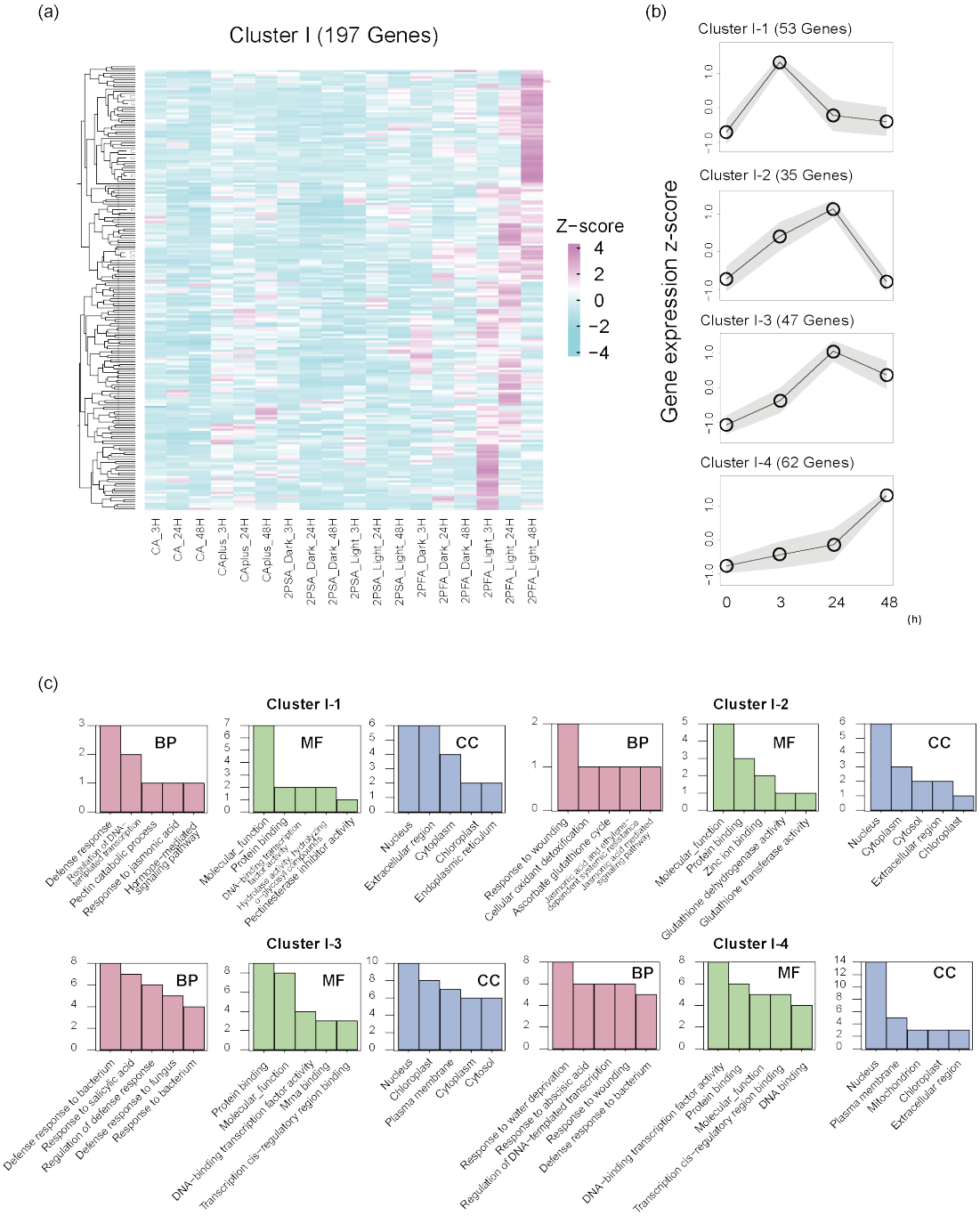


Fig. 4.4. Heatmap showing expression patterns among groups (CA, CA+, 2PSA, and 2PFA) by *k-means* analysis and GO analysis in cluster I.

(a) Heatmap of time-series expression levels of genes belonging to cluster I (n=197) in **Fig. 4.2.a** among groups (CA, CA+, 2PSA, and 2PFA). The gene expression of time series was transformed into a Z-score. (b) Cluster analysis of 2PFA plants under light conditions in cluster I. The Z-score-transformed time-series expression levels of the genes were classified into 4 clusters (Cluster I-1 to I-4) using the *k*-means method. Line graphs showed the kinetics of

changes in gene expression profile in all clusters in (a). The gray range represents SE (n=3). (c) GO enrichment analysis of genes derived from (b).

Cluster I-1 (CC category)

Chloroplast
GO:0009507

Gene	Computational_description	FRKM (2PFA_Light) 3H	24H	48H
AT1G02810	Plant invertase/pectin methylesterase inhibitor superfamily	5.2	2.0	0.0
AT5G45890	senescence-associated gene 12	3.8	2.4	2.2

Cluster I-2 (CC category)

Chloroplast
GO:0009507

Gene	Computational_description	FRKM (2PFA_Light) 3H	24H	48H
AT1G19550	Glutathione S-transferase family protein	17.3	25.6	3.6

Cluster I-3 (CC category)

Chloroplast
GO:0009507

Gene	Computational_description	FRKM (2PFA_Light) 3H	24H	48H
AT2G29350	senescence-associated gene 13	3.2	6.4	4.2
AT3G48850	phosphate transporter 3	0.8	5.5	3.5
AT3G50480	homolog of RPW8 4	1.9	4.2	4.1
AT4G04510	cysteine-rich RLK RECEPTOR-like protein kinase 38	2.4	9.8	4.9
AT4G21840	methionine sulfoxide reductase B8	2.1	7.6	3.5
AT4G23810	WRKY family transcription factor	2.2	9.8	4.7
AT4G32810	carotenoid cleavage dioxygenase 8	1.3	8.4	1.9
AT5G39520	hypothetical protein	3.5	4.3	5.0

Cluster I-4 (CC category)

Chloroplast
GO:0009507

Gene	Computational_description	FRKM (2PFA_Light) 3H	24H	48H
AT1G72770	HYPERSENSITIVE TO ABA1	1.3	5.4	13.2
AT3G49570	response to low sulfur 3	1.6	4.0	11.8
AT4G15280	UDP-glucosyl transferase 71B5	1.3	2.4	3.6

Table. 4.1. Genes determined as enriched in "Chloroplast" in cluster I.
Time-series gene expression in the 2PFA plants under light conditions against CAplus_0h. The list showed the genes belonging to the cluster I-1 to I-4 shown in **Fig. 4.4.a,b** and determined as enriched shown in "Chloroplast" in **Fig. 4.4.c**. Gene expression levels were normalized by using Fragments Per Kilobase of exon per Million mapped fragments (FPKM), which is the expression level normalization method.

Cluster 4 (BP category)

Response to water deprivation GO:0009414				
		FRKM (2PFA_Light)		
Gene	Computational_description	3H	24H	48H
AT1G07900	LOB domain-containing protein 1	6.4	22.1	61.0
AT2G18340	late embryogenesis abundant domain-containing protein / LEA domain-containing protein	1.4	2.2	3.9
AT2G47190	myb domain protein 2	1.5	3.2	6.0
AT3G49570	response to low sulfur 3	1.6	4.0	11.8
AT4G15280	UDP-glucosyl transferase 71B5	1.3	2.4	3.6
AT4G22620	SAUR-like auxin-responsive protein family	2.8	1.5	3.8
AT4G37370	cytochrome P450, family 81, subfamily D, polypeptide 8	1.2	4.7	6.3
AT5G02580	argininosuccinate lyase	2.5	12.9	23.7

Table. 4.2. Genes determined as enriched in "Response to water deprivation" and belonging to cluster I-4.
Time-series gene expression in the 2PFA plants under light conditions against CAplus_0h. The list showed the genes belonging to the cluster I-1 to I-4 shown in **Fig. 4.4.a,b** and determined as enriched shown in "Response to water deprivation" in **Fig. 4.4.c**. Gene expression levels were normalized by using Fragments Per Kilobase of exon per Million mapped fragments (FPKM), which is the expression level normalization method.

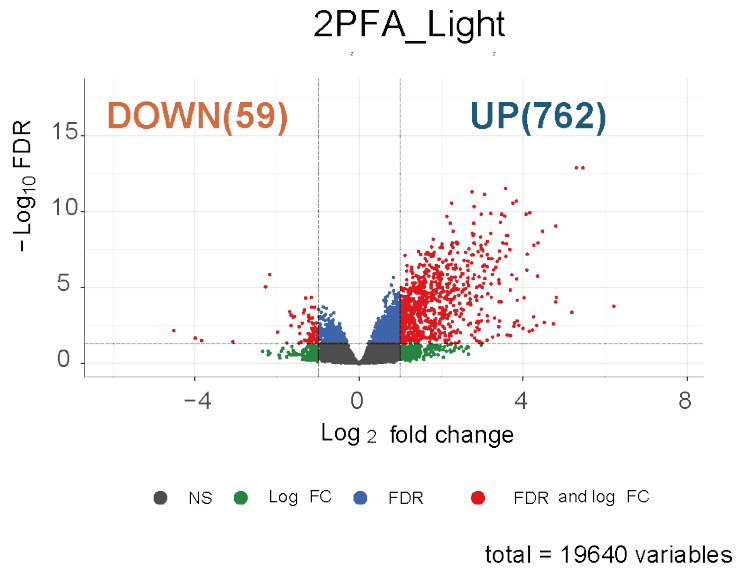
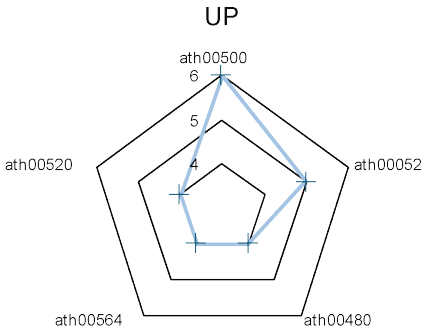


Fig. 4.5. Volcano plot showing up-or down-regulated genes in the 2PFA treatments under light conditions.

Volcano plots showing differential gene expression in the 2PFA plants under light conditions against CA+ plants with an FDR of < 0.05 and a $\log_2$ fold-change > 1, < −1.

(a)

Path ID	Description
ath00500	Starch and sucrose metabolism
ath00052	Galactose metabolism
ath00480	Glutathione metabolism
ath00564	Glycerophospholipid metabolism
ath00520	Amino sugar and nucleotide sugar metabolism



(b)

Path ID	Description
ath00941	Flavonoid biosynthesis
ath01250	Biosynthesis of nucleotide sugars
ath00480	Glutathione metabolism
ath00562	Inositol phosphate metabolism
ath00944	Flavone and flavonol biosynthesis

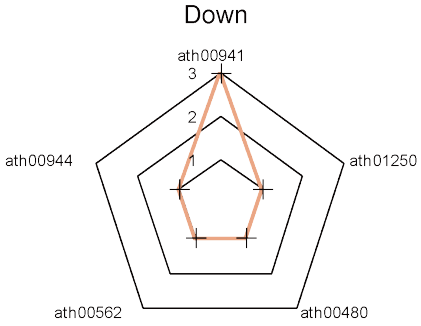


Fig. 4.6. KEGG pathway enrichment analysis of DEGs in the 2PFA plants under light conditions.

KEGG analysis of the 821 DEGs in the 2PFA plants under light conditions relative to CA+ plants (762 upregulated, 59 downregulated, shown in **Fig. 4.5**). In the (a) upregulated and (b) downregulated genes, highly enriched KEGG term was listed.

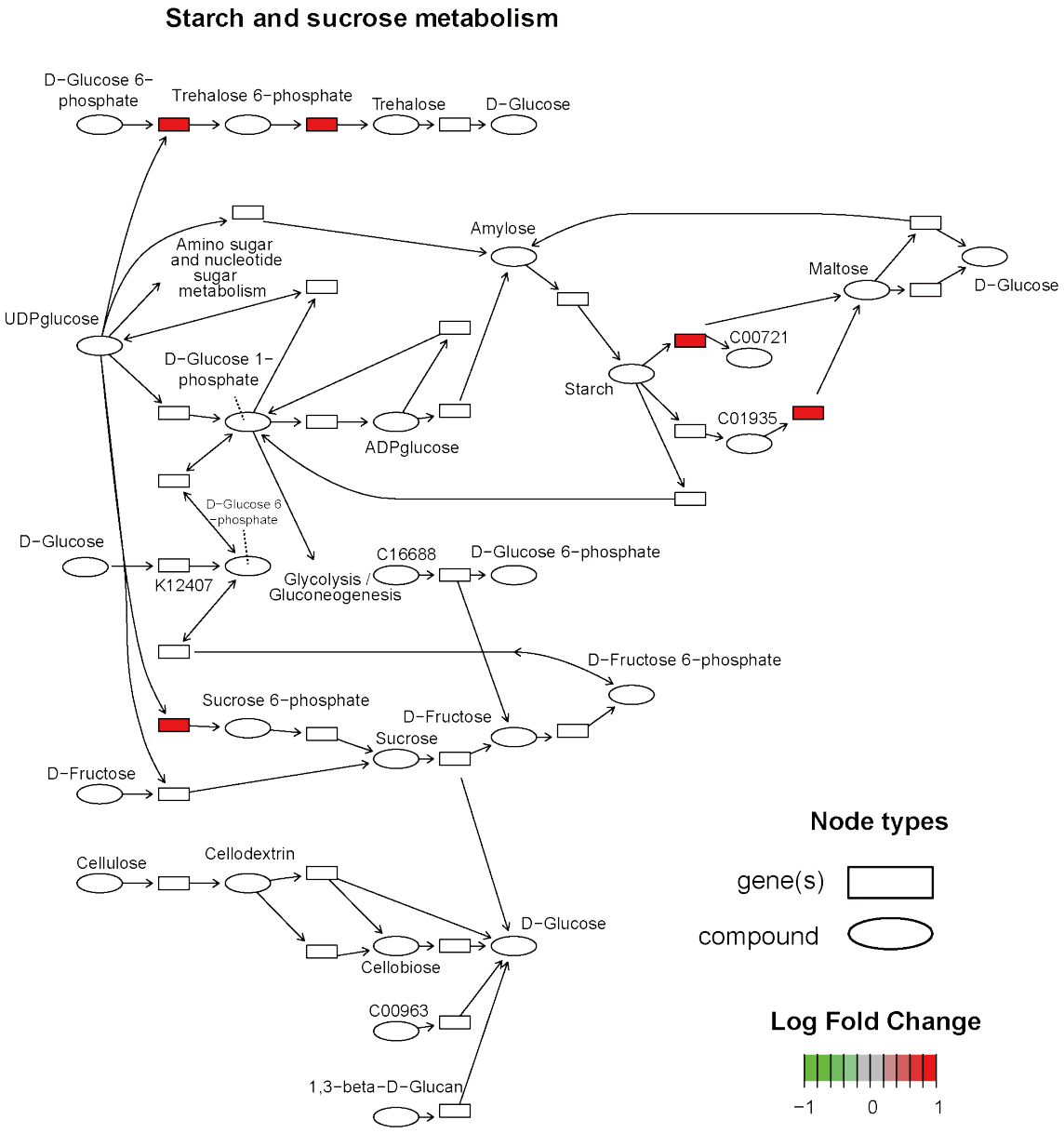


Fig. 4.7. Expressions of genes involved in starch and sucrose metabolism, were up-regulated by 2PFA treatment under light conditions.

KEGG metabolic map of starch and sucrose metabolism. The color bar indicates $\log_2$ fold change of gene expression, and red indicates elevated expression and green indicates suppressed expression. The heatmaps showed expression levels of genes in the 2PFA treatment under light conditions, relative to CA+ treatment.

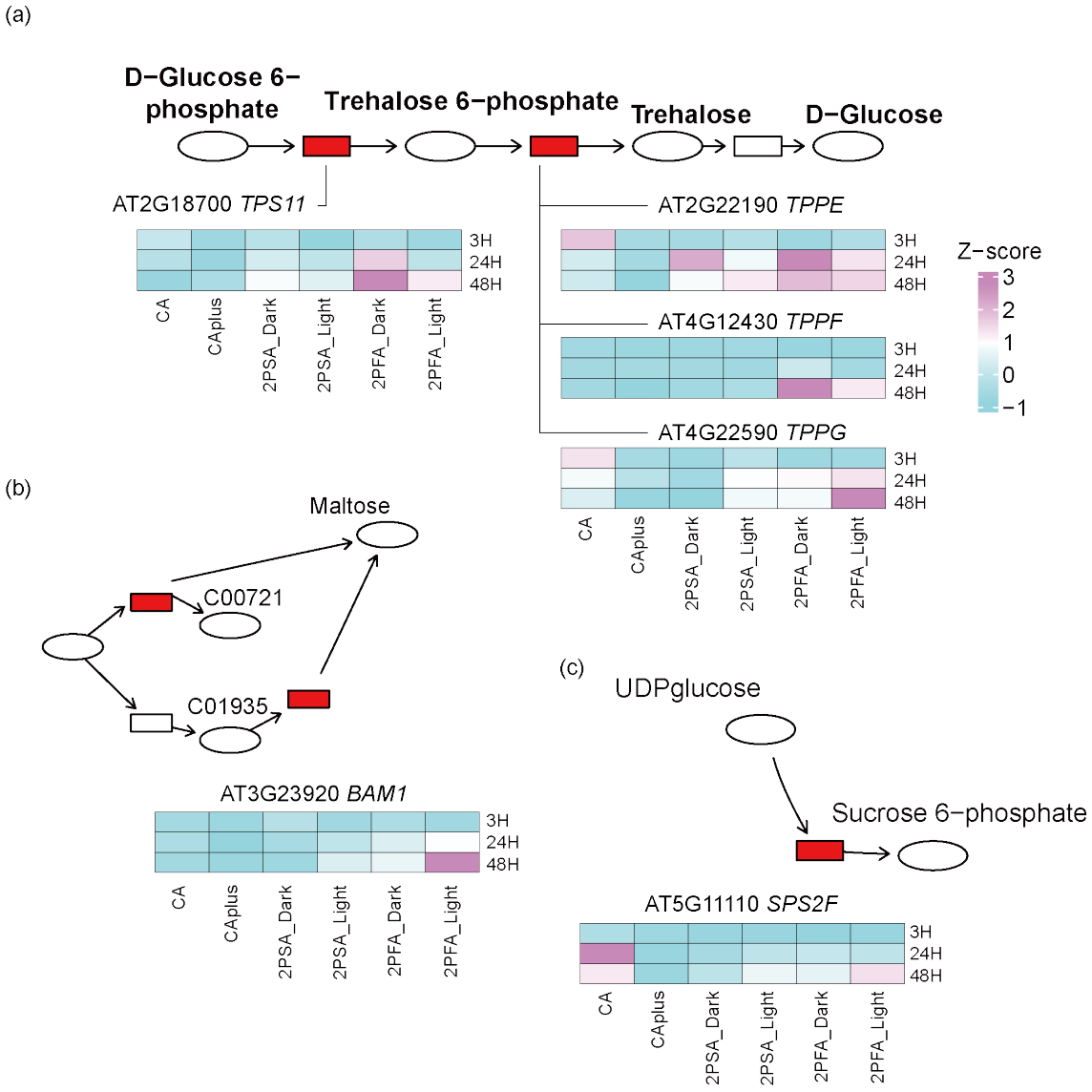


Fig. 4.8. Expression of *TPS11*, *TPPs*, *SPS2F*, and *BAM1* genes under various treatments at 3, 24, and 48 h.

The heatmaps showed time-series expression levels of genes enriched in "Starch and sucrose metabolism". The heatmaps showed time-series expression levels of genes, (a) AT2G18700; *trehalose phosphatase/synthase 11* (*TPS11*), AT2G22190; *TREHALOSE-6-PHOSPHATE PHOSPHATASE E* (*TPPE*), AT4G12430; *TREHALOSE-6-PHOSPHATE PHOSPHATASE F* (*TPPF*), and AT4G22590; *TREHALOSE-6-PHOSPHATE PHOSPHATASE G* (*TPPG*), (b) AT3G23920; *beta-amylase 1* (*BAM1*), and (c) AT5G11110; *sucrose phosphate synthase 2F* (*SPS2F*) were expressed relative to CA_0h (CA_3, 24, 48h) and CA+_0h (CA+, 2PSA, 2PFA_3, 24, 48h). The color bar indicates gene expression transformed into Z-score.

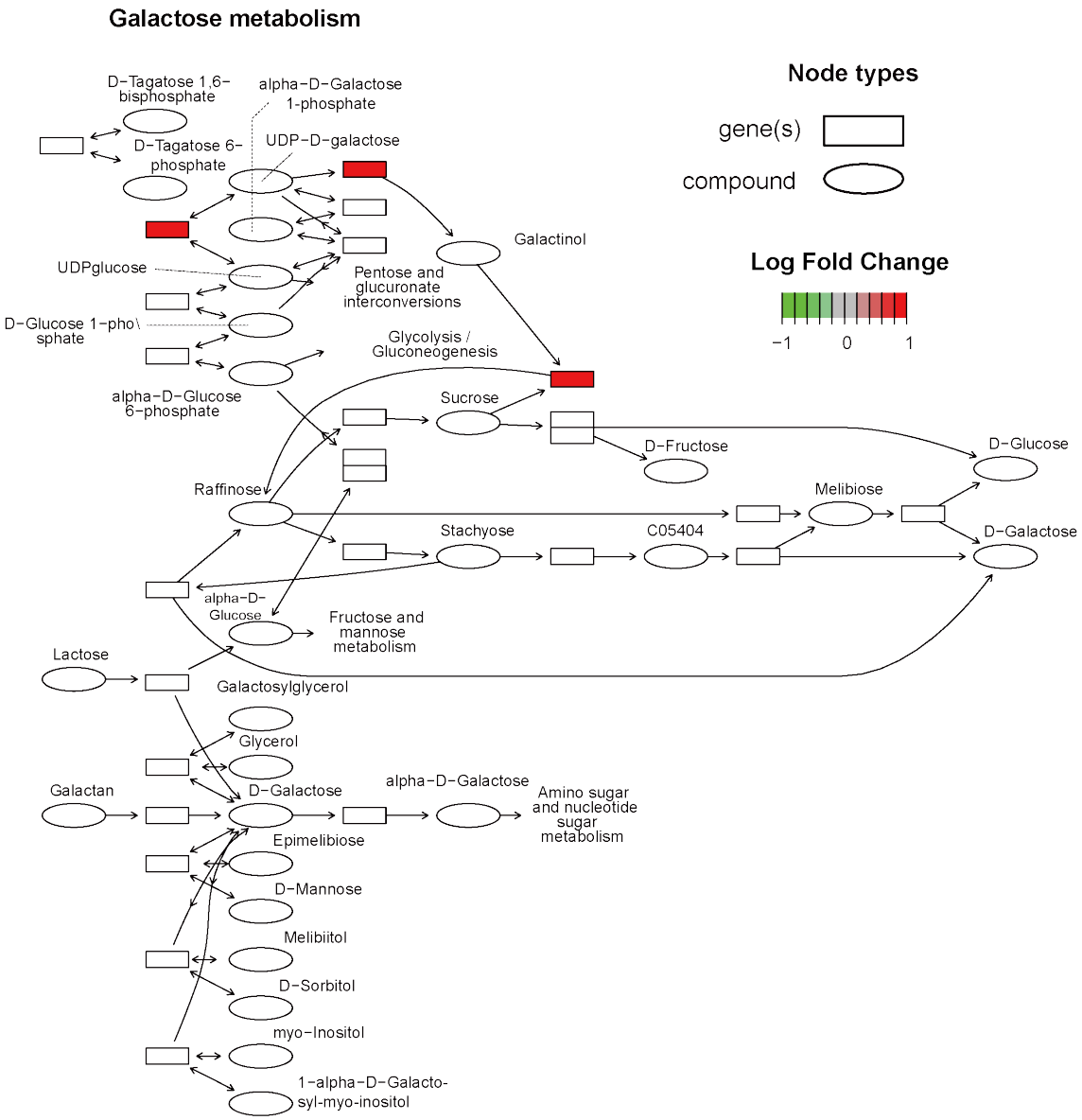


Fig. 4.9. Expressions of genes involved in galactose metabolism, were up-regulated by 2PFA treatment under light conditions.

KEGG metabolic map of galactose metabolism. The color bar indicates $\log_2$ fold change of gene expression, and red indicates elevated expression and green indicates suppressed expression. The heatmaps showed expression levels of genes in the 2PFA treatment under light conditions, relative to CA+ treatment.

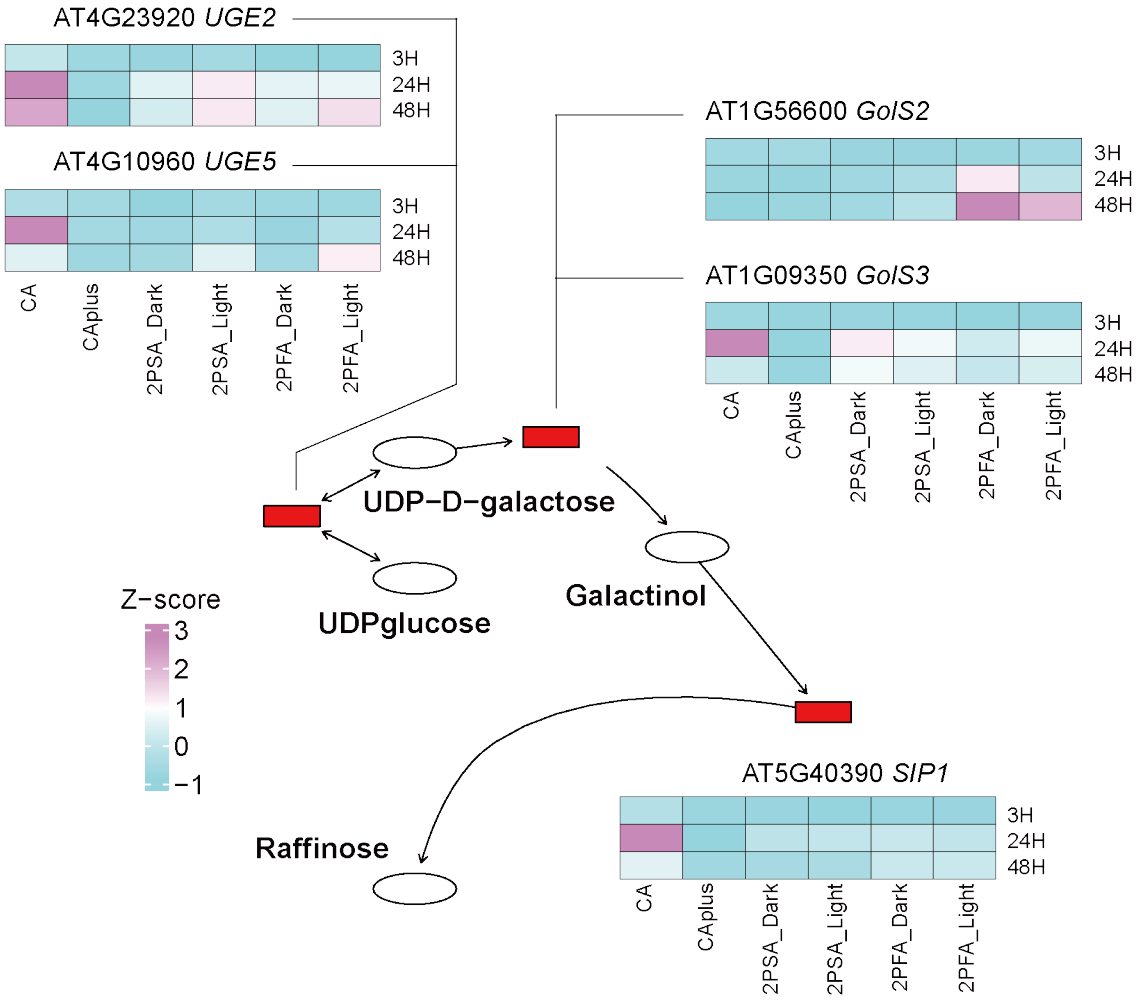


Fig. 4.10. Expression of *UGEs*, *GolSs*, and *SIP1* genes under various treatments at 3, 24, and 48 h.

The heatmaps showed time-series expression levels of genes enriched in "Galactose metabolism". The heatmaps showed time-series expression levels of genes, (a) AT4G23920; *UDP-D-glucose/UDP-D-galactose 4-epimerase 2* (*UGE2*), AT4G10960; *UDP-D-GLUCOSE/UDP-D-GALACTOSE 4-EPIMERASE 5* (*UGE5*), AT1G56600; *galactinol synthase 2* (*GolS2*), AT1G09350; *galactinol synthase 3* (*GolS3*), and AT5G40390; *raffinose synthase family protein* (*SIP1*) were expressed relative to CA_0h (CA_3, 24, 48h) and CA+_0h (CA+, 2PSA, 2PFA_3, 24, 48h). The color bar indicates gene expression transformed into Z-score.

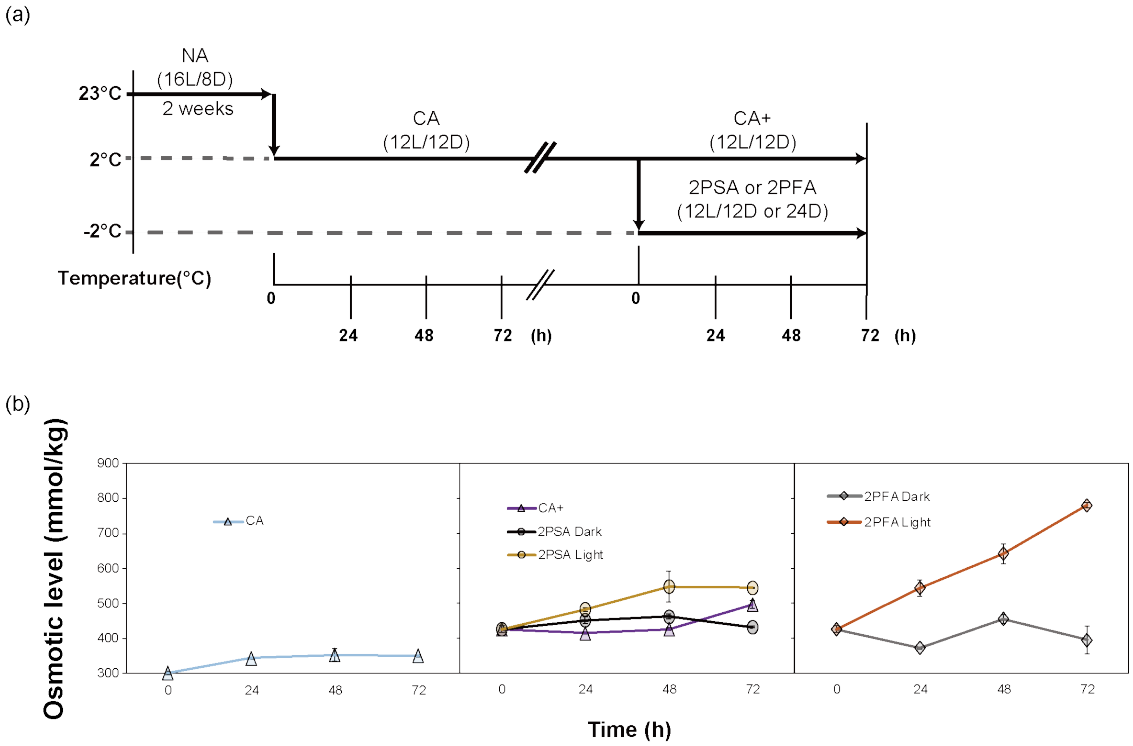


Fig. 4.11. Measurement of the osmolality in the plants under various treatments (CA, CA+, 2PSA, and 2PFA).

(a) Overview of experimental design. Plants at 0, 24, 48, and 72 h after transferring to a growth chamber at 2°C from 23°C (CA samples), and in cold-, freezing- and supercooling-acclimated plants (CA+, 2PFA, and 2PSA, respectively) after 0, 24, 48, and 72 h.

(b) Osmolality was measured in the plants under various treatments. Error bars indicate SE (n=3).

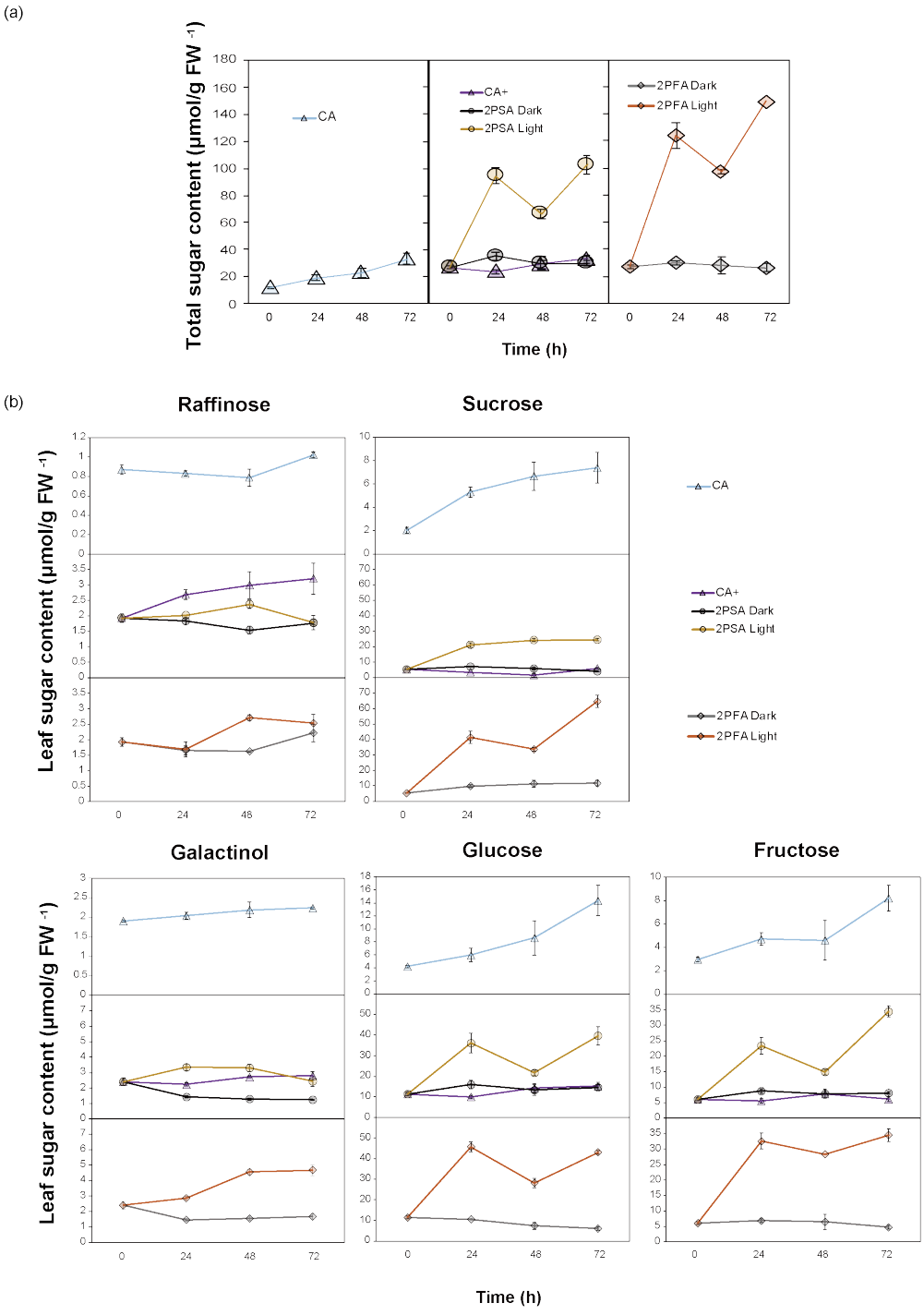


Fig. 4.12. Measurement of sugar content in the plants under various treatments (CA, CA+, 2PSA, and 2PFA).

(a) Total soluble sugar content and (b) each sugar of raffinose, sucrose, galactinol, glucose, and fructose were measured in the plants at 0, 24, 48, and 72 h after transferring to a growth chamber at 2°C from 23°C (CA samples), and in cold-, freezing- and supercooling-acclimated plants (CA+, 2PFA, and 2PSA, respectively) after 0, 24, 48, and 72 h.

(b) Osmolality was measured in the plants under various treatments. Error bars indicate SE (n=3).

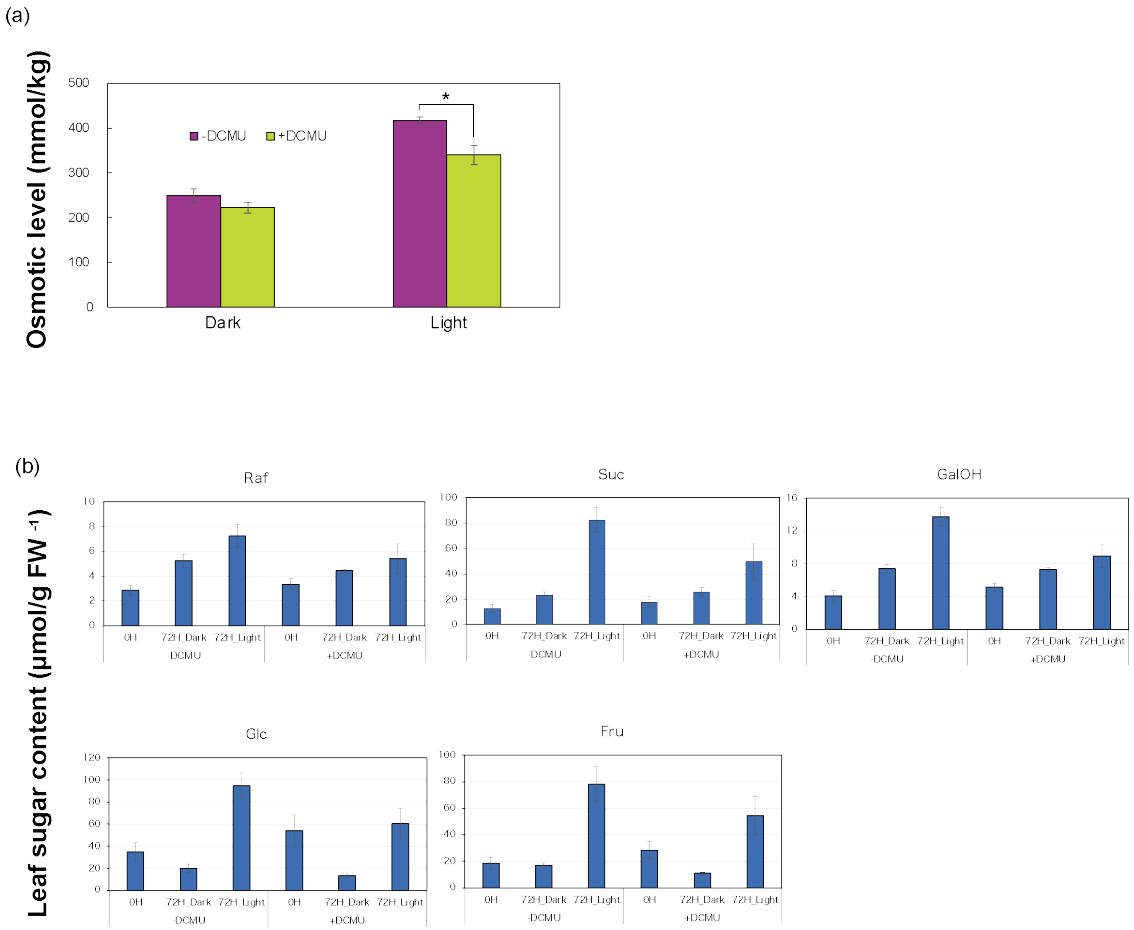


Fig. 4.13. Measurement of osmolality and sugar content in the 2PFA plants either in the dark or light conditions pretreated with or without DCMU.
CA was treated with 50 μM DCMU and kept at room temperature for 1 h before 2PFA treatment (−2°C with ice for 3 days in the dark or under a 12-h light/12-h dark photoperiod). (a) Osmolality and (b) soluble sugars were measured in the 2PFA plants either in the dark or light conditions pretreated with or without DCMU at 0 and 72 h. Error bars indicate SE (*n*=2-3).

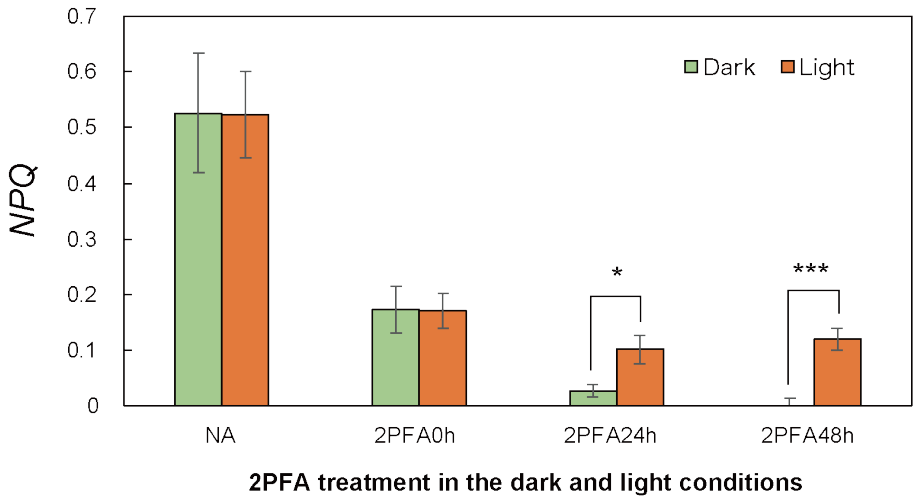


Fig. 4.14. NPQ of plants during 2PFA in the dark and light conditions.
NPQ was measured in the 2PFA plants in the dark or light conditions at 0, 24, and 48 h. As a control, NPQ of the same samples used for the 2PFA in the dark and light conditions were measured when the plants were grown at 23°C for 2 weeks (NA) before CA treatment started. Error bars indicate SE (n=7-8). Asterisks at each temperature show a significant difference (Student's t-test, * $p < 0.05$, *** $p < 0.001$).

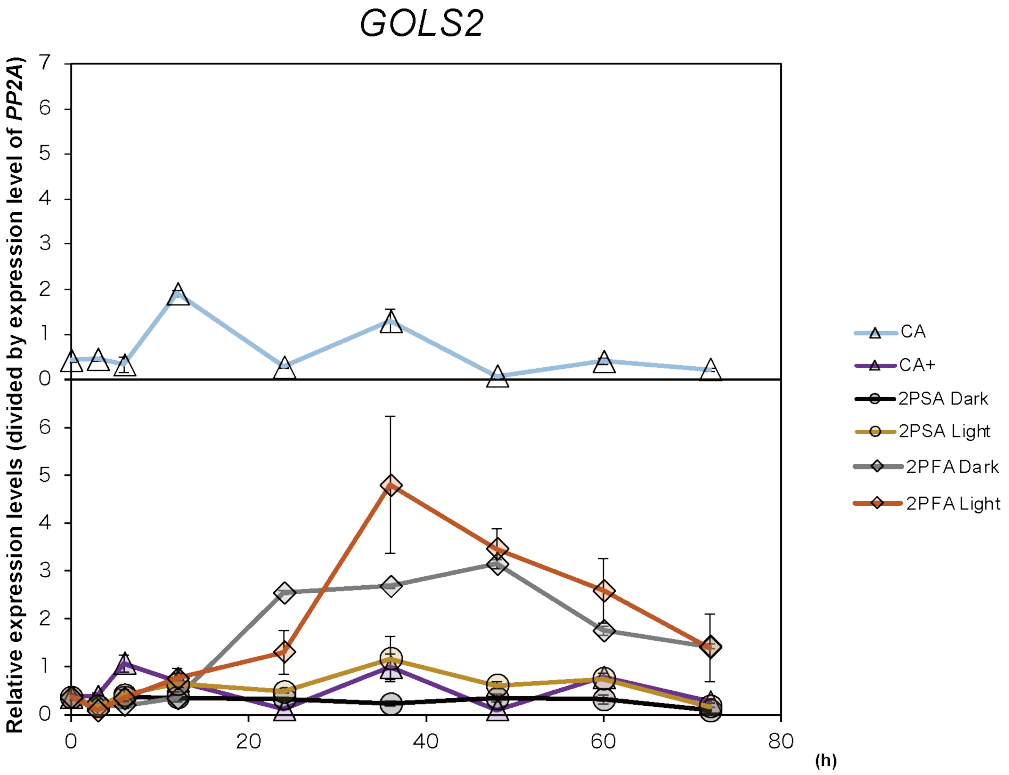


Fig. 4.15. Gene expression of *GolS2* under various treatments (CA, CA+, 2PSA, and 2PFA).

The expression of *GolS2* genes in plants at 0, 3, 6, 12, 24, 36, 48, 60, and 72 h after transferring to a growth chamber at 2°C from 23°C (CA samples), and in cold-, freezing- and supercooling-acclimated plants (CA+, 2PFA, and 2PSA, respectively) after 0, 3, 6, 12, 24, 36, 48, 60, and 72 h. Transcript levels were determined after normalization against that of *PP2A* (n=3).

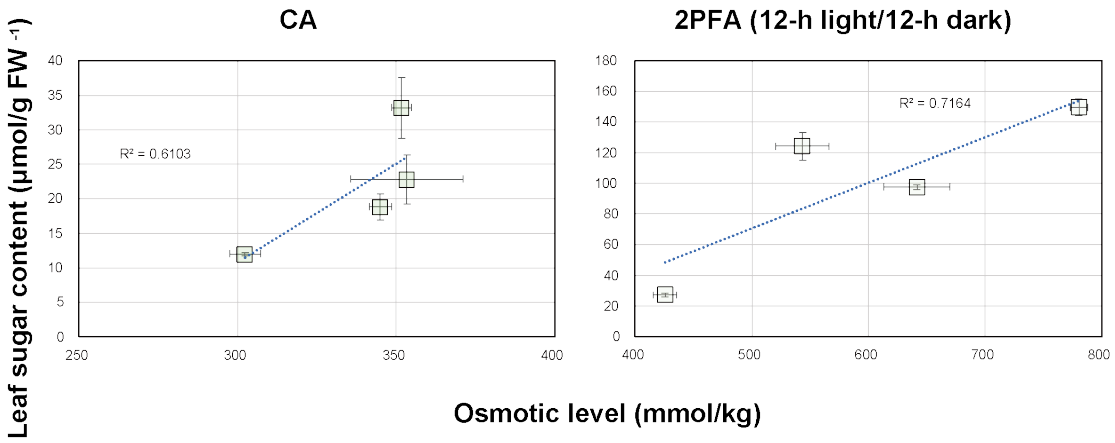


Fig. 4.16. Correlation between the osmotic pressure and sugar content in the CA and 2PFA plants under light conditions.

Osmotic pressure and sugar content was measured in the CA plants and 2PFA plants (−2°C for 3 days with ice crystal) under light conditions at 0, 24, 48, and 72 h.

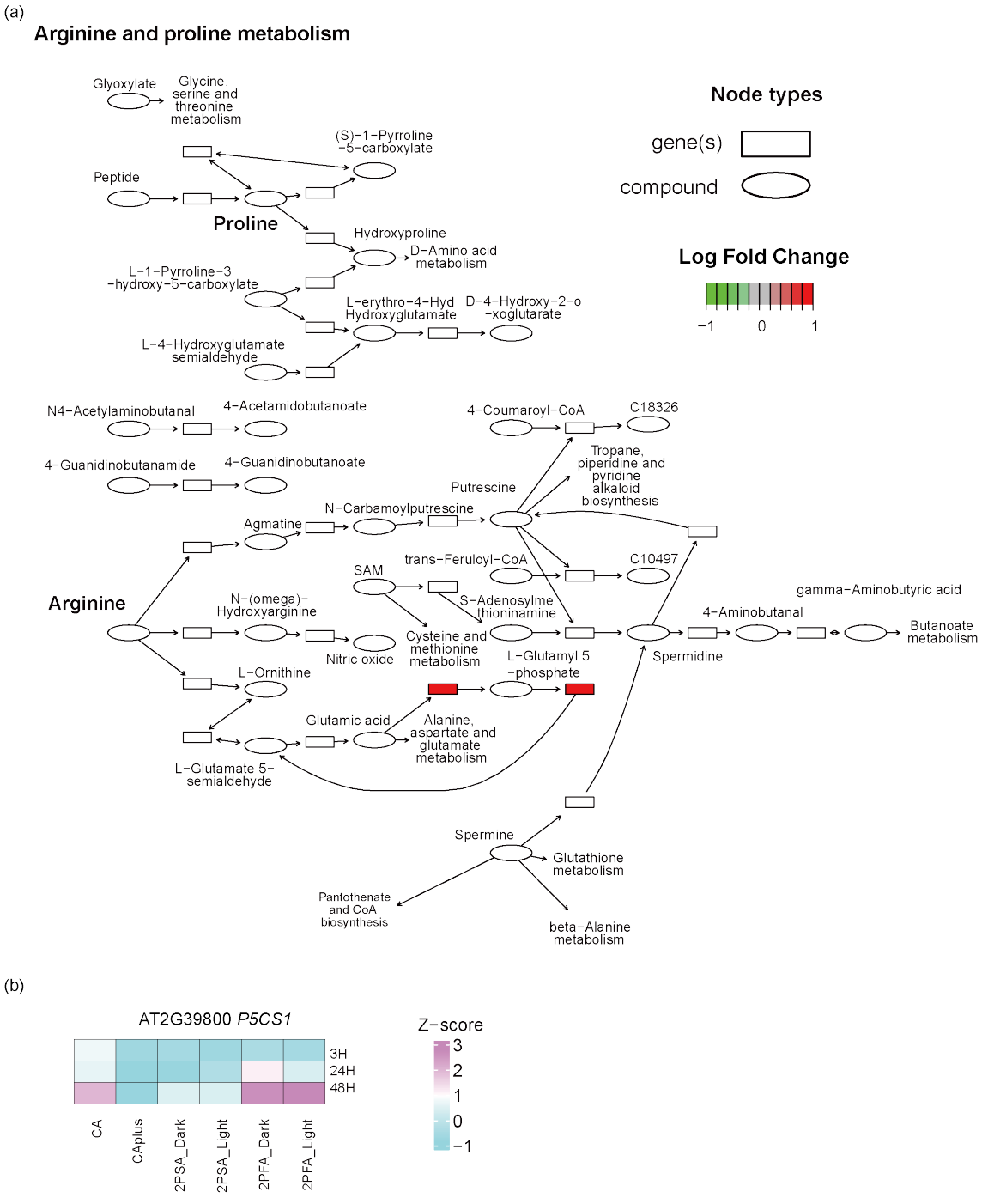


Fig. 4.17. Expression of *P5CS1* gene under various treatments at 3, 24, and 48 h.
The heatmaps showed time-series expression levels of genes enriched in "Arginine and proline metabolism". The heatmaps showed time-series expression levels of genes, AT2G39800; *delta1-pyrroline-5-carboxylate synthase 1* (*P5CS1*) was expressed relative to CA_0 h (CA_3, 24, 48 h) and CA+_0h (CA+, 2PSA, 2PFA_3, 24, 48h). The color bar indicates gene expression transformed into Z-score.

Chapter5

A multifaceted approach of RNA-seq for a comprehensive understand of the molecular mechanism of second phase freezing acclimation under light conditions

Abstract

Overwintering plants survive at sub-zero temperatures through, first, cold acclimation (CA) by sensing short-day and low temperatures above 0℃ in autumn, and, next, second phase cold hardening (2PH) by sensing sub-zero temperatures in winter. A previous study revealed that during 2PH, there are four patterns, in which CA plants are exposed to sub-zero temperatures without ice (i.e. second phase supercooling acclimation; 2PSA) in the (1) dark and (2) light conditions, and in which plants are exposed to sub-zero temperatures with ice (i.e. second phase freezing acclimation; 2PSA) in the (3) dark and (4) light conditions. In a previous chapter (Chapter 4), I revealed that 2PFA plants showing maximum freezing tolerance in the four patterns in the 2P process increased soluble sugars by regulating the genes related to sugar synthesis. Here I will focus on other factors for enhancing freezing tolerance in the 2PFA plants under light conditions other than soluble sugars by a multifaceted approach of RNA-seq. Transcriptome analysis by RNA sequencing was conducted in this study. *k-means* analysis showed that the transcript levels of genes related to cell wall modification was regulated in the 2PFA plants under light conditions. Furthermore, LRT and GO analysis revealed that 2PFA plants under light conditions regulate transcript levels of genes related to various plant hormone signaling pathways. These results suggested that cell wall cross-linking and plant hormone signaling pathways may be involved in increasing freezing tolerance in the 2PFA plants under light conditions.

Introduction

In cooler parts of the world such as the temperate zone (Cfa) and the subarctic zone (Dfa and Dfb) in the Köppen climate classification, overwintering plants sense low temperatures and shorter daylengths in fall and increase their freezing tolerance, which is known as cold acclimation (CA). In mid-winter, plants are exposed to freezing by frost. During freezing, plants prevent tissue injury by allowing ice to form outside the cell.

During CA, changes in cell wall composition and its properties affect freezing tolerance (Kutsuno et al., 2023; Takahashi et al., 2024). In addition, cross-linking by polysaccharide modification of cell wall composition ingredients plays an important role in increasing freezing tolerance (Chen et al., 2018; Panter et al., 2019).

Cold acclimated-plants get further enhanced freezing tolerance when they are exposed to mild-freezing temperatures (Le et al., 2008, 2014; Sugita et al., 2024; Takahashi et al., 2019, 2021). This phenomenon is called second phase cold hardening (2PH) or sub-zero acclimation (SZA). A previous study revealed that there are four patterns during 2PH, in which plants are exposed to sub-zero temperatures without ice (i.e. second phase supercooling acclimation; 2PSA) in the (1) dark and (2) light conditions, and in which plants are exposed to sub-zero temperatures with ice (i.e. second phase freezing acclimation; 2PSA) in the (3) dark and (4) light conditions (Sugita et al., 2024). It has been reported that changes in cell wall composition and its structure affect freezing tolerance in *Arabidopsis* leaves at sub-zero temperature without freezing in the dark conditions (i.e. supercooling) after CA (Takahashi et al., 2021). In a previous chapter (Chapter 4), I revealed that soluble sugars such as sucrose and galactinol increased in the 2PFA plants under light conditions. Since the osmotic pressure was also elevated with the accumulation of sugars, sugars may function as a compatible solute. Conversely, there is a possibility that they function as components of cell wall polysaccharide synthesis and thus contribute to increased freezing tolerance through cell wall modification.

Furthermore, the link between plant hormones and the CA process was well studied. *aba* mutants with low abscisic acid (ABA) content showed reduced freezing tolerance after cold acclimation (Gilmour and Thomashow, 1991). Furthermore, in Arabidopsis, ethylene biosynthesis and signaling decrease freezing tolerance after CA by regulating negative regulators of cytokinin signaling (Shi et al., 2012). Thus, to increase freezing tolerance, various plant hormone signaling networks and their crosstalk were regulated in response to low temperatures (Shi et al., 2015).

Although in the previous chapter (Chapter 4), I revealed that soluble sugars were accumulated in the 2PFA plants under light conditions, there is still a possibility that a variety of

factors could increase freezing tolerance. Here, I conducted an RNA-seq analysis and performed a multifaceted approach for a comprehensive understanding of the molecular mechanism of 2PFA under light conditions. These results obtained suggested that cell wall cross-linking and plant hormone signaling pathways were regulated at the transcript levels.

Materials and Methods

Plant materials and growth conditions

See “Plant materials and growth conditions” in Chapter4.

RNA extraction

See “RNA extraction” in Chapter4.

RNA-Seq analyses

See “RNA-Seq analyses” in Chapter4.

Results

Cell wall remodeling during the 2PFA process was regulated at the transcript levels.

It has been reported that structural changes in the cell wall were involved in increasing freezing tolerance (Chen et al., 2018, Panter et al., 2019, Takahashi et al., 2019). Thus, firstly, I investigated whether the transcript levels of genes associated with cell wall such as cell wall polysaccharide synthase genes, sugar nucleotide synthase genes, and polysaccharide modification enzyme genes are regulated in the 2PFA plants under light conditions. In Chapter 4, the *k*-means method was performed to detect genes that were regulated in the 2PFA under light conditions at the transcript levels (**Figs. 4.3,4, Table. 4.1**). Interestingly, the transcript levels of *PMEI*, which inhibits the activity of pectin methylesterase (PMEs) in the plant cell wall were up-regulated in the 2PFA plants under light conditions (**Fig. 5.1, Table. 5.1**). In addition, the transcript levels of *UDP-glycosyltransferase*, which involves in cell wall biosynthesis, was upregulated (**Fig. 5.1, Table. 5.1**).

Next, KEGG pathway enrichment analysis of DEGs in 2PFA plants under light conditions was conducted to identify the metabolic pathways related to the cell wall during the 2PFA process under light conditions. “Amino sugar and nucleotide sugar metabolism (ath00520)” was highly enriched as shown in **Fig.4.6a** and **Fig. 5.2** in the genes derived from 2PFA plants under light conditions relative to CA+ plants. Thus, time-course heatmap analysis was performed to

gain a further understanding of expression patterns (**Fig. 5.3**). The results showed that in the up-regulated genes, in addition to *UGE2*, *UGE5* encoding enzyme that catalyzes the conversion of UDP-glucose to UDP-galactose, which was shown in a previous chapter (Chapter 4), *Chitinase family protein*, which encodes enzyme that breaks down to N-acetylglucosamine from chitin, *MUR4* involving in UDP-β-L-arabinose biosynthesis I (from UDP-α-D-xylose) were detected. While the expression of *UGE2* and *UGE5* genes was up-regulated in the 2PFA plants under light conditions at 48 h, their expression was substantially regulated in the CA plants at 24 h. The transcript level of *MUR4* was increased at 24 h and 48 h in the 2PSA and 2PFA treatment under both dark and light conditions compared with CA and CA+. The expression of *MIOX2* gene that encodes the enzyme in the inositol route was down-regulated in not only the 2PFA plants under light conditions but also the other 2P treatments at 24 h and 48 h.

These analyses of gene transcript profiles using RNA-seq show that cell wall remodeling during the 2PFA process was regulated at transcript levels.

2PFA plants under light conditions regulate the transcript levels of genes related to various plant hormone signaling pathways.

Next, the false-discovery rate (FDR) approach was implemented to identify differentially expressed genes (DEGs) that were regulated by 2PFA treatment under light conditions. A volcano plot between the 2P plants (2PSA treatment or 2PFA treatment (−2°C without ice) under dark or light conditions) and CA+ plants displayed differentially expressed genes, DEGs (FDR < 0.05, log fold-change > 1, < −1) (**Fig. 5.4a**). The analysis by likelihood ratio test (LRT) revealed, in 2PSA treatment, there were 466 up-regulated and 170 down-regulated genes when plants were incubated under dark conditions, and 642 upregulated and 94 downregulated genes when plants were incubated under light conditions (**Fig. 5.4b**). Conversely, in 2PFA treatment, 994 genes were up-regulated and 126 genes were down-regulated in the dark conditions, and 762 genes were up-regulated and 59 were down-regulated under light conditions (**Fig. 5.4b**). The DEGs in each of the four 2P treatments were analyzed to determine the expression-2P treatment relationship and plotted into the Venn diagram (**Fig. 5.4c**). The results showed that 105 genes were up-regulated and 17 genes were down-regulated solely in response to the 2PFA treatment under light conditions. These results suggested that 2PFA treatment is dramatically higher than the number of down-regulated genes.

Next, gene ontology (GO) enrichment analysis was conducted to provide biological attributes of the 122 DEGs (105 upregulated and 17 downregulated genes) that were found solely in response to 2PFA treatment under light conditions (**Fig. 5.5**). The GO enrichment

analysis classified into three GO categories: 1) biological process (BP), 2) molecular functions (MF), and 3) cellular component (CC). In the BP category, interestingly, GO terms associated with abscisic acid such as “Response to abscisic acid” in the upregulated DEGs and “Abscisic acid-activated signaling pathway” in the downregulated DEGs were enriched. In the MF category, “Protein binding” was the top enriched GO term in the 122 DEGs. In the CC category, “Nucleus” was the top enriched GO term in the 122 DEGs. In the DEGs enriched “Response to abscisic acid”, the transcript level of *ABA-INDUCIBLE BHLH-TYPE TRANSCRIPTION FACTOR* (*AIB*), which encodes BHLH-type domain containing transcription activator in the regulation of ABA signaling was increased at 3 and 48 h during 2PFA treatment under light condition (**Table. 5.2**). Conversely, in the DEGs enriched “Abscisic acid-activated signaling pathway”, the transcripts levels of *REGULATORY COMPONENTS OF ABA RECEPTORs*, *PYL4*, and *PYL6*, which encode proteins with functions as abscisic acid sensors were gradually decreased in the 2PFA plants under light conditions (**Table. 5.3**). These analyses showed that the expression of genes related to ABA signaling pathway including signal perception, signal transduction were regulated in the 2PFA plants under light conditions.

Next, in 122 DEGs, the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed to comprehensively examine the genes associated with signaling in the plant hormones. The analysis revealed that 8 out of 122 DEGs were mapped into “Plant hormone signal transduction” and “MAPK signaling pathway”. ABA signaling pathway genes such as *PYRs* and *abscisic acid responsive elements-binding factor 2* (*ABF2*), gibberellin receptor *GA INSENSITIVE DWARF1B* (*GID1B*), salicylic-acid responsive gene *PATHOGENESIS-RELATED GENE 1* (*PR1*), and jasmonic acid-responsive gene *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) were upregulated in the 2PFA plants (**Fig. 5.6,7**). These results suggested that the plants during the 2PFA process under light conditions regulated the transcript levels of genes related to various plant hormone signaling pathways.

Expression of genes is regulated transiently or longer at least between 24 and 48 h in the 2PFA plants under light conditions.

Next, to gain a further understanding of expression patterns of differential genes in the 2PFA plants under light conditions, the genes of CA, CA+, and 2P treatments in time-course series (3, 24, and 48 h) were identified by the LRT (**Fig. 5.8a**). In the 2P treatments, genes were regulated in their transcript levels at 24 and 48 h more than the treatments at 3 h. At 3h, 2PFA plants in the dark conditions affected the expression of genes more than the plants treated differently. At 24 and 48 h, more genes were regulated at the transcriptional levels in the 2PFA plants under

the light conditions. Next, the genes in their transcript levels in each treatment were analyzed and plotted into the Venn diagram to examine the overlapped genes among the treatments (**Fig. 5.8b, c**). The results showed that in 2PFA treatment under light conditions at 3h, 98 genes were up-regulated and 63 genes were down-regulated. Conversely, there were 242 up-regulated and 239 down-regulated genes at 24 h, and 400 up-regulated and 456 down-regulated genes (**Fig. 5.8**). To count the number of genes that were up-or down-regulated in the 2PFA plants under light conditions, using this comparison among the five groups in time-course series, transcription of genes was regulated only by 2PFA plants under light conditions in time-course series were represented in the Venn diagram (**Fig. 5.9**). At 3 h, 87 and 57 genes in their transcript levels were transiently up- and down-regulated, respectively. Conversely, at 24 and 48 h, 198 and 358 genes were up-regulated respectively. At both 24 and 48 h, 37 genes were downregulated. In the downregulated genes at 3 h, most genes (57 out of 63) were transiently down-regulated. In addition, 202 and 417 genes were down-regulated at 24 and 48 h, respectively. At both 24 and 48 h, 35 genes were down-regulated. These results suggested that genes regulated in the 2PFA plants under light conditions were divided into two groups: those transiently regulated at 3, 24, or 48 h and those regulated longer at least between 24 and 48 h.

Discussion

Cell wall cross-linking might increase freezing tolerance in 2PFA plants under light conditions.

In this study, I conducted a large-scale gene expression analysis using RNA-seq to understand the molecular mechanism of how 2PFA plants under light conditions dramatically enhance freezing tolerance. The cluster analysis by the *k*-means method shown in Chapter 4 (**Figs. 4.3,4**) showed that the transcript level of *PMEI* was upregulated in the 2PFA plants (**Table. 5.1**). The heatmap showing time-series FRKM expression levels of the gene revealed that in addition to 2PFA plants under light conditions, other 2P plants increased the transcript level of *PMEI* at early stage during the treatment. Homogalacturonan (HG), which is the most abundant polymer of pectin, is demethylated by pectin methylesterases (PME). Biomechanical properties of the cell wall change through calcium crosslinking of HG (Ridley et al., 2001, Wormit and Usadel. 2018). This activity is regulated by pectin methylesterase inhibitors (PMEIs). A previous study showed that overexpression lines of *PMEIs* in *Arabidopsis* decreased freezing tolerance, but increased salt stress (Chen et al., 2018). In addition, boron (B)-dependent dimerization through fucosylation of rhamnogalacturonan II (RGII), pectin domains, was involved in the enhancement of freezing tolerance (Panter et al., 2019). Thus, cross-linking by polysaccharide

modification such as methylation and fucosylation of pectin domains is important in increasing freezing tolerance. However, based on these previous studies, the result obtained in this study cannot explain how increased transcript levels of *PMEI* in 2PFA plants can lead to the acquisition of freezing tolerance. In the 2PFA process in light conditions, ice nucleation and propagation through inhibiting the activity of cell wall cross-linking might be important signals for increasing freezing tolerance. Future work will determine the chemical and physical properties of the cell wall in the 2PFA plants under light conditions.

Expression of genes related to plant hormone signaling pathways in 2PFA plants under light conditions regulates.

I showed that by various analyses such as LRT, GO, and KEGG, the transcript levels of genes related to the abscisic acid (ABA) signaling pathway including signal perception and signal transduction, were regulated in the 2PFA plants under light conditions (**Figs. 5.4,5, Table. 5.2,3**). ABA is one of the most studied plant hormones associated with not only cold but also drought and salt stress (Gilmour and Thomashow, 1998; Zhang et al., 2006). ABA signaling in guard cells induces stomatal closure (Ma et al., 2009). Under the conditions at sub-zero temperatures with freezing, plants are expected to be exposed to drought stress induced by extracellular freezing and need to enhance ABA-mediated stomatal closure to inhibit the reduction of water loss. RNA-seq data in this study obtained contrasting results, i.e., the transcript levels of *PYRs* decreased in the 2PFA plants under light conditions, indicating that the 2PFA plants suppressed the activity of ABA-mediated stomatal closure (**Fig. 5.6**). In Chapter 4, I revealed that the photosynthetic electron transport pathway is involved in increasing soluble sugars during the 2PFA under light conditions. Thus, the 2PFA plants under light conditions might inhibit ABA-mediated stomatal closure to maintain gas exchange for photosynthesis at freezing temperatures.

Gibberellin receptor *GID1B* encodes the protein that binds to DELLA and induces destruction of it through the ubiquitin-proteasome pathway (Colebrook et al., 2014). DELLA plays a role in regulating other hormonal signaling pathways such as jasmonic acid, ethylene, and ABA by interacting with each signaling component (Colebrook et al., 2014; Hou et al., 2010). In *della* mutants, freezing tolerance decreased after cold acclimation in *Arabidopsis* (Achard et al.,2008). Based on these previous studies, the 2PFA plants under light conditions might accumulate DELLA and regulate various hormonal signaling pathways for increasing freezing tolerance (**Fig. 5.6,7**). It is reasonable that for controlling the accumulation of DELLA, the transcript level of *GID1B* increased in the 2PFA plants under light conditions (**Fig. 5.6**).

Chloroplast GO:0009507				
		FRKM (2PFA_Light)		
Gene	Computational_description	3H	24H	48H
AT1G02810	Plant invertase/pectin methylesterase inhibitor superfamily	5.2	2.0	0.0
AT4G15280	UDP-glucosyl transferase 71B5	1.3	2.4	3.6

Table. 5.1. Genes enriched "Chloroplast" in cluster I as shown Chapter 4.
Time-series FRKM expression levels 2PFA plants under light conditions based on CAplus_0h.

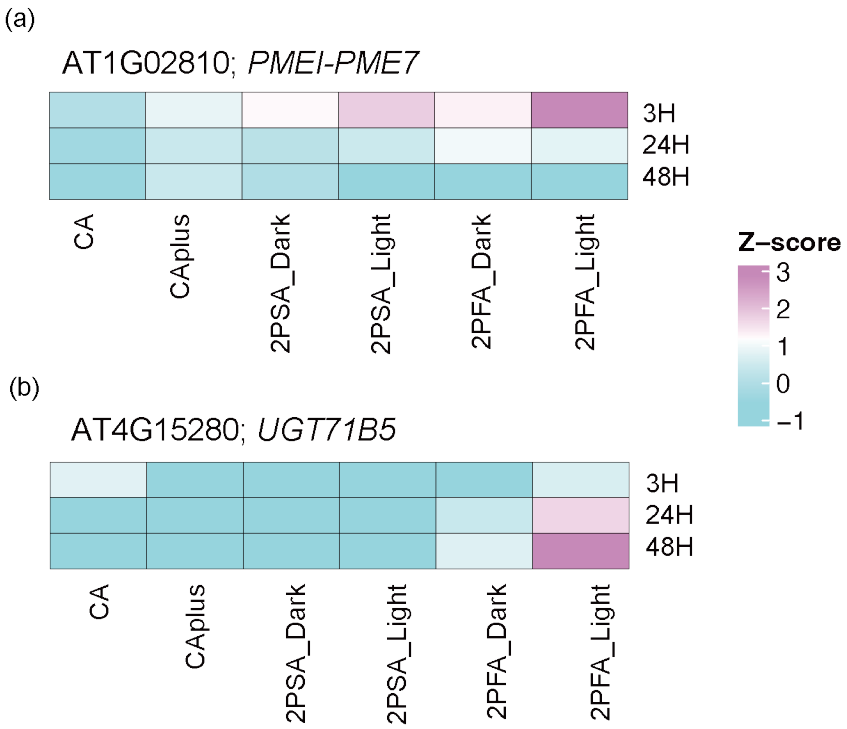


Fig. 5.1. Transcript levels of *PMEI* and *UGT71B5* under various treatments at 3, 24, and 48 h.

The heatmaps showed time-series expression levels of genes, (a) AT1G02810; *PECTIN METHYL ESTERASE INHIBITOR-PECTIN METHYLESTERASE7* (*PMEI-PME7*), and (b) AT4G15280; *UDP-GLUCOSYL TRANSFERASE 71B5* (*UGT71B5*) were expressed relative to CA_0h (CA_3, 24, 48_h) and CA+_0h (CA+, 2PSA, 2PFA_3, 24, 48_h). The color bar indicates gene expression transformed to Z-score.

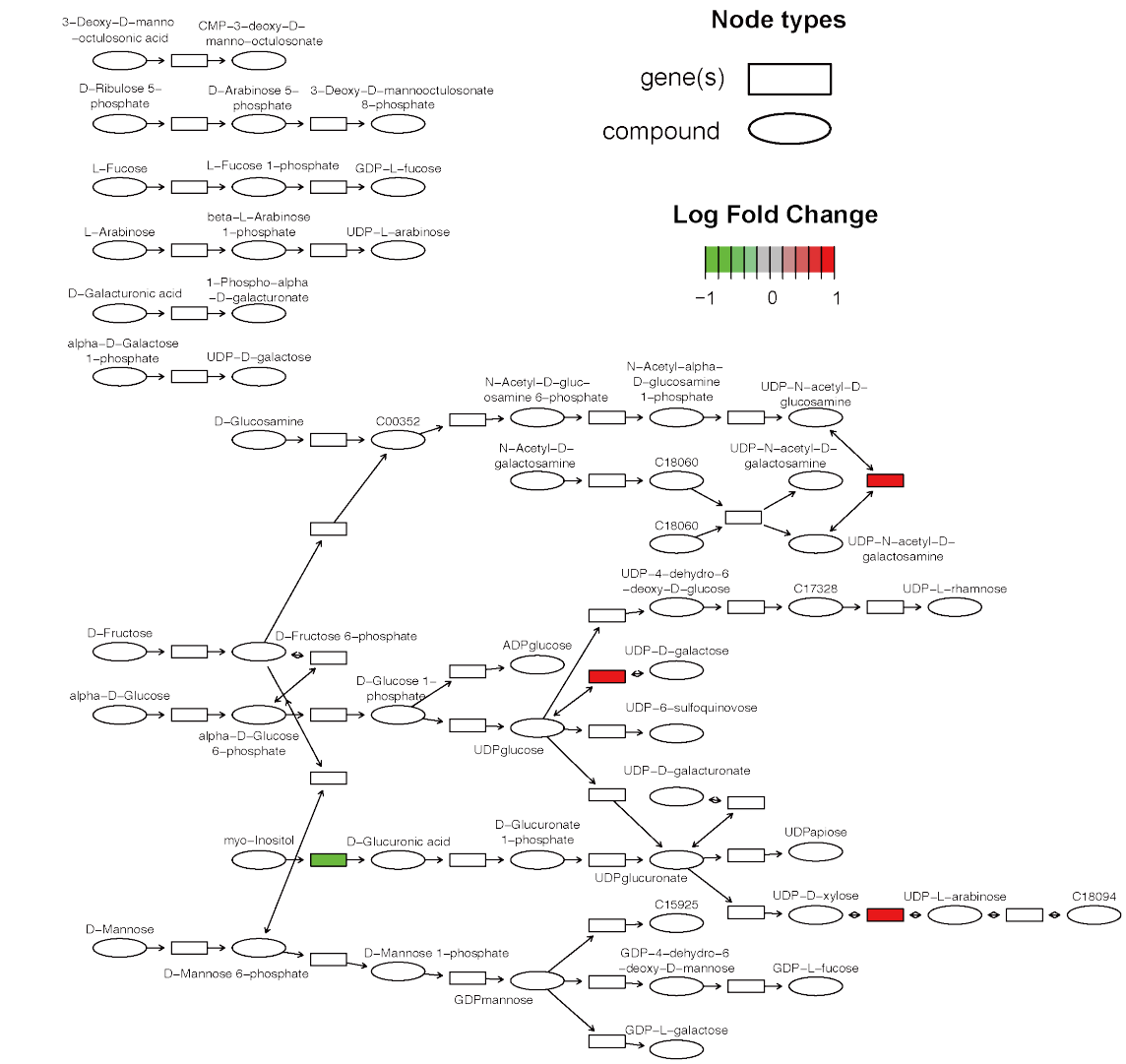


Fig. 5.2. Expressions of genes involved in amino sugar and nucleotide sugar metabolism, was up regulated by 2PFA treatment under light conditions.

KEGG metabolic map of amino sugar and nucleotide sugar metabolism. The color bar indicates $\log_2$ fold change of gene expression, and red indicates elevated expression and green indicates suppressed expression. The heatmaps showed expression levels of genes in the 2PFA treatment under light conditions, relative to CA+ treatment.

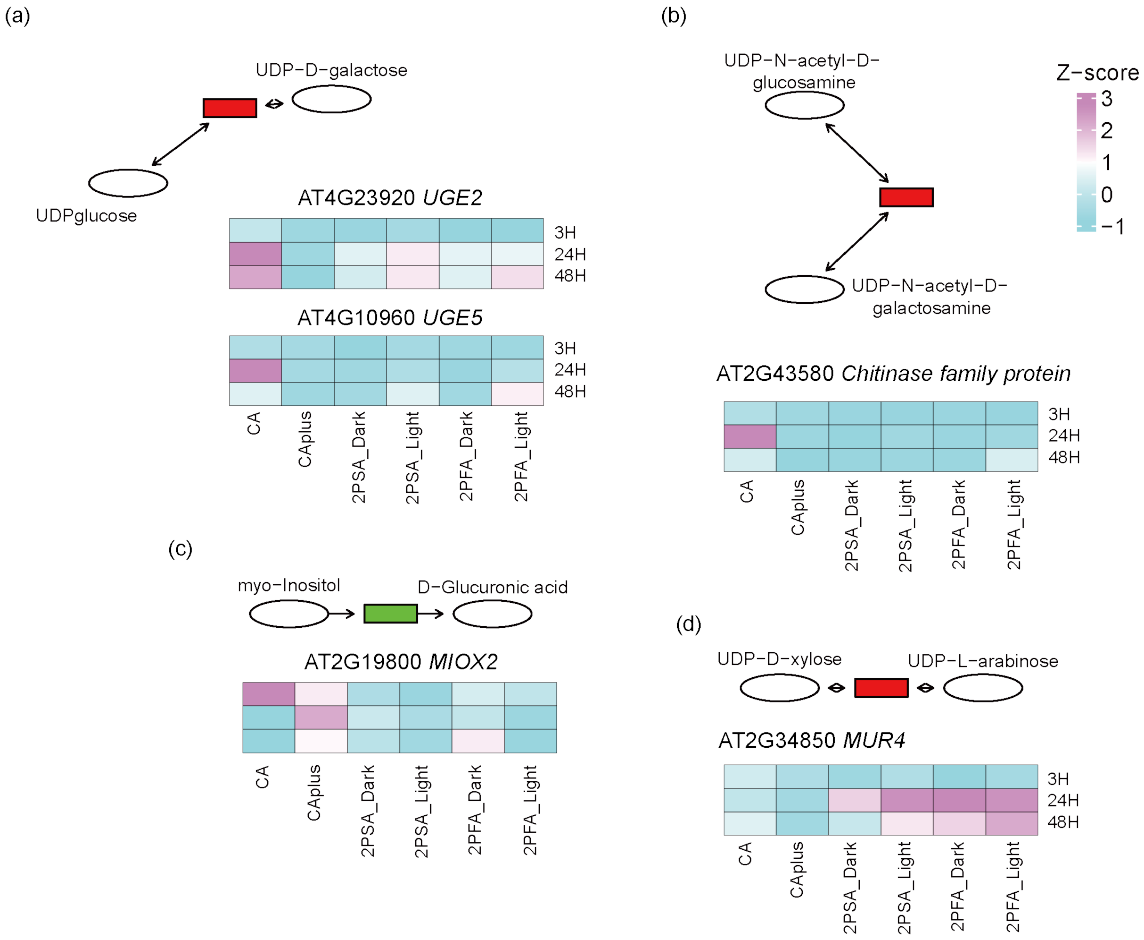


Fig. 5.3. Expressions of genes of *UGE2*, *UGE5*, *Chitinase gamily protein*, *MIOX2* and *MUR4* under various treatments at 3, 24, and 48 h.

The heatmaps showed time-series expression levels of genes enriched "Amino sugar and nucleotide sugar metabolism". Time-series expression levels of genes, (a) AT4G23920; *UDP-D-glucose/UDP-D-galactose 4-epimerase 2* (*UGE2*), AT4G10960; *UDP-D-GLUCOSE/UDP-D-GALACTOSE 4-EPIMERASE 5* (*UGE5*), (b) AT2G43580; *Chitinase family protein*, (c) AT2G19800; *MYO-INOSITOL OXYGENASE 2* (*MIOX2*), and (d) AT2G34850; *NAD(P)-binding Rossmann-fold superfamily protein* (*MUR4*) were expressed relative to CA_0h (CA_3, 24, 48_h) and CA+_0h (CA+, 2PSA, 2PFA_3, 24, 48_h). The color bar indicates gene expression transformed to Z-score.

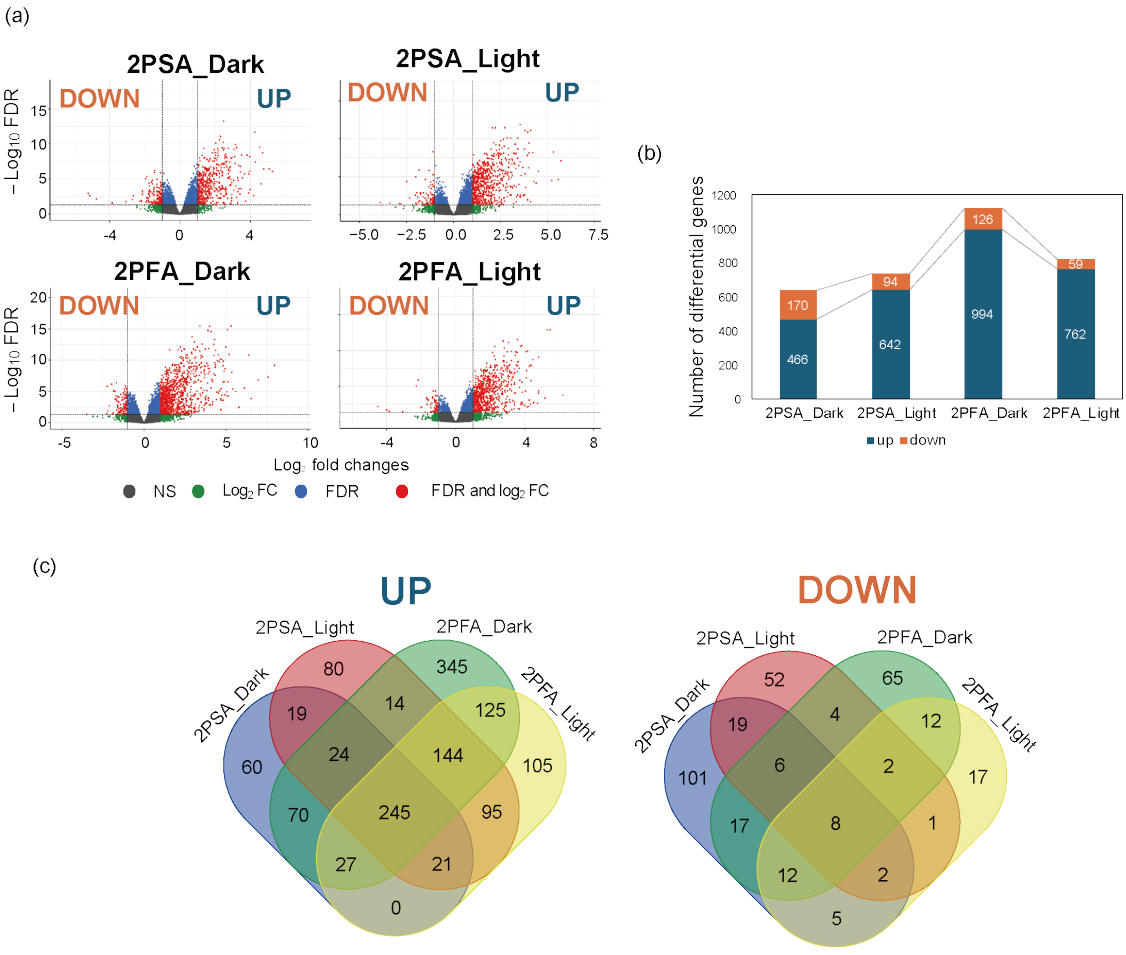


Fig. 5.4. The number of regulated genes by 2P treatment compared with CA plus treatment.

(a) Volcano plots between the 2P plants (2PSA treatment or 2PFA treatment (−2°C without ice) under dark or light conditions) and CA+ plants displayed differentially expressed genes (DEGs) (the false-discovery rate (FDR) < 0.05, $\log_2$ fold-change > 1, < −1). (b) The number of up-or down-regulated genes by likelihood ratio test (LRT) (FDR < 0.05, $\log_2$ fold-change > 1, < −1) in 2P plants against CA+. (c) Venn diagram showing the numbers of the overlapped genes of that were detected in Figure (b) among the treatments.

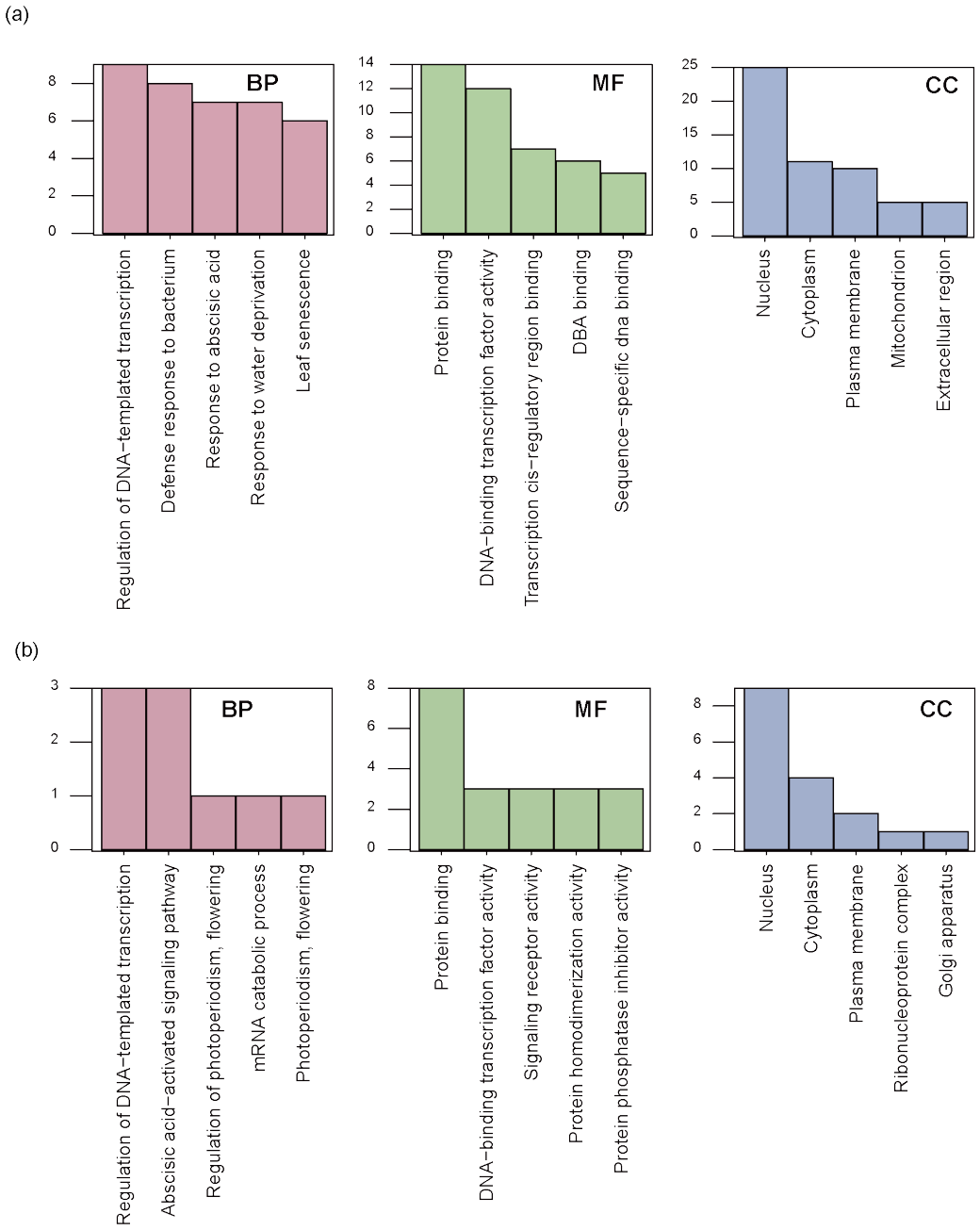


Fig. 5.5. GO enrichment analysis of 122 DEGs (105 upregulated and 17 downregulated genes) that were found solely in response to 2PFA treatment under light conditions. (a) GO enrichment analysis of 105 upregulated DEGs in only the 2PFA plants under light conditions (**Fig. 5.2c**). (b) GO enrichment analysis of 17 downregulated DEGs in the 2PFA plants under light conditions in (**Fig. 5.2c**).

Response to abscisic acid GO:0009737				
		FRKM (2PFA_Light)		
Gene	Computational_description	3H	24H	48H
AT1G05100	mitogen-activated protein kinase kinase kinase 18	0.9	2.8	6.3
AT2G46510	ABA-inducible BHLH-type transcription factor	2.0	2.3	2.1
AT4G00700	C2 calcium/lipid-binding plant phosphoribosyltransferase family protein	1.5	2.4	5.2
AT4G02380	senescence-associated gene 21	0.9	2.5	5.3
AT4G23220	cysteine-rich RECEPTOR-like kinase	1.3	5.5	5.6
AT4G37370	cytochrome P450, family 81, subfamily D, polypeptide 8	1.2	4.7	6.3
AT5G59550	zinc finger C3HC4-type RING finger family protein	2.6	3.7	2.4

Table. 5.2. Genes enriched in "Response to abscisic acid" that were upregulated in the 2PFA plants under light conditions.
Time-series FRKM expression levels of genes that were upregulated in the 2PFA plants under light conditions at 3, 24, and 48 h, relative to CAplus_0h. The genes were enriched "Response to abscisic acid" by GO enrichment analysis as shown in **Fig. 5.5**.

Abscisic acid-activated signaling pathway GO:0009738				
		FRKM (2PFA_Light)		
Gene	Computational_description	3H	24H	48H
AT2G38310	PYR1-like 4	0.5	0.1	0.1
AT2G40330	PYR1-like 6	0.5	0.1	0.1
AT5G05440	Polyketide cyclase/dehydrase and lipid transport superfamily protein	0.6	0.1	0.2

Table. 5.3. Genes enriched in "Abscisic acid-activated signaling pathway" that were downregulated in the 2PFA plants under light conditions.
Time-series FRKM expression levels of genes that were upregulated in the 2PFA plants under light conditions at 3, 24 and 48 h, relative to CAplus_0h. The genes were enriched "Abscisic acid-activated signaling pathway" by GO enrichment analysis as shown in **Fig. 5.5**.

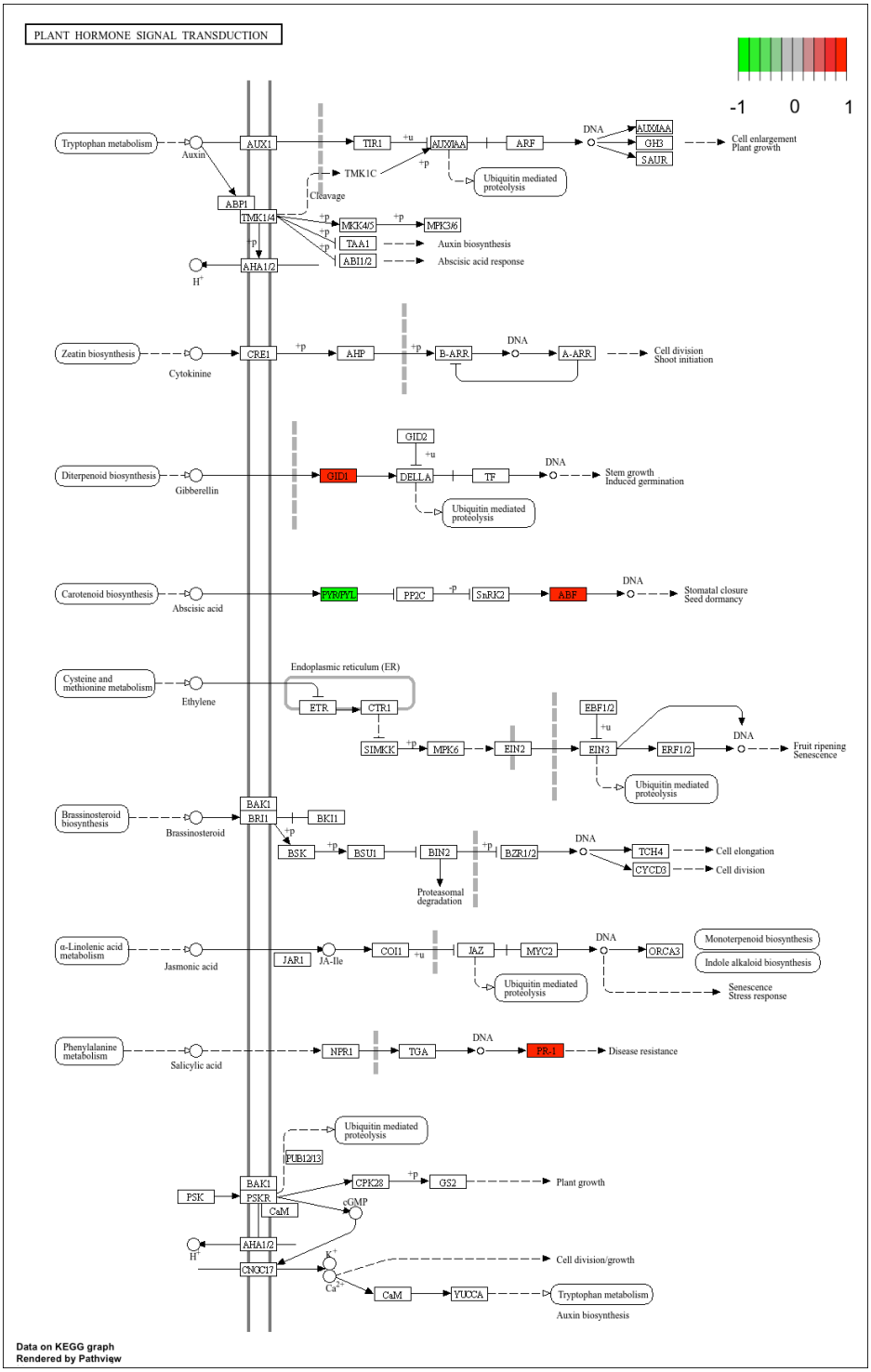


Fig. 5.6. Gene mapped into "Plant hormone signal transduction" that were up-or down-regulated by 2PFA treatment under light conditions.

KEGG metabolic map of "Plant hormone signal transduction". The color bar indicates $\log_2$ fold change of gene expression, and red indicates elevated expression and green indicates suppressed expression. The color showed mapped genes into the pathway out of 122 DEGs (105 upregulated and 17 downregulated genes) that were found solely in response to 2PFA treatment under light conditions.

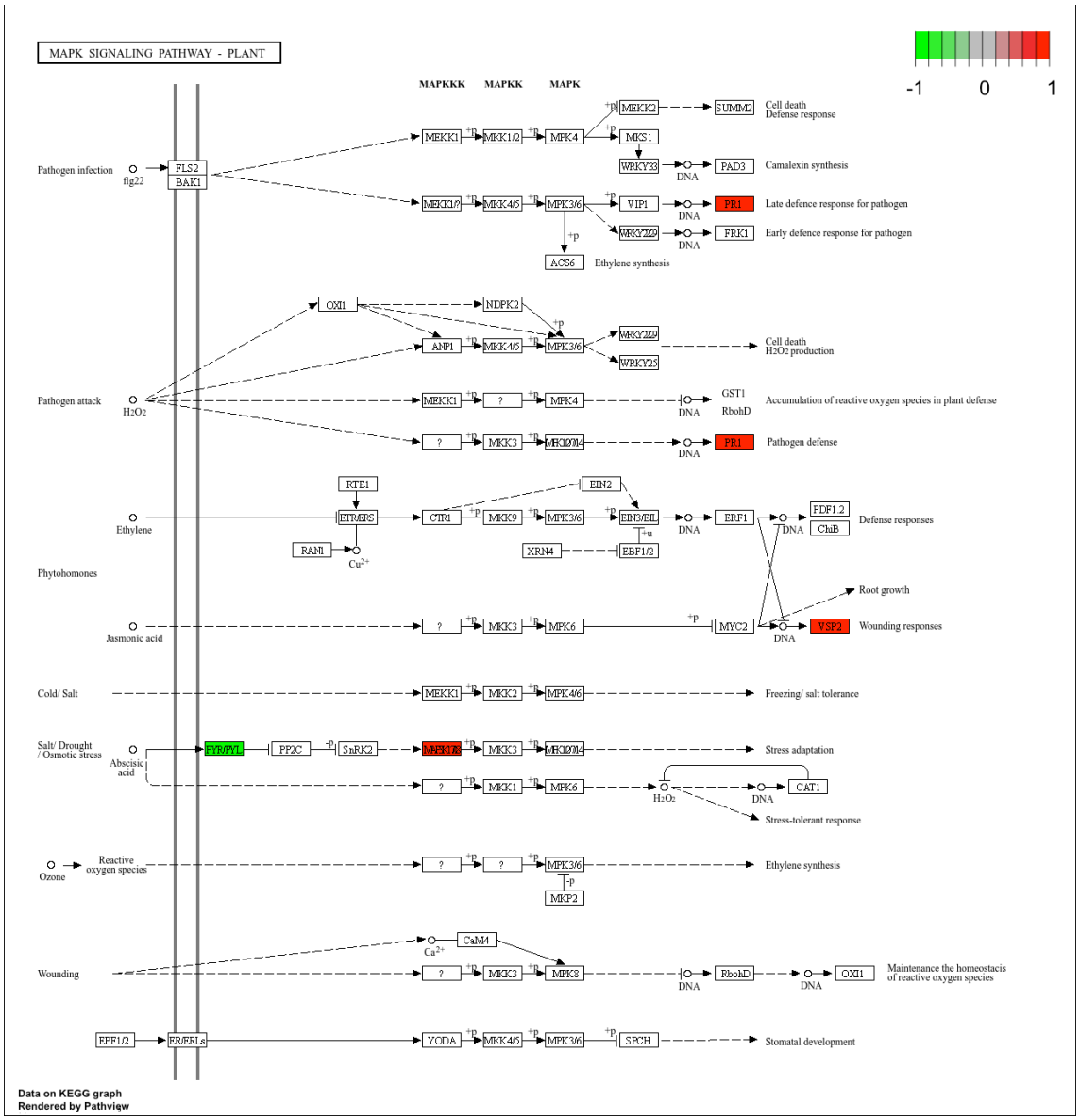


Fig. 5.7. Gene mapped into "MAPK signaling pathway" that were up-or down-regulated by 2PFA treatment under light conditions.

KEGG metabolic map of "MAPK signaling pathway". The color bar indicates $\log_2$ fold change of gene expression, and red indicates elevated expression and green indicates suppressed expression. The color showed mapped genes into the pathway out of 122 DEGs (105 upregulated and 17 downregulated genes) that were found solely in response to 2PFA treatment under light conditions.

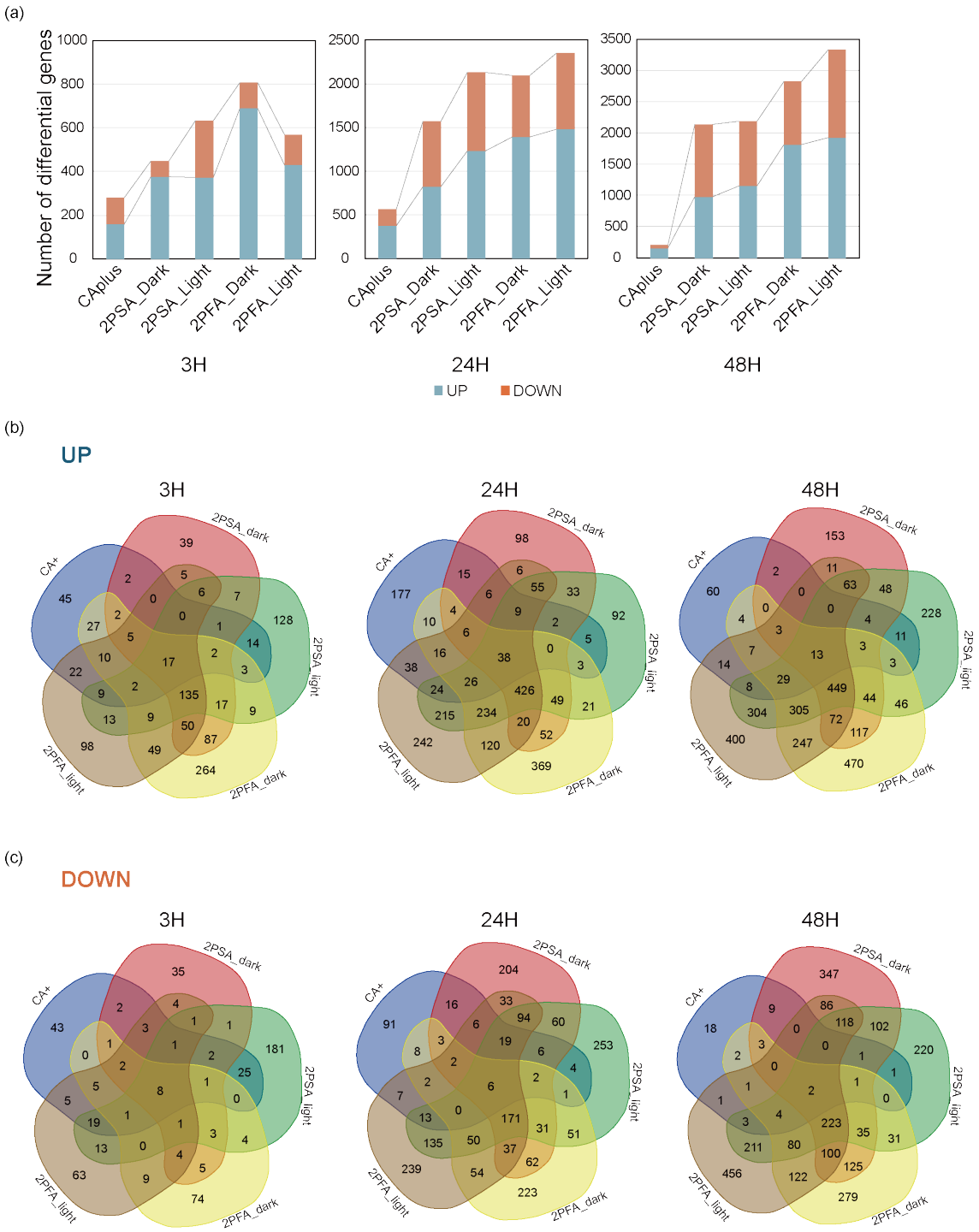


Fig. 5.8. Time-series expression levels of genes that were regulated by 2P treatment.
(a) The up-or down-regulated genes in CA+ and 2P plants in time-course series (3, 24, and 48 h) identified by likelihood ratio test (LRT) (FDR < 0.05, log fold-change > 1, < −1) against CA+_0h. (b) Venn diagram showing the numbers of the overlapped genes of that were detected in Figure (a) among the treatments.

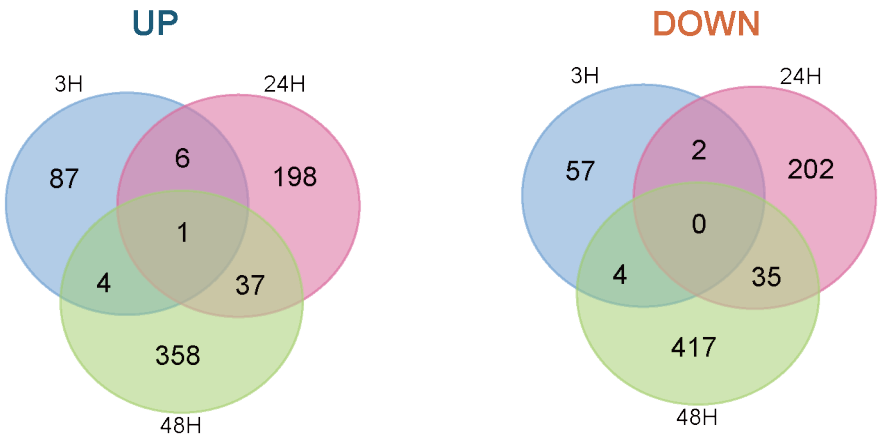


Fig. 5.9. Venn diagram showing the numbers of the DEGs that were found to be regulated only in the 2PFA plants under light conditions.
Three-group comparison of time-series expression levels of the DEGs that were up-or down-(up: 98 genes at 3 h, 242 genes at 24 h, and 400 genes at 48 h, down: 63 genes at 3 h, 239 genes at 24 h, and 456 genes at 48 h) regulated in 2PFA plants under the light conditions from **Fig. 5.8.b.c**.

Chapter6

General discussion

In this thesis, I sought to elucidate the molecular mechanisms of 2PH in order to understand the overwintering mechanisms of plants. Specifically, the study revealed the following.

1) Physiological conditions to induce 2PH with ice crystallization.

Ice nucleation in the process of 2PH significantly increased freezing tolerance. I named them either second-phase freezing acclimation (2PFA) or second-phase supercooling acclimation (2PSA) to distinguish the 2PH process with and without ice, respectively. In this study, in particular, I focused on 2PFA mechanism since it exhibits higher freezing tolerance than 2PSA after 2PH process.

1) Physiological conditions to induce 2PH with ice crystallization.

[1] The effect of duration of 2PH with ice crystallization on freezing tolerance (Chapter2).

During 2PFA treatment, dark conditions increased plant freezing tolerance within 1 days, and light conditions required for 3-day treatment.

[2] The effect of light and changing in photoperiod during 2PH with ice crystallization on freezing tolerance (Chapter2).

Light illumination during 2PFA treatment increased further freezing tolerance compared with dark conditions, while changing in photoperiods had no effect on freezing tolerance.

2) Molecular mechanism of 2PH with ice crystallization under light conditions.

[3] The expression of the *CBF* regulon in 2PH (Chapter3).

Cold-inducible CBF regulon was not up-regulated in 2PFA plants under light conditions.

[4] Role of the electron transport system in chloroplast in the process of 2PH with ice crystallization under light conditions (Chapter3).

Electron transport system is active during 2PFA, and this signal increase their freezing tolerance.

[5] Role of photoreceptors in the process of 2PH with ice crystallization under light conditions (Chapter3).

Phototropin and phytochrome were involved in the process of 2PFA under light conditions.

[6] Sugar content during 2PH process and regulation of transcript levels of genes (Chapter4).

In 2PFA under light conditions, sugar contents increased, and the transcript levels of genes related to sugar synthesis was regulated.

[7] Large-scale expression analysis for comprehensive understanding of 2PH mechanisms (Chapter4,5).

The transcript levels of genes related to sugar synthesis cell wall polysaccharide synthesis and cross-linking, and signaling pathway of plant hormones such as abscisic acid and gibberellin was regulated in the 2PFA plants under light conditions.

The results of these experiments are discussed.

There are four patterns in 2PH

In the previous experiment, two 2PH treatment conditions were tested in the dark conditions: one in which agar was frozen and the other in which it was not frozen. In this thesis, I tested the effect of two more 2PH treatment conditions i.e., light conditions on freezing tolerance. The light conditions increased the effects of 2PH. Thus, freezing and light play important roles for accelerating the effect of 2PH, indicating that there are four patterns in 2PH. The four patterns are: no freezing (2PSA), in the (1) dark and (2) light conditions, and with freezing (2PFA), in the (3) dark and (4) light conditions. From September 2022 to April 2023 in Morioka as shown in **Fig.5.1**, temperatures began to gradually drop from late September to December. During this period, overwintering plants increase their tolerance to the first phase of freezing through CA process (**Fig.5.1**). Over December, minimum temperatures at sub-zero temperatures. Presumably, overwintering CA plants may have acquired freezing tolerance through the 2P patterns beginning this time of the year. Since December often has mild freezing temperatures during the night, it is likely to start with pattern (1), "2PSA in the dark condition," among the 2P patterns. As the temperature drops below freezing during even morning, plants increase their freezing tolerance through the pattern 2 of the 2PH, "2PSA in the light conditions". Also, as **Fig1.1** shows, freezing due to radiative cooling, causing the pattern (3), "2PFA in the light

condition" and (4), "2PFA in the dark condition". Thus, various patterns of the 2PH occur in response to these temperature changes and freezing environments.

In this doctoral dissertation, in the four patterns, I focused on the physiological conditions under which 2PFA occurs and the molecular mechanisms. As mentioned in the discussion of Chapter 2, the results of **Figs. 2.2, 2.3** show that freezing tolerance was similar in the light and dark conditions after 1 day of 2PFA treatment, suggesting that the freezing tolerance mechanism acquired during 1 day of 2PFA treatment is common between the light and dark conditions. Since freezing tolerance furtherer increased in light condition 2PFA plants after 3 days of 2PFA treatment, freezing tolerance is enhanced by mechanisms that differs from dark conditions. Indeed, osmotic pressure measurements and RNA-seq results show that in 2PFA plants, osmotic pressure increased and genes, related to sugar synthesis were up regulated at 24 and 48 h, indicating that sugar accumulation is expected. These results suggest that there are two steps in the process of 2PFA. First step is that it takes place over within one day during 2PFA process, which is a common mechanism between treatment under light and dark conditions. Osmotic pressure increased in the 2PFA plants under the light conditions, but not in plants under the dark conditions compared to CA+ plants although there was no significant difference in freezing tolerance between the two treatment plants. Thus, the 1-day 2PFA treatment may not be a process that is associated with the accumulation of sugars. The second step is a process that occurs after 1 day, which requires light conditions. This step involves the accumulation of soluble sugars such as galactinol, raffinose, trehalose, and glucose. Although there are many unknowns about the function of soluble sugars under freezing stress, sugars are expected to function as (1) an osmotic pressure regulator to prevent rapid dehydration under low temperature and freezing stress, (2) a membrane protectant against mechanical stress caused by ice crystals, (3) a reactive oxygen scavenger, (4) a cell wall constituent sugar. Through these functions, 2PFA plants under light conditions after 1 day may further increase freezing tolerance.

In this doctoral dissertation, during 2PH, experimental conditions such as temperature and freezing were kept constant. During CA, the freezing tolerance to be acquired depends on changes in temperature, light intensity, and light quality. In the future, I would like to further elucidate the 2PH conditions by performing at different sub-zero temperatures and under various light conditions such as changing in light intensity and light quality during 2PH. Furthermore, in an outdoor environment, one would expect repeated freezing and thawing, accompanied by temperature changes. A previous study showed that the experience with freeze-thaw cycles is involved in increasing freezing tolerance in winter wheat (Skinner and Bellinger,

2016). Therefore, it will be interesting to determine the effect of freezing- thaw cycle during 2PFA.

In blueberries, which have high freezing tolerance, plant-derived epidermal nucleation substances have been isolated (Kishimoto et al., 2014). Thus, some woody plants can freeze spontaneously at sub-zero temperatures (Ishikawa 2014). Conversely, in herbaceous plants, plant-derived surface nucleation substances have not yet been founding so far (Ishikawa 2014). Therefore, for herbaceous plants, ice propagation into the plants starts from snow or frost on the leaf surface (Ishikawa 2014). It is also quite possible that soil frost as shown in **Fig. 1.1**, may propagate freezing into plants root. In this doctoral dissertation, it is not known if that plant was frozen during 2PFA treatment. It would be interesting to investigate if the specific tissues to be frozen and the order in which they are frozen are important for induction of 2PFA induction.

As mentioned in the discussion of Chapter 2, the results of Fig. 2 show that the difference in LT50 between the light and dark conditions of 2PSA and the light and dark conditions of 2PFA is different, indicating that different mechanisms are occurring in the 2PFA light and 2PSA light conditions. In fact, 2PSA is not subjected to freeze-derived desiccation, dehydration, or osmotic stress, and the increase in gene expression of *GOLS2* derived from desiccation stress does not occur in the 2PSA treatment. In addition, osmotic pressure measurements indicate that the osmotic pressure in the 2PFA light condition is higher than that in the 2PSA light condition. Freezing is trigger for the accumulation of soluble sugar when CA plants are exposed to light.

Behavior of woody and herbaceous plants at sub-zero temperatures

Previous studies have reported that in woody plants including apple and *Cornus stolonifera*, cold acclimation is followed by a second stage of increased freezing tolerance by frost (Huystee et al., 1967; Kuroda et al., 1990; Weiser, 1970). This phenomenon is similar to the 2PFA that occurred in the herbaceous plant, Arabidopsis thaliana, in this study. CA plants acquired freezing tolerance by exposure to sub-zero temperature with or without ice nucleation, and this effect was enhanced by freezing treatment in Arabidopsis (**Fig. 1.3, Fig. 2.4,** Sugita et al., 2024).

Regarding the second stage of acclimation with freezing in woody plants, the plants are exposed to both short-day and freezing conditions in the field at this time (Weiser, 1970). For this study, the results of the freezing tolerance test of 2PFA under light conditions showed that changes in photoperiod had no effect on freezing tolerance (**Fig. 2.3**). This suggested that freezing signals, but not changing in photoperiods, are important for increasing freezing

tolerance in cold-acclimated herbaceous plants, regardless of daylength, but I do not know the case of woody plants.

Can photosynthesis take place in a frozen environment?

The results in this study indicate that under 2PFA light conditions, the plants may accumulate sugars through photosynthesis. However, it is highly likely that the stomata is closed before freezing to prevent desiccation due to freezing in the first place. Therefore, the gas exchange necessary for photosynthesis cannot take place via the stomata. There is a possibility is that the gas exchange and photosynthesizing leaves are dependent on leaf age during 2PFA process. In previous studies revealed that young leaves are less likely to freeze than older leaves in wheat, barley and shoots of *Buxus sempervirens* (Hacker and Neuner 2007; Livingston et al., 2018; Pearce and Fuller; 2001). Therefore, young leaves rather than old leaves may be able to maintain a supercooled state, which enable to be exchange gases, and photosynthesize to produce sugars and energy. In fact, in 2PFA plants under light conditions after the freezing test, the outer leaves, i.e., the older leaves, are damaged more and the central leaves, i.e., the younger leaves, are less damaged (**Fig. 3.10**). Osmotic pressure measurement, sugar analysis and RNAseq analysis results indicate that freeze-induced drought stress induces the accumulation sugars in 2PFA plants under light conditions through regulation of the expression such as *GOLS2*. The rapid sensing of freezing signals in old leaves may lead to accumulation of sugars in young leaves through gene regulation via signaling such as calcium. Alternatively, sugar may be produced in the old leaves and accumulated in the young leaves through transporters. To verify this, it would be interesting to investigate the differences in gene expression of *GOLS2*, sugar accumulation and freezing tolerance in old and young leaves, respectively.

However, regardless of leaf age, the 2PFA process still results in the closure of the stomata in many leaves, which exposes them to low carbon dioxide (CO_2) stress. Previous studies have reported that under low CO_2 conditions, the green alga *Chlamydomonas reinhardtii* maintain photosynthesis by converting carbonate ions to CO_2 in chloroplasts, which is termed as CO_2-concentrating mechanism (CCM) (Wang et al., 2016). Thus, there is a possibility that 2PFA plants are able to produce the CO_2 needed for photosynthesis by CCM without gas exchange through stomata cells.

Conversely, photosynthesis during 2PFA process under the light conditions may be dependent on the freezing step. It has been reported that there are two freezing steps in various plants: first, vascular bundles and second, ice propagation in the intercellular spaces or intracellularly. Even potatoes, which are less tolerant to freezing, show that gas exchange was

occurred when ice was formed in vascular bundles, i.e., step 1 before ice propagation in the intracellularly (Stegner et al., 2022). Thus, plants that freeze extracellularly, which means high freezing tolerance plants, during ice propagation in Step 1 may be undergoing gas exchange. In addition, it is expected that the Antarctic liverwort *Marchantia berteroana and* spinach perform photosynthesis under freezing conditions (Davey and Rothery, 1997; Murakami et al., 2021) and spinach, it cannot be completely ruled out that Arabidopsis also performs photosynthesis at sub-zero temperatures.

In conclusion, I have demonstrated that in Arabidopsis, plants increase freezing tolerance by perceiving the signal of freezing and light through accumulation of soluble sugars. This is likely to be occurred not only in the laboratory, but also in the outdoors, as shown in **Fig. 1.1**. Understanding the molecular mechanism of 2PFA will help lead to increased crop yields by reducing frost damage and expanding the range and timing of crop growth.

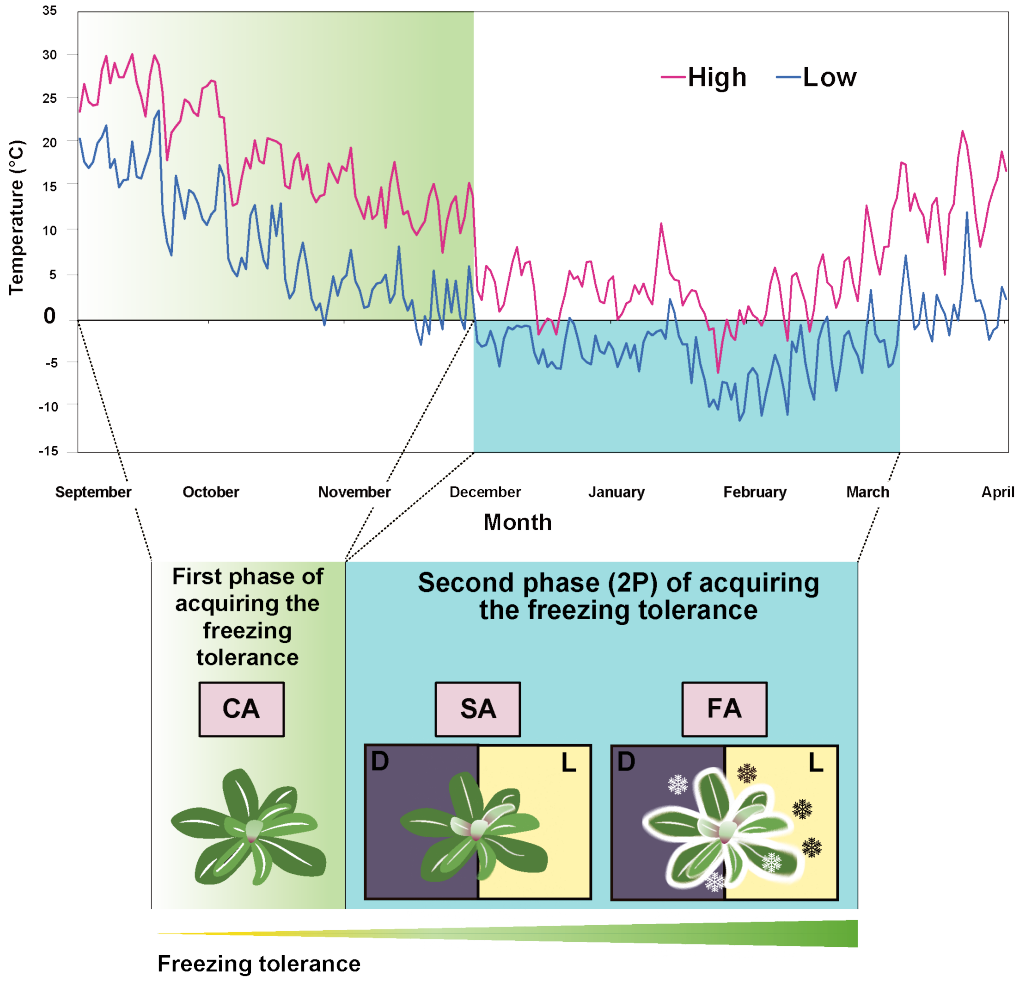


Fig.6.1. Proposed model of second phase processes in herbaceous plants at sub-zero temperatures during overwintering.
Freezing tolerance of plants is established through exposure to non-freezing temperatures from about fall as the first phase (cold acclimation, CA). From late fall and early winter, as in previous reports, the second phase (2P) process works at sub-zero temperatures, and our experiments revealed that there are four patterns in how the 2P process works. The effects of the 2P process are stronger when combined with ice crystal formation (2PFA) than when supercooled (2PSA), and the molecular pathways between them are also different. Furthermore, while these two 2P processes also works in the dark, the presence of light further enhances freezing tolerance via different molecular pathways than in the dark. As an example of temperature data, which were obtained from the Japan Meteorological Agency (https://www.jma.go.jp/jma/index.html), the maximum (highest) and minimum (lowest) temperatures from September 2022 to April 2023 in Morioka are shown in the figure.

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