STUDIES ON THE VARIETAL DIFFERENTIATION OF FROST RESISTANCE OF THE TEA PLANT

IV. The effects of sugar level combined with protein in chloroplasts on the frost resistance

Noriko Sugiyama and Takasi Simura
Faculty of Agriculture, Nagoya University

Synopsis. In connection with frost resistance on the role of sugars in the plant cells, it becomes necessary to investigate the relation among chloroplasts soluble protein content, damage of the protein by freezing and sugar level in this protein, during the frost-hardening season. It was found that the chloroplast soluble protein content, the sugar level in this protein, and the resistance against freezing of this protein increased with the increase of frost resistance, but the injury of chloroplasts was almost constant despite of the increase of frost resistance.

Introduction
The phenomenon of frost resistance in the plant has been held the interest of many investigators for a number of years. The typical component of hardening process is the sugar which leads to high osmotic concentration and resistance of cells against freezing. On the other hand, the soluble protein in the cytoplasm increases with the increase of frost resistance (Siminovitch et al., 1953; Parker, 1960; Sugiyama and Simura, 1965). A large portion of soluble protein consists of chloroplast soluble protein (Wilmsdorff and Jorgendorf, 1952). Chloroplast soluble protein content is coincident with frost resistance (Sugiyama and Simura, 1966).

The present studies have been designed to elucidate the reason why the chloroplast soluble protein is resistant against freezing.

Materials and Methods
As described in the previous papers (1965 and 1966), Yabukita, U-22 and Y-3 of Japanese origin, Benihomare and Kyann of Indian origin which were grown in the field were used for these experiments.

Chloroplast soluble protein, chloroplast injury and frost resistance of the plant were determined by the method of Sugiyama and Simura (1966).

Determination of chloroplast soluble protein injury: 3 ml of chloroplast soluble protein was frozen at -20°C for 17 hours and the remainder of it was kept at 6°C for 17 hours as control. They were centrifuged at 5000g/15 min. The supernatant and residue were determined by Kjeldahl digestion. We considered the residue as protein injured by freezing and the supernatant as not injured protein.

Determination of sugars content in chloroplast soluble protein: The chloroplast soluble protein was hydrolyzed by 2 N-HCl at 120°C for 17 hours and then was determined by usual method using colorimetric method.

Determination of sugar content in leaves: 1 g of dry weight was heated boiling with 80 % of ethanol with a trace of CaCO₃ for an hour. This procedure was repeated 5 times until the residue was colorless. Collected extracts were concentrated in vacuum at 36°C up to 10 ml. 0.3 g of Pb-acetate was added into this 10 ml extracts and kept at 0°C to remove tannin. The supernatant was mixed with 1.5 g of IR-4B and 1.5 g of IR-120, and stirred in ice for 30 minutes and then adjusted with HCl to pH 7.0. These were passed through Buchner funnel and concentrated in vacuum at 36°C up to 5 ml. 4 ml of this extract was added to 0.01 M Borax. This sample was applied to Dowex 1 × 8 column (0.9 × 9 cm) which were washed with 100 ml of 0.1 N HCl, 120 ml of 0.1 M Borax, and 100 ml of H₂O. Sucrose, raffinose, stachyose, glucose, and fructose were fractionated by various concentration of Borax ( Sodium Borate). Sucrose, stachyose and raffinose were determined by concentrated sulfuric acid-5 % phenol.

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Fig. 1. Seasonal change of chloroplast soluble protein contained in leaves coming from five varieties.

Fig. 2. Seasonal change of the resistance of chloroplast soluble protein against freezing coming from five varieties.

Fig. 3. Seasonal change of the resistance of chloroplasts against freezing.

Experimental Results

As shown in Fig. 1, the ratio of chloroplast soluble protein to total soluble protein increased from the end of October to the beginning of November in five varieties of the different frost resistance. This period almost agreed with that of increase of frost resistance. Up to April the ratio was constant and then decreased with frost resistance.

On the contrary, denaturation of chloroplast soluble protein by freezing was proportional to frost injury, and furthermore, there were some differences of denaturation among varieties, and the resistant varieties showed lower denaturation than the less resistant ones (Fig. 2).

The resistance of chloroplasts against freezing also increased from the end of October. However, the difference of frost resistance was not so remarkable among five varieties (Fig. 3).

Our previous paper (1966) suggested that the role of sugars was important to be protective against freezing. Both the chloroplast soluble and the chloroplast insoluble proteins contained sugars, although the latter protein was sensitive against freezing. Sugar content in chloroplast soluble protein increased with the increase of frost resistance (Fig. 4 and 5). But sugar content of chloroplast insoluble protein did not increase and was constant for
a year (Fig. 5). Sugar content in chloroplast soluble protein was more abundant in resistant varieties than in less resistant varieties. However, sugar content in chloroplast insoluble protein was not so different among five varieties. Nevertheless, the ratio of sugars in chloroplast soluble protein to total sugars increased with the increase of frost resistance, although the ratio is of low value (Fig. 6). The results of determination revealed that the total sugars began to increase about 2 weeks before the increase of sugars in chloroplast soluble protein.

During the period of higher frost-hardening in the plant, the rate of sucrose to total sugars is higher than the other sugars, such as glucose, fructose, stachyose and raffinose, all of which did not increase with the increase of frost resistance (Fig. 7, 8, 9, and 10). These phenomena appeared in all five varieties. Furthermore the frost resistant varieties were inclined to contain higher concentration of sugars.

**Discussion**

Chloroplast soluble protein increased with the increase of frost resistance (Table 1, Fig. 1). On the other hand, HEBER (1959) found that the volume of chloroplast increased with the increase of frost hardness, according to his observation by microscope. SMURA (1961) also has observed that in comparison of frost resistant varieties with frost sensitive varieties, the volume of the chloroplast was larger in the former than in the latter. The authors' observations of the tea plant agreed with
their results. Therefore, the authors are able to propose that the increase of the volume of chloroplast is induced by the increase of content of chloroplast soluble protein, and the increase of protein is coincident with that of the frost resistance.

By our experiment, chloroplast soluble protein was stable against low temperature (−20°C), and with the increase of frost resistance the stability of chloroplast soluble protein gradually increased (Fig. 2). According to HEBER (1964), the part of soluble enzyme in chloroplast in wheat leaves, may be included in chloroplast soluble protein, and is stable against freezing. However, the chloroplasts themselves are not stable against freezing even during the period of increasing frost resistance (Fig. 3). As it is supposed that there is another protein in chloroplasts and this protein is very unstable against low temperature. LOVELOCK (1959) reported that unstable protein against low temperature was lipoprotein in animal cells. It is supposed that the chloroplast insoluble protein may be lipoprotein. 70% of a chloroplast protein is consisted of insoluble protein (GRANIC 1960). Although the chloroplast soluble protein (resistant protein against freezing) increased, chloroplasts themselves were not so resistant against freezing. However, it is not clear the relation between frost resistance and lipoprotein.

Certainly, the leaves frozen at −20°C for 5 hours and then kept at 14°C for 2 days look brown–black, and this results may be caused by the destruction of chlorophyll.

On the other hand, it arises a question why the chloroplast soluble protein is stable against freezing. The sugars are said to play a protective action against freezing, and this protein is supposed to be combined with sugars. In fact, this protein was combined with sugars (Fig. 4 and 5). The content of sugars in chloroplast insoluble protein was very low. From this view, it is suggested that the role of sugars is important to be protective against freezing in chloroplasts.

Nevertheless, the ratio of sugars in chloroplast soluble protein to total sugars in cells was very low. It remains a question where sugars as is coincident with frost resistance accumulated in a cell or a tissue.

The content of sucrose increased with the increase of the frost resistance (Figs. 7, 8, 9 and 10). This result agreed with SAKAI's (1960), LEVITT's (1956), and HEBER's (1958). Both raffinose and stachyose also increased, although glucose and fructose decreased with the increase of frost resistance.

It is an interesting problem where large part of sucrose in the cells is distributed. The sugars may be accumulated in cytoplasm or vacuoles. The cell with frost resistance has grown fully mature, so it has a number of vacuoles.

**Summary**

The major protein of tea leaves, namely chloroplast soluble protein was extracted from five varieties of different frost resistance, Yabukita, U–22, Y–3, Benihomare and Kyann.

The chloroplast soluble protein is characterized as follows: This protein increased with the frost resistance and was not injured by freezing. The resistance of chloroplast soluble protein against freezing was essentially similar to the frost resistance of the tea plant. This protein is combined with sugars and the sugars in this protein increased with the increase of frost resistance.

On the other hand, the sugars combined with chloroplast insoluble protein were lowerly accumulated and not related to the increase of frost resistance. The chloroplasts which contain both insoluble and soluble proteins were severely injured by freezing.

In tea leaves sucrose content forms a large percentage of total sugars, while both raffinose and stachyose were little contained. The increase of all these sugars was coincident with the increase of frost resistance. However, glucose and fructose content decreased with the increase of frost resistance.
茶樹の耐凍性の品種間差異に関する研究

IV. 業木体中の蛋白質と結合している糖含量が耐凍性に及ぼす影響

杉山範子・志村篤
（名古屋大学農学部）

筆者らは、先の実験により耐凍性の増大と共に業木体の形態及び構成成分が変化することを報告した。業木蛋白質である業木体水溶性蛋白質が、耐凍性の異なる品種の宇都波、U-22、Y-3、ベニホマト、Kyannから抽出された。その結果業木体水溶性蛋白質がそのような特性を持ち耐凍性に関与することがわかった。

（1）この蛋白質含量は耐凍性ともに増加する。この蛋白質は -20℃ に 3 時間放置しても変性しなかった。しかし、業木体水溶性蛋白質の凍結に対する抵抗性の強い品種は耐凍性も強いことが観察される。

（2）この蛋白質と結合している糖は耐凍性増大時期に増加しはじめめる。耐凍性の弱い品種は、この糖含量が少ない。

（3）業木体の凍結抵抗性は耐凍性とともに増加する。業木体は凍結（-20℃）によって被害をうけやすい。業木体の凍結抵抗性の品種間差異は全くみられなかった。

（4）業木体を構成する分子の一つの蛋白質として、業木体不溶性蛋白質が厚い凍結層を形成する。焦糖及びラフノースが不溶性蛋白質と強く結合している。これらの糖の不溶性は、耐凍性増大に影響を及ぼす。これらの糖含量が多い品種ほど耐凍性が強い。一方 glucose と fructose 含量は耐凍性の増大に伴って減少した。