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

III. With special emphasis on the relation between their frost resistance and chloroplast soluble protein

Noriko SUGIYAMA and Takasi SIMURA

Faculty of Agriculture, Nagoya University

Synopsis. A considerable attention has been paid to elucidate the relation between frost resistance and chloroplast soluble protein which is 70% of total soluble protein of leaves in the tea plant. The authors have found that the ratio of chloroplast soluble protein to total soluble protein is higher in resistant variety than in less resistant variety, and damage of chloroplasts by freezing is proportional to frost injury. Additionally, chloroplasts suspended in sugar solutions are not injured by freezing.

Introduction

In recent ten years, studies of the cause of frost resistance of the plants have been repeated by measuring different biochemical components of the plant cells such as sugars, nitrogens, SH-group, activity of enzymes and adenosine triphosphate, and further by observing different conditions of chloroplasts. In our laboratory, SUGIYAMA and SIMURA (1966) have found the following interesting relations between the frost resistance and soluble protein of the tea leaves:

- 1) With the increase of frost resistance, soluble protein increases in quantity,
- 2) By artificial hardening, it is also increased,
- 3) It is more abundant in the resistant variety than in the moderately resistant and less resistant.

Biochemical studies in combination with electron microscopy have elucidated that a large portion -75%- of soluble protein in the cytoplasm is represented by protein of the stroma of chloroplasts (WILDMAN and BONNER 1947; WILDMAN and JARGENDORF 1952). The soluble protein, which we have previously examined in the leaves of the tea plant, may be also supposed to represent soluble protein of the stroma of chloroplasts. The present authors intend to elucidate the relation be-

tween the quantity of soluble protein of chloroplasts and the frost resistance of the tea plant.

Materials and Methods

Leaves from 5 varieties of the tea plant which have different frost resistance were used. The resistant variety, Yabukita of Japanese origin, the moderately resistant variety, Benihomare and slightly resistant variety, X-10 of Indian origin were grown in the field. N and T varieties (less resistant variety) which originated from seeds harvested in Burma in 1962 were grown in the green house. The experiment was performed from February to August 1965.

The quantitative determination of chloroplast soluble protein was made by the method of AKAZAWA et al. (1964). Chloroplast soluble protein and total protein were determined in the supernatant fraction by the usual method of Kjeldahl digestion.

Isolation of chloroplast was performed according to the method of KROGMANN and JARGENDORF (1957). Hill reaction was used to examine the *in vivo* and *in vitro* injuries by freezing of chloroplasts. For the *in vitro* injury experiments, chloroplasts prepared were suspended again into the same medium and frozen at -20°C for 3 hours and the others as control were kept at 6°C for 3 hours. For the *in vivo* injury experiment, chloroplasts

Received May 24, 1966

were prepared in the same manner from the leaves frozen at -20°C for 3 hours, and the control chloroplasts were prepared from the leaves kept at 6°C for 3 hours. The isolation of chloroplast to be examined for this reaction was performed according to the method of KROGMANN and JARGENDORF (1957).

In vitro and *in vivo* experiments to examine the role of sugar in freezing of chloroplasts were additionally performed. For the *in vitro* experiment, chloroplasts prepared were suspended to solutions of different sugars and to distilled water as control, and frozen at -20°C for 3 hours. For the *in vivo* experiment, leaves were immersed in vacuum in the same solution for artificial absorbing of the sugars, and then frozen at -20°C for 3 hours, while control leaves were immersed in distilled water in vacuum and then frozen at -20°C for 3 hours. Chloroplasts were prepared in the same manner from leaves, and Hill reaction

was examined on both treated and control chloroplasts.

Frost resistance of leaves of the tea plant was determined according to the method of SIMURA and SUGIYAMA (1965). Total sugars contained in the leaves were also analyzed by their method.

Experimental results

The relation between the quantity of chloroplast soluble protein and frost resistance: The quantity of chloroplast soluble protein was most abundant in Yabukita and was decreased in order in moderately resistant Benihomare and less resistant X-10, while it was more abundant in very slightly resistant N and T varieties than in Benihomare and X-10 (Table 1). However, the ratio of the chloroplast soluble protein content to total soluble protein in leaves of the 5 varieties were ranked in the following order: Yabukita, Benihomare, X-10,

Table 1. Chloroplast soluble protein contained in 5 varieties of different frost resistance

	Feb.-5			Feb.-23			Mar.-16			Mar.-27			Apr.-13			Apr.-23		
	C.S.* P.	T.P.** T.P.	C.S.P. T.P.	C.S.* P.	T.P.** T.P.	C.S.P. T.P.	C.S.* P.	T.P.** T.P.	C.S.P. T.P.	C.S.* P.	T.P.** T.P.	C.S.P. T.P.	C.S.* P.	T.P.** T.P.	C.S.P. T.P.	C.S.* P.	T.P.** T.P.	C.S.P. T.P.
			%			%			%			%			%			%
Yabu.	1.46	2.36	62	1.30	2.13	61	1.32	2.24	59	1.27	2.09	61	1.20	2.00	60	1.15	1.98	58
Beni.	0.99	1.70	58	0.92	1.65	56	0.90	1.64	55	0.89	1.62	55	0.91	1.65	55	0.77	1.45	53
X-10	0.80	1.46	55	0.85	1.52	56	0.70	1.32	53	0.78	1.40	56	0.71	1.31	54	0.67	1.28	52
N.	0.86	1.83	47	0.96	1.96	49	0.86	1.90	45	0.84	1.75	48	0.81	1.65	49	0.79	1.52	46
T.	0.90	1.96	46	0.97	2.03	48	0.89	1.93	46	0.91	1.89	48	0.80	1.70	47	0.71	1.48	48

* Chloroplast soluble protein. mgN/g fresh weight.

** Total protein. mgN/g fresh weight.

Table 2. Hill reaction of chloroplasts freezed after isolation (*in vitro*) and Hill reaction of chloroplasts isolated from frozen leaves (*in vivo*) in 3 varieties

	Yabukita				Benihomare				X-10			
	<i>in vitro</i>		<i>in vivo</i>		<i>in vitro</i>		<i>in vivo</i>		<i>in vitro</i>		<i>in vivo</i>	
	+6°C	-20°C	+6°C	-20°C	+6°C	-20°C	+6°C	-20°C	-6°C	-20°C	+6°C	-20°C
May-20	236*	54*	196*	12*	218*	60*	210*	15*	196*	52*	226*	18*
June-10	218	68	183	13	225	62	232	10	213	56	193	20
June-20	184	52	257	24	200	48	230	18	212	48	218	23
July-10	156	70	223	19	216	46	221	16	169	49	223	20
July-23	128	48	195	19	159	51	216	17	143	46	186	19
Aug.-11	120	45	198	21	140	48	218	20	171	51	209	17

* μ moles ferricyanide reduction /mg chlorophyll per 1 hour.

T. and N. varieties (Table 1). This relation was coincident with their frost resistance.

Injury of whole chloroplasts by freezing: The influence of freezing on the whole chloroplast was examined by Hill reaction. Table 2 shows *in vitro* and *in vivo* that the value of ferricyanide reduction of frozen chloroplasts was smaller than that of the controls, but that the value was greater *in vitro* than *in vivo*. Further more, Table 2 indicated that the frost resistance of the different varieties was not always coincident with their value of ferricyanide reduction.

The role of sugars in freezing: Table 3 shows that isolated chloroplasts were always protected *in vitro* by sugars from damage of freezing. By artificial absorption of sugars, leaves actually increased the content of sugars (Table 5). The leaves, when they were frozen, did not increase the frost resistance (Table 4). The chloroplast isolated from artificially sugar-absorbing leaves decreased ferricyanide reduction (Table 3).

Discussion

As described in the authors' papers (1965 and

Table 3. The relation between role of sugars and frost injury of chloroplasts in Benihomare expressed by Hill reaction

	Glucose		Fructose		Sucrose		Xylose		Raffinose	
	<i>in vivo</i>	<i>in vitro</i>	<i>in vivo</i>	<i>in vitro</i>	<i>in vivo</i>	<i>in vitro</i>	<i>in vivo</i>	<i>in vitro</i>	<i>in vivo</i>	<i>in vitro</i>
H ₂ O	23 *	58 *	29 *	53 *	25 *	49 *	28 *	47 *	28 *	46 *
0.1 M	32	236	32	210	32	295	29	223	22	200
0.3 M	38	246	25	212	36	288	33	236	20	220

control. *in vivo* : 35 *in vitro* : 48.

* μ moles ferricyanide reduction /mg chlorophyll per hour.

in vitro : Chloroplast were suspended in sugar solution and then frozen at -20°C for 3 hours.

in vivo : Leaves were artificially absorbed sugar solutions and then frozen at -20°C for 3 hours. After freezing, chloroplasts were isolated.

Table 4. Frost injury of leaves which were artificially absorbed sugar solutions by vacuum infiltration in 2 varieties

	Glucose		Fructose		Sucrose		Xylose		Raffinose	
	Yabu	Beni	Yabu	Beni	Yabu	Beni	Yabu	Beni	Yabu	Beni
H ₂ O	4 *	4 *	4 *	4 *	4 *	4 *	5 *	5 *	4 *	4 *
0.1 M	4	4	3	3	3	3	5	5	4	5
0.3 M	4	3	4	4	4	4	5	5	5	5

control (Natural condition) Yabu. 4, Beni. 4.

* Leaves were frozen at -20°C for 3 hours. Frost injury is graded from 0 to 10.

Table 5. The content of total sugars in leaves which were absorbed artificially sugar solutions by vacuum infiltration

	Glucose		Fructose		Sucrose		Xylose		Raffinose	
	Yabu	Beni	Yabu	Beni	Yabu	Beni	Yabu	Beni	Yabu	Beni
H ₂ O	59 *	56 *	50 *	53 *	48 *	40 *	58 *	52 *	41 *	43 *
0.1 M	68	61	58	61	60	52	61	59	58	55
0.3 M	85	78	81	88	85	83	85	88	80	79

control (Natural condition) Yabu 42, Beni 40.

* mg/g dry weight.

1966) the increase of soluble protein of tea leaves is coincident with the increase of frost resistance in the varieties. The present results showed that 55% of soluble protein related to the frost resistance is chloroplast soluble protein (Table 1). WILDMAN and BONNER (1947) and WILDMAN and JARGENDORF (1952) earlier reported in green plants that a large portion of total soluble protein in the cytoplasm consists of chloroplast soluble protein and HEBER (1960) also reported in wheat that 50% of total soluble protein is so. Recent electron microscopic observations have elucidated that the chloroplast is divisible into the stroma and grana, and further, combined with biochemistry, that chloroplast soluble protein is contained in the stroma which is the site of the dark reaction of photosynthesis (MENKE 1962; GRANIK 1960; PARK and PON 1961). As shown in the Table 1, the ratio of chloroplast soluble protein to total protein is proportional to the frost resistance of the varieties. This is suggested that chloroplast soluble protein is stable against frost and freezing. HEBER (1959) found in the wheat two kinds of proteins, frost resistant protein and frost sensitive protein, by fractionating by centrifugation at $28,000 \times G$. The present authors suppose that frost resistant protein found by him may correspond to the chloroplast soluble protein examined here.

In rice leaves, chloroplast soluble protein which was purified with sephadex G 75 and G 200, namely fraction 1 protein has been found to be glycoprotein (AKAZAWA, SAIO and SUGIYAMA 1965). From this, it is supposed that chloroplast soluble protein as isolated from tea leaves may be glycoprotein. JERMINAS (1965) found in wheat leaves that glycoprotein content is higher in frost resistant variety than in the slightly resistant. HEBER (1959) also observed that the glycoprotein in wheat leaves is more stable against freezing than the other proteins. These results may support indirectly the present observation of dependence of the frost resistance of the tea plant upon the quantity of chloroplast soluble protein in their leaves.

Table 3 shows that whole chloroplasts are injured by freezing and this agrees with HEBER's (1961). Frost injury of chloroplast has

been found to occur in the grana which contains insoluble protein, namely lipoprotein (GRANIK 1960; MENKE 1963). The lipoprotein has been reported also in animal cells to be sensitive to low temperature (LOVELOCK 1959). HEBER (1964) reported that the alternation of lipoprotein is caused by the frost injury and that the loss of adenosine triphosphate occurs owing to the alternation of lipoprotein and therefore phosphorylation is inactivated. SUGIYAMA and SIMURA (1966) determined insoluble protein content has not direct relation with the frost resistance. The present experiments shows that the content of chloroplast soluble protein which may be supposed to contain glycoprotein is proportional to the frost resistance in the five varieties (Table 1), and that the frost resistant variety Yabukita which contain most abundant chloroplast soluble protein is not injured by freezing. It is probable that the glycoprotein may protect lipoprotein from damage by frost.

It is well known that the increase of frost resistance depends upon the increase of sugar content in leaves of plants. Table 3 shows that the isolated chloroplast which were suspended in different sugar solutions and frozen at $-20^{\circ}C$, have still considerable activity even after 3 hours. However Table 3 and 4 show that the leaves which absorbed sugars by vacuum infiltration do not show frost resistance. As for difference of the role of sugars *in vitro* and *in vivo*, HEBER (1964) suggested that sugar contained in vacuoles are ineffective, or at best only of indirect influence. From this it is supposed that the sugars absorbed artificially by vacuum infiltration are accumulated in vacuoles, although LEVITT (1959) revealed that the dehydration of the cytoplasm is dependent on the sugar level in vacuoles. The results of present *in vitro* experiments agree with HEBER's results (1964), that sucrose is best protective against damage by freezing (Table 4), and it is supposed that the sugars absorbed into leaves might be contained in cytoplasm or vacuoles and be ineffective to the frost resistance (Table 5 and 6).

Summary

As a part of the studies on the frost resistance of the tea plant, the relation between their frost resistance and chloroplast soluble protein as well as chloroplasts was examined. The varieties of different frost resistance Yabukita (resistant variety), Benihomare (moderately resistant variety), X-10 (slightly resistant variety), N and T (very slightly resistant varieties) were used.

The ratio of chloroplast soluble protein to total protein are proportional with the frost resistance of the 5 varieties.

Hill reaction shows that the frost resistance of whole chloroplast both *in vitro* and *in vivo* in the different varieties was not always proportional with that of intact leaves.

Whole chloroplast suspended in sugar solutions are not injured by freezing, while intact leaves are injured by freezing, even though the leaves absorb sugars by vacuum infiltration.

It is a pleasure to thank Dr. T. AKAZAWA and Dr. T. TAKAHASHI, Nagoya University for helpful suggestions and encouragements during the course of this work.

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チヤの耐凍性の品種間差異に関する研究

Ⅲ 葉緑体蛋白質含量が耐凍性に及ぼす影響

杉山 範子・志村 喬

(名古屋大学農学部)

さきに著者らは、チヤの各品種が耐凍性の増大とともに、葉の水溶性蛋白質含量が増加することを報告した。一般にいろいろの緑色植物の葉で、水溶性蛋白質の多くが葉緑体中に含まれていることが明かにされ、著者らのチヤの葉の実験でも同じ知見を得ている。一方この蛋白質は光合成能に関与していることが多くの生化学者らによって実証されている。そこで著者らは、この葉緑体蛋白質含量と耐凍性との関係、ならびにこの蛋白質を含有する葉緑体の凍結による被害程度と耐凍性との関係、さらに蛋白質の凍結に対する糖の保護作用について実験を行つた。供試品種は、ヤブキタ(耐凍性強、静岡種)、ベニホマレ(耐凍性中位、インド種)、X-10(耐凍性弱、インド種)、これらは農場に栽培されている7~8年生の株である。NおよびTは、耐凍性が甚だ弱く、1962年ビルマで採取した種子を温室で栽培したものである。

耐凍性の強い品種は、葉緑体水溶性蛋白質含量の全水

溶性蛋白質含量に対する割合が高かつた。その順序は、ヤブキタ、ベニホマレ、X-10、NおよびTの順で、耐凍性の大きさの順序と一致している。

葉緑体の凍結による被害の程度の品種間差異は、耐凍性の品種間差異と常に一致しているとは限らない。また抽出した葉緑体を -20°C においたとき生じる被害より、葉を -20°C に放置した後抽出した葉緑体の方が、凍結による被害が大きかつた。

葉を切りとり、減圧して、葉柄から、glucose, sucrose, fructose, xylose および raffinose を吸収させたとき、葉の全糖含量は、対照区に比べて増加するが、耐凍性は増大しなかつた。しかし抽出した葉緑体を糖液につけ、 -20°C においたところ、葉緑体は凍結によつて殆ど害がなかつた。糖類中では、sucrose が凍結による葉緑体の被害を最も保護した。