

## Isozyme Analysis in Higo chrysanthemum (*Dendranthema grandiflora* Tzvelev)

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### Summary

Glutamate-oxaloacetate transaminase, phospho-gluco isomerase, and peroxidase in Higo chrysanthemum were electrophoretically analyzed in order to provide information for cultivar identification.

In all the 3 enzymes of the 29 cultivars used, a high degree of polymorphisms in isozymes were observed and each cultivar showed different zymogram from every other cultivar. In some cultivars zymograms exhibited the same numbers and positions of bands but had different staining intensities. Therefore, isozyme analysis of these 3 enzymes is available to identify the cultivars. It will be practically useful for the advancement of the preservation, propagation, and breeding of Higo chrysanthemum cultivars.

### Introduction

Higo chrysanthemum (*Dendranthema grandiflora* Tzvelev, Higo-giku in Japanese) is in nature a bedding flower and was once cultivated exclusively in Higo (the old name of Kumamoto Prefecture) of Kyushu Island during the regime of Shigetaka Hosokawa, the 8th feudal lord (Murayama, 1974). He encouraged the cultivation of Higo chrysanthemum to promote the moral education and to uplift the character of his clansmen and retainers. This peculiar chrysanthemum is characterized by the unique techniques of cultivation and arrangement which were strictly and originally established by the Hideshima School during the latter part of the 17th century. On the basis of the principles of Confucianism, the cultivars are planted in 3 rows in a zigzag style which resemble Heaven (back row), Man (middle row), and Earth (front row). The cultivars with large, medium, and small flowers correspond to these 3 rows, respectively. Moreover, in each row, yellow-, white-, and red-flowered cultivars with flat, tubular, or spoon-like ray florets are alternately arranged in the same sequence. Every 3 closely situated flowers on a plant

are arranged to form a right triangle.

Even now, the chances to cultivate and aesthetically appreciate Higo chrysanthemum are limited to Kumamoto Prefecture although these cultivars with elegant and dainty flowers have been cultivated continuously for almost 200 years. The establishment of convenient methods for the classification and preservation of Higo chrysanthemum cultivars is significant not only because they are a part of the products of the long history of Japanese horticulture but also because they are potential genetic resources for the improvement of modern chrysanthemums. However, it is difficult to identify many cultivars solely by their morphological characteristics. The emergence of new cultivars or mislabelling could also mislead breeders and growers during long period of cultivation.

It is the aim of the present study to analyze isozymes in order to provide information for identification of Higo chrysanthemum cultivars.

### Materials and Methods

Twenty-nine cultivars (Table 1) selected from the collection of Higo chrysanthemum in Kumamoto Prefectural Agricultural Research Center were used in this study. Different tissues, apical buds, young and old leaves, young and old stems, and

**Table 1.** Flower characteristics and chromosome numbers of Higo chrysanthemum cultivars.

Number	Cultivar		Flower		Chromosome number <sup>2</sup>
	Name	Size	Color	Petal shape	
1	Chihiro No Umi	Large	White	Flat, straight	54
2	Chiyo No Kotobuki	Large	Light magenta	Flat, twist	52,53,54
3	Hatsu Shigure	Large	White	Tubular, straight	54
4	Horen	Large	Rouge	Tubular, twist	54
5	Kin Kujaku	Large	Bright yellow	Flat, bilge-like	55
6	Kuni No Hikari	Large	Rouge	Tubular, twist	54
7	Tennyo No Mai	Large	Magenta	Flat, twist, bilge-like	55
8	Inkunshi	Medium	Golden yellow	Flat, twist	54
9	Kinyo	Medium	Golden yellow	Tubular, straight	55
10	Kojo No Tsuki	Medium	White	Flat, straight	56
11	Mado No Yuki	Medium	White	Tubular, straight	54
12	Miyuki No Matsuo	Medium	Lemon-yellow	Spoon-like, straight	54
13	Shiranui	Medium	Vermilion	Flat, twist	53
14	Tagoto No Tsuki	Medium	White	Flat, straight	54
15	Takaragasa	Medium	Lemon-yellow	Flat, straight	54
16	Yumeji Guruma	Medium	Snow-white	Flat, straight	54
17	Akebono No Hikari	Medium	Light rouge	Tubular, straight	54
18	Banshu	Medium	White	Flat, twist	54
19	Kimi Ga Sode	Small	Lemon-yellow	Flat, straight	54,55
20	Mikakimori	Small	Light rouge	Tubular, straight	54
21	Mine No Matsu	Small	Bronze	Tubular, straight	55
22	Momiji Gari	Small	Vermilion	Flat, straight	54
23	Nogio	Small	Lemon-yellow	Flat, straight	54
24	Nowake	Small	Vermilion	Flat, straight	55
25	Shuko	Small	Purplish red	Flat, straight	54
26	Yuki No Karasaki	Small	White	Tubular, straight	54
27	Zuisei	Small	Snow-white	Flat, straight	54
28	Beni Kosode	Small	Light magenta	Flat, straight	54
29	Harugasumi	Small	Pink	Tubular, straight	54

<sup>2</sup> Data taken from Miyazaki et al. (1982) except for cultivar Nos. 17, 18, 28, and 29 which are of present data.

roots from green-house grown plants, were used for the extraction of enzymes. Fresh tissues at 0.2~0.3g were ground using a pre-cooled mortar and pestle with 1 ml of extraction buffer. Centrifugation of ground tissues was from 10~15 min at 12,000 rpm. Supernatants obtained were loaded directly into the wells of the gels with a syringe at 20  $\mu$ l for each sample. Three enzyme systems were used: glutamate-oxaloacetate transaminase (E.C.2.6.1.1, GOT), phospho-gluco isomerase (E.C.5.3.1.9, PGI), and peroxidase (E.C.1.11.1.7, PER). In all the electrophoretic systems, Tris-glycine and Tris-HCl were the major components used in electrode and gel buffers, respectively. Polyacrylamide gels were all prepared at 7% concentration and electrophoretic flows were done in 4°C room. Extraction buffer, electrophoretic con-

dition, and isozyme activity staining solution for each enzyme are described below.

### 1. GOT

A buffer solution (0.3M Tris, 0.1M NaCl, 0.2M ascorbic acid, 2% Triton X-1000) was used for the extraction medium. Electrophoretic flow was allowed at 200V for 90 min and later at 300V for 4 hr. Bromophenol blue dye was used as marker. Gels were treated with the foregoing staining solutions. Potassium phosphate, monobasic and potassium phosphate, dibasic at 0.1M each were separately prepared in a 100 ml distilled water 15 min before the staining time. The former solution was added to the latter solution until pH reached to 7.4. Just before staining, 100 ml of the solution obtained was poured into a beaker containing

100 mg of aspartic acid, 100 mg of  $\alpha$ -ketoglutaric acid, and 150 mg of fast blue BB salt. Gels were incubated in the dark at 25°C for 60 min or until blue bands appeared. The reaction was stopped and fixed with 7% acetic acid. The method mentioned above was according to Hirai (personal communication).

2. *PGI*

The extraction buffer used was according to Roose and Gottlieb (1978). Electrophoresis of the extracts were carried out at 200V for 1 hr and later at 300V for 3 hr. The enzyme activity staining was according to Vallejos (1983).

3. *PER*

The extraction procedure and buffer were the same in GOT. Electrophoresis of supernatants were done continuously at 200V for 4 hr. Staining procedure was according to Etoh and Ogura (1980) except that 4-chloro-naphthol was used instead of 5-amino-2 naphthol.

**Results**

Of the tissues used, the youngest fully expanded leaves, apical buds, and young stems gave good results for GOT and PGI, while matured leaves for PER. Zymograms of the 3 enzymes from 29 cul-

tivars of Higo chrysanthemum are shown in Figs. 1-3. They showed considerable variations among cultivars. The details of the results in each enzyme are as follows:

1. *GOT*

Most bands were heavily concentrated on both cathodal and anodal portions of the gel and few were found in the intermediate portion. The lowest number of bands in a zymogram was 2 while the highest was 8. Twenty-one different bands were totally detected but only 1 band (band No. 18) was common to all cultivars. There were two cultivar groups (cultivar Nos. 9, 12, 13, 21, 25, 26 and 3, 4) in which cultivars showed the same numbers and positions of bands. Those cultivars, however, differed in band intensities. Thus, it was possible to find out the differences among all cultivars with this system.

2. *PGI*

Bands were intensely concentrated in the intermediate portion of the gel. The lowest and highest number of bands in a zymogram were 3 and 9, respectively. Fourteen different bands were observed but only one band (band No.9) was common to all cultivars. There were four cultivar groups (cultivar Nos. 8, 9; 2, 25, 26; 5, 29; and 10, 20) in

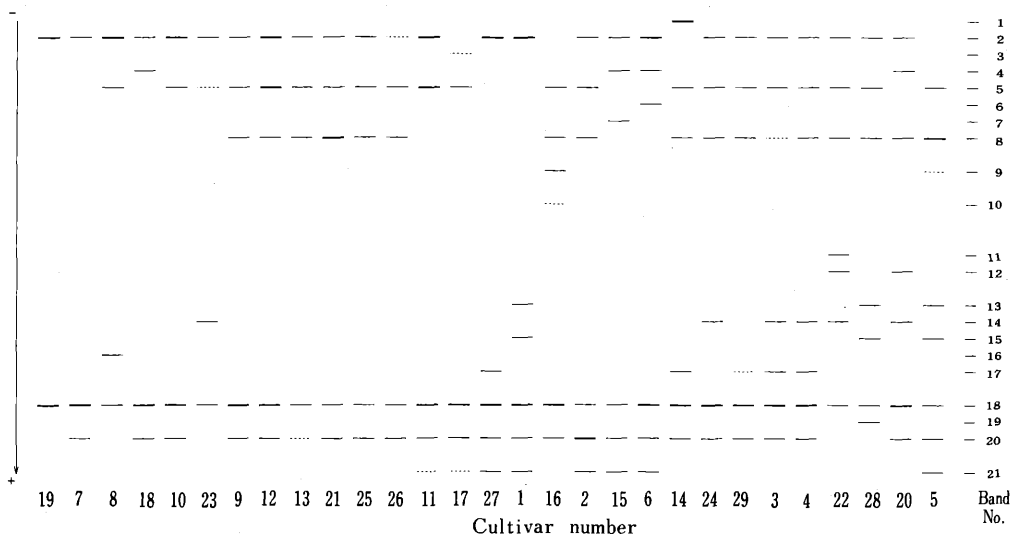


Fig. 1. Schematic presentation of GOT zymograms from 29 cultivars of Higo chrysanthemum. Zymograms are arranged from simple to complex according to numbers of bands. Cultivar names corresponding to cultivar numbers are shown in Table 1.

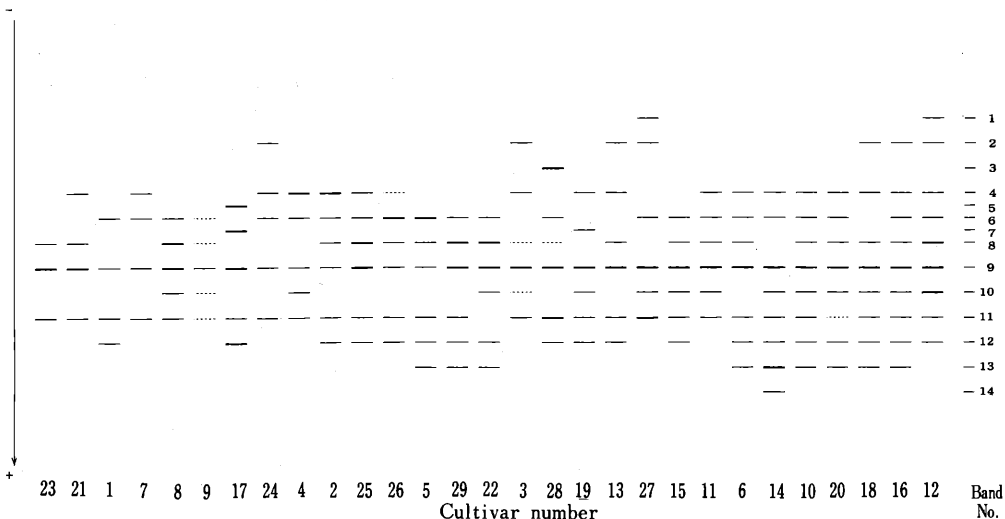


Fig. 2. Schematic presentation of PGI zymograms from 29 cultivars of Higo chrysanthemum. Zymograms are arranged from simple to complex according to numbers of bands. Cultivar names corresponding to cultivar numbers are shown in Table 1.

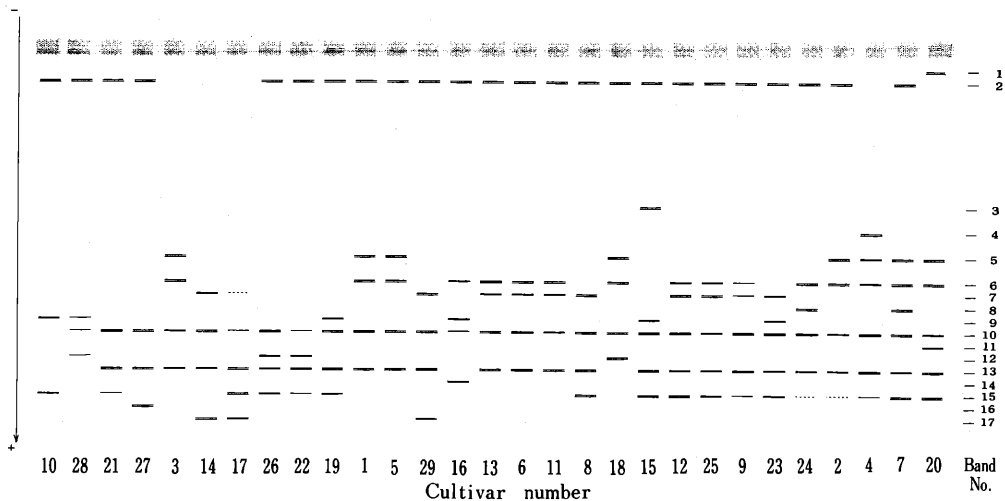


Fig. 3. Schematic presentation of PER zymograms from 29 cultivars of Higo chrysanthemum. Zymograms are arranged from simple to complex according to numbers of bands. Cultivar names corresponding to cultivar numbers are shown in Table 1.

▨ : Indefinite band.      : Wide but lightly stained band.

which cultivars showed the same numbers and positions of bands. Those cultivars, however, differed in band intensities. In detail, each cultivar showed different banding patterns from every other cultivar.

### 3. PER

Bands were concentrated more on the anodal but less on the cathodal and in the intermediate. A group of indefinite bands were observed near the origin of the zymograms. The total number of

bands was 17 except for the indefinite bands. No band was common to all cultivars. Three was the lowest while 7 was the highest number of bands in a zymogram. There were four cultivar groups (cultivar Nos. 26, 22; 1, 5; 13, 6, 11; and 12, 25, 9) in which cultivars showed the same numbers and positions of bands. Those cultivars, however, differed in band intensities. It was also possible to find out the differences of zymograms among cultivars with this system.

### Discussion

In all the 3 enzymes used in this study there were polymorphisms in isozymes. Although some cultivars exhibited the same numbers and positions of bands, they had different staining intensities. It was, therefore, possible to identify the zymogram differences among cultivars in all the 3 enzymes. A high degree of polymorphism of isozyme observed in Higo chrysanthemum is an evidence that several ancestors and repeated crossings have contributed to the establishment and development of the cultivars during their long history of cultivation. The hexaploidy of this plant seems to involve the complex numbers, positions, and intensities of bands detected in all enzymes in conjunction with the dosage effect of alleles. Further analysis of isozyme banding patterns, including additional enzymes in the hybrids, their progenies and related species, are necessary to study the genetics of isozymes and to elucidate the origin of the cultivars.

Ikeda et al. (1990) carried out a study of GOT isozyme patterns in *Dendranthema* and the related species and grouped all isozyme patterns into 7 types. Their results suggested that there is a relationship between the isozyme patterns of species and their geographic distribution. In the same study, 100 cultivars of *Dendranthema grandiflora* were investigated and all of their isozyme patterns were classified under the same type. Since differences among these cultivars were not mentioned in detail, it is difficult to refer their results to the present results. To investigate the relationships between Higo chrysanthemum cultivars and other cultivars in *Dendranthema grandiflora*, accumulation of additional studies will be necessary.

In all enzymes, zymograms obtained from the 3 flower-sized groups (large, medium, small) of Higo

chrysanthemum cultivars showed considerable variations on the numbers, positions, and intensities of bands within each group (Table 1 and Figs. 1-3). Flower color, petal shape, and chromosome number also had no relationships with the isozyme banding pattern.

In this study it is shown that zymograms of GOT, PGI, and PER isozymes are available to identify Higo chrysanthemum cultivars. The numbers and positions of bands in cultivar Nos. 9, 12, and 25 were the same in GOT and PER but different in PGI. On the other hand, those of cultivar Nos. 25 and 26 were the same in GOT and PGI but different in PER. A combined use of the 3 enzymes must, therefore, make the identification of the cultivars through isozyme analysis more exact and easier. Isozyme analysis is a quick, complete, cheap, and easy alternative method for the identification of the cultivars. It can be used even at the early stages of the plant growth when morphological characters are not available. It will be practically useful to breeders, especially in the identification of the mistakable cultivars or hybrids.

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肥後ギク (*Dendranthema grandiflora* Tzvelev) のアイソザイム分析ニューイェン J.L. ロハス<sup>1</sup>・田代洋丞<sup>1</sup>・宮崎貞巳<sup>1</sup>・竹下昭人<sup>1</sup>・大島唯由<sup>2</sup><sup>1</sup>佐賀大学農学部 840 佐賀市本庄町<sup>2</sup>熊本県農業研究センター 861-11 熊本県菊池郡合志町

## 摘 要

約200年前から熊本地方で育成・保存されて来た肥後ギク29品種を供試してアスパラギン酸トランスアミナーゼ (GOT), グルコースリン酸イソメラーゼ (PGI) およびパーオキシダーゼ (PER) のアイソザイム分析を行った。

GOT, PGI および PER ザイモグラムはそれぞれ2~8, 3~9 および3~7本のバンドを持ち, 全品種でそれぞれ合計21, 14 および17本の異なるバンドが観察された。また, 全品種に共通するバンドは GOT および PGI ザイモグラムでそれぞれ1本のみ観察され, PER ザイモグラムではまったく観察されなかった。いずれ

の酵素でも多くの品種のザイモグラムはバンドの数あるいは位置が異なっていた。さらに, バンドの数および位置が同じ品種間でもバンドの濃度(比活性)に違いが観察された。したがって, バンドの数, 位置および濃度によってすべての品種のザイモグラムを区別することができた。

以上の結果から, GOT, PGI および PER のアイソザイム分析は肥後ギクの品種同定に有効であり, 特に未開花の苗を用いて同定が可能であることから, 肥後ギクの品種保存, 増殖および育種を進める上で役立つと考えられる。