

Inheritance of albinism in grapefruit (*Citrus paradisi* Macf.; $al_2^+al_2^-$) and 'Hanayu' (*C. hanaju* hort. ex Shirai; $al_1^+al_1^-$).

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Abstract

Albinism seen in very young seedlings from seeds infected with *Alternaria* fungi and that seen in variegated leaves is known in *Citrus*. However, genetic control of albinism has not been reported in *Citrus* accessions probably because of their apomixis, self-incompatibility, long generation time and cross breeding. In the present study, albino seedlings were observed for the first time in the zygotic seedlings obtained from the crosses or backcrosses with grapefruit and Hanayu with the segregation ratio of 1 albino:3 green and 1 albino:7 green. The 1:3 ratio indicated that Hanayu and grapefruit have independent single recessive gene for the albino seedlings. The albino genes detected in Hanayu and grapefruit designated al_1 and al_2 respectively. On the other hand, the 1:7 ratio indicated that Hanayu or Yuzu has independent single dominant gene designating R_2 a green-restorer for restoration of genetic albinism in the crosses with grapefruit. Albinism observed in the seedlings showed the stability in terms of the two albino genes and all the albino seedlings showing slow growth died several months after seed germination. When grapefruit (pummelo×sweet orange?) was crossed to 46 monoembryonic *Citrus* accessions including 28 pummelo accessions, albino seedlings did not segregate in the progenies of all crosses. The albino seedlings found in this study may be useful as markers in various experiments and contribute to chloroplast research in *Citrus*.

Keywords: Albino gene alleles al_1^+ al_2^+ , Grapefruit, Green-restorer R_2 , Hanayu.

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Introduction

Citrus is one of the most important commercial and nutritional fruit crops in the world, and thus, molecular genetic analysis are in great progress [1]. However, genetic analysis of traits governed by recessive genes is especially difficult in *Citrus* accessions because of their apomixis, self-incompatibility, long generation time and cross breeding [2]. In the studies for self-incompatibility [3], male sterility [4] and parthenocarpy [5] in *Citrus* accessions, we carried out more than hundred self-pollinations, crosses and backcrosses to find out their inheritance. During this study, we happened to find out albino seedlings from self-pollination of Hanayu (*Citrus hanaju* hort. ex Shirai) and grapefruit (*C. paradisi* Macf.).

In Satsuma mandarin (*C. unshiu* Marcow.), variegated shoots with a white over green periclinal chimera structure occurred in the trees with a frequency of 10 shoots /1 ha /20 years. The variegated shoots with albinism in their germ layer 2 were also seen in many groups of *Citrus* plants such as citron, pummelo, calamondin, lemon, sweet orange and sour orange as well as trifoliolate orange (*Poncirus trifoliata* (L.) Raf.) and kumquat (*Fortunella* spp.) [2]. Some of these variegated *Citrus* accessions are valuable as ornamental plants. Albino nucellar seedlings were steadily produced from polyembryonic seeds of these variegated accessions with white over green structure. However, one albino seedling steadily appeared with a certain

rate in a polyembryonic seed of self-pollinated Hanayu, suggesting that albino seedling formation was not due to fungi infection that reported in *Citrus* seeds [6].

In this study, the attempt was focused on the segregation of seedlings showing genetic albinism in various crosses with grapefruit and Hanayu to extend our knowledge as a basis for cross breeding. Coincidentally, albinism was widely surveyed with grapefruit as a pollen parent in various crosses with many *Citrus* accessions.

Materials and Methods

Plant materials

Plant materials used were about 30-year-old trees of Hanayu, 'Foster Pink' grapefruit, 'Mash seedless' grapefruit, 'Variegated Daidai' sour orange (*C. aurantium* L.), 'Variegated Buddha's Hand' citron (*C. medica* L.). During our previous studies for inheritance of male sterility and parthenocarpy [4,5], we conducted various crosses and backcrosses from which the seedlings with various age were produced. From these hybrid seedlings, we selected monoembryonic materials useful for genetic analysis in the present study. The monoembryonic hybrids between Hanayu and Yuzu (*C. junos* Sieb. ex Tanaka) and those between Hanayu-Yuzu hybrids and grapefruit were about 10-year-old trees. Forty-six monoembryonic *Citrus* accessions containing 28 pummelos, 11 pummelo-relatives, four

yuzu-relatives and three mandarin-relatives used for crosses with grapefruit were 20- to 30-year-old trees of the 28 pummelo accessions, 15 were collected from more than 100-year-old trees grown in southwest Japan while 13 were introduced from National Institute for Fruit Tree Science (NIFTS) and Kagoshima Prefectural Fruit Tree Experimental Station in Japan. These accessions were grown in the Experimental Farm of Kyushu University, Fukuoka, Japan.

Crosses with Hanayu

Thirteen accessions including polyembryonic Hanayu, Yuzu, 'Variegated Daidai', seedless 'Variegated Buddha's Hand' and nine monoembryonic Hanayu hybrids were hand-pollinated with/to Hanayu. Pollination was made in May of 2008 to 2016 with at least three replicates in each cross. The flower buds one day before anthesis were emasculated, hand-pollinated with pollen of target accessions, bagged to prevent pollination with alien pollen, and harvested in November of the year.

Crosses with grapefruit

Ten accessions including Hanayu, 'Foster Pink' and 'Marsh seedless' grapefruit, (Hanayu×Yuzu)-No.16 and six monoembryonic hybrids between 'Foster Pink' and (Hanayu×Yuzu)-No.16 were pollinated with grapefruit. Since two grapefruit cultivars are highly polyembryonic, they were used as pollen parents. Three hybrids between 'Foster Pink' and (Hanayu×Yuzu)-No.16 were self-pollinated. Pollination was made in May of 2011 to 2018 with at least three replicates in each cross. All the pollinated flowers were bagged to prevent outcross, and harvested in November of the year.

Forty-six monoembryonic *Citrus* accessions including 28 pummelo accessions, 11 pummelo hybrid accessions, four Yuzu relative accessions and three mandarin accessions were pollinated with 'Foster Pink' grapefruit with the same procedure as mentioned above.

Seedling production and observation

The seeds were extracted from mature fruit in November of the year. The zygotic embryos extracted from the seeds were germinated under fluorescent light in a room maintaining at 25°C and allowed to grow for about six months in a greenhouse (13-30°C). The rate of zygotic or monoembryonic seeds was about 80% in crosses with polyembryonic 'Hanayu' as a seed parent and about 10% in crosses with polyembryonic 'Yuzu' as a seed parent. The polyembryonic seeds containing two or more embryos per seed were not used, and only monoembryonic seeds were selected for this study. In each of the 46 monoembryonic *Citrus* accessions pollinated with grapefruit, more than 100 seeds were used for the detection of albino seedlings.

As a control for genetic albinism in this research, open pollinated sour orange seeds were collected in November and sown in soil without disinfection and albinism in the infected seedlings were observed.

Assessment of albinism

Morphology of albino seedlings in various crosses with Hanayu and grapefruit was observed in comparison to the green seedlings. The rates for albino seedlings observed in various crosses was repeatedly examined for at least three years, and the result in each year was combined in each cross combination.

Statistical analysis

Segregation rates for albino seedlings were calculated and Chi-

square test was performed to determine the probability.

Results

Morphology of albino seedlings

Sour orange seedlings grown in disinfected soil showed various degree of albinism and root development (Figure 1). In comparison to this non-genetic albinism, genetic albinism seen in zygotic seedlings was stable in morphology, i.e., white leaf color, white stem color, white cotyledon color, seedling high and root development (Figures 2 and 3). In comparison to the green zygotic seedlings in each cross, albino zygotic seedlings were small in height, leaf size, cotyledon size and root size (Figures 2 and 3). There was no year-to-year variation for the morphology of albino seedlings, and they did not turn green during their growth period and died several months after germination.

Segregation of albino seedlings in the crosses with Hanayu

In the self-pollination of Hanayu, albino seedlings and green seedlings segregated in a ratio of 123:380 (1:3), while in the reciprocal crosses between Hanayu and Yuzu only green seedlings appeared (Table 1). The 1:3 ratio for the segregation of



Figure 1. Various degree of albinism seen in seedlings from polyembryonic sour orange seeds sown in soil spoiled with fungi such as *Alternaria*. Note the correlation between the degree of root development and the degree of albinism.



Figure 2. Albino (upper) and green (lower) seedlings derived from the cross of [(Hanayu×Yuzu)-16×Foster Pink]-No.3×Foster Pink grapefruit three weeks after germination under the plant growth fluorescent light at 25°C.



Figure 3. Segregation of albino seedlings (A) and morphology of albino and green seedlings (B) derived from the cross of Hanayu x (Hanayu x Yuzu)-No.14 under greenhouse conditions.

Table 1. Segregation of albino seedlings in crosses and backcrosses with/to Hanayu ($al_1^+al_1^-$).

Cross	Genotypes	Observed ratio		Expected ratio	X ²	p-value
		Albino seedling	Green seedling			
Hanayu x Hanayu	$al_1^+al_1^- \times al_1^+al_1^-$	123	380	01:03	0.183	0.669
Hanayu x Yuzu	$al_1^+al_1^- \times al_1^-al_1^-$	0	117	00:01	-	-
Yuzu x Hanayu	$al_1^-al_1^- \times al_1^+al_1^-$	0	101	00:01	-	-
Hanayu x (Hanayu x Yuzu)-3	$al_1^+al_1^- \times al_1^-al_1^-$	0	76	00:01	-	-
Hanayu x (Hanayu x Yuzu)-10	$al_1^+al_1^- \times al_1^+al_1^-$	16	35	01:03	1.105	0.293
Hanayu x (Hanayu x Yuzu)-24	$al_1^+al_1^- \times al_1^+al_1^-$	32	79	01:03	0.868	0.352
Hanayu x (Hanayu x Kabosu)-22	$al_1^+al_1^- \times al_1^-al_1^-$	0	129	00:01	-	-
Hanayu x (Hanayu x Kabosu)-31	$al_1^+al_1^- \times al_1^+al_1^-$	39	138	01:03	0.831	0.362
(Hanayu x Yuzu)16 x Hanayu	$al_1^-al_1^- \times al_1^+al_1^-$	0	200	00:01	-	-
(Hanayu x Yuzu)16 x Yuzu	$al_1^-al_1^- \times al_1^-al_1^-$	0	100	00:01	-	-
(Hanayu x Kabosu)14 x Hanayu	$al_1^+al_1^- \times al_1^+al_1^-$	19	52	01:03	0.117	0.732
(Hanayu x Kabosu)34 x Hanayu	$al_1^-al_1^- \times al_1^+al_1^-$	0	17	00:01	-	-
(Mexican lime x Hanayu)-2 x Hanayu	$al_1^+al_1^- \times al_1^+al_1^-$	29	90	01:03	0.025	0.874
Hanayu x Variegated Daidai	$al_1^+al_1^- \times al_1^-al_1^-$	0	349	00:01	-	-
Hanayu x Variegated Buddha's Hand	$al_1^+al_1^- \times al_1^-al_1^-$	0	65	00:01	-	-

albino seedlings were constant for four years observations (data is not presented but combined in Table 1). When Hanayu was backcrossed, albino seedlings and green seedlings segregated in five of nine backcross combinations with a ratio of 1:3, while only green seedlings appeared in four of the nine backcrosses (Table 1). Albino seedlings did not segregate in the cross of Hanayu x Variegated Daidai and that of Hanayu x Variegated Buddha's Hand.

Segregation of albino seedlings in the crosses with grapefruit

In two crosses of Hanayu x Foster Pink grapefruit and (Hanayu x Yuzu)-No.16 x Foster Pink grapefruit, albino seedlings did not segregate. In the three self-pollinations of 'Foster Pink'

grapefruit hybrids, [(Hanayu x Yuzu)-No.16 x Foster Pink]-No.3 generated albino and green seedlings with a ratio of 1:3, while in two of the three, albino seedlings did not segregate (Table 2).

When 'Foster Pink' grapefruit hybrids [(Hanayu x Yuzu)-No.16 x Foster Pink] was backcrossed with 'Foster Pink' grapefruit, albino seedlings and green seedlings segregated in two of four backcross combinations with a ratio of 1:3 and in one of the four back cross combinations with a ratio of 1:7 (Table 2). On the other hand, only green seedlings appeared in one of the four backcrosses (Table 2). When 'Foster Pink' grapefruit hybrids [(Hanayu x Yuzu)-No.16 x Foster Pink]-No.2 was backcrossed with 'Marsh seedless' grapefruit, albino seedlings and green seedlings also segregated with a ratio of 1:7 (Table 2).

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Table 2. Segregation of albino seedlings in crosses and backcrosses with grapefruit, and genotypes for albino genes al_1 and al_2 and green restoration gene R_2 for al_2 .

Cross	Albino genotype for three genes al_1 , al_2 and R_2	Observed ratio		Expected ratio	X ²	p-value
		Albino seedling	Green seedling			
Hanayu×Foster Pink	$al_1^+al_1^-al_2^-al_2^-R_2^{-or+}R_2^- \times al_1^-al_1^-$ $al_2^+al_2^-R_2^-R_2^-$	0	800	00:01	-	-
(Hanayu×Yuzu)-16×Foster Pink	$al_1^-al_1^-al_2^-al_2^-R_2^{-or+}R_2^- \times al_1^-al_1^-$ $al_2^+al_2^-R_2^-R_2^-$	0	200	00:01	-	-
[(Hanayu×Yuzu)-16×Foster Pink]-3×selfing	$al_1^-al_1^-al_2^-al_2^-R_2^{-or+}R_2^- \times al_1^-al_1^-$ $al_2^+al_2^-R_2^-R_2^-$	38	117	01:03	0.019	0.889
[(Hanayu×Yuzu)-16×Foster Pink]-3×selfing	$al_1^-al_1^-al_2^-al_2^-R_2^{-or+}R_2^- \times al_1^-al_1^-$ $al_2^-al_2^-R_2^-R_2^-$	0	21	00:01	-	-
[(Hanayu×Yuzu)-16×Foster Pink]-5×selfing	$al_1^-al_1^-al_2^-al_2^-R_2^{-or+}R_2^- \times al_1^-al_1^-$ $al_2^-al_2^-R_2^-R_2^-$	0	24	00:01	-	-
[(Hanayu×Yuzu)-16×Foster Pink]-1×Foster Pink	$al_1^-al_1^-al_2^+al_2^-R_2^-R_2^- \times al_1^-al_1^-$ $al_2^+al_2^-R_2^-R_2^-$	27	84	01:03	0.027	0.869
[(Hanayu×Yuzu)-16×Foster Pink]-2×Foster Pink	$al_1^-al_1^-al_2^+al_2^-R_2^+R_2^- \times al_1^-al_1^-$ $al_2^+al_2^-R_2^-R_2^-$	67	474	01:07	0.004	0.948
[(Hanayu×Yuzu)-16×Foster Pink]-2×Marsh	$al_1^-al_1^-al_2^+al_2^-R_2^+R_2^- \times al_1^-al_1^-$ $al_2^+al_2^-R_2^-R_2^-$	35	250	01:07	0.008	0.929
[(Hanayu×Yuzu)-16×Foster Pink]-3×Foster Pink	$al_1^-al_1^-al_2^+al_2^-R_2^-R_2^- \times al_1^-al_1^-$ $al_2^+al_2^-R_2^-R_2^-$	66	200	01:03	0.005	0.944
[(Hanayu×Yuzu)-16×Foster Pink]-6×Foster Pink	$al_1^-al_1^-al_2^-al_2^-R_2^{-or+}R_2^- \times al_1^-al_1^-$ $al_2^+al_2^-R_2^-R_2^-$	0	34	00:01	-	-

In the pollination with 'Foster Pink' grapefruit as a pollen parent, no segregation of albino seedlings was observed in 45 monoembryonic *Citrus* accessions including 28 pummelos, 11 pummelo hybrids, four Yuzu relatives and three mandarins.

Discussion

Genetic studies in different crops show that albinism is a recessive trait governed by many loci [7]. The present study in *Citrus* crop also show that albinism is a recessive trait governed by at least two loci. In the self-pollinations and backcrosses with Hanayu, the albino seedlings and green seedlings segregated in a ratio of 1:3. This indicates that the genetic albinism is governed by single recessive gene designated al_1 . The nuclear gene al_1 consists of functional allele al_1^+ and nonfunctional allele al_1^- . In the cross of Hanayu×Foster Pink grapefruit, albino seedlings did not segregate, while in the self-pollinations of grapefruit hybrids and backcrosses with grapefruit, the albino seedlings and green seedlings segregated in a ratio of 1:3. This indicates that the genetic albinism is also governed by single recessive gene designated al_2 . The nuclear gene al_2 consists of functional allele al_2^+ and nonfunctional allele al_2^- .

The present study in *Citrus* crop also shows that albinism is restored by a green-restorer gene on at least one locus. In the backcrosses with grapefruit, the albino seedlings and green seedlings segregated in a ratio of 1:7. This indicates that the genetic albinism is restored by single dominant gene designated R_2 . The nuclear gene R_2 consists of functional allele R_2^+ and nonfunctional allele R_2^- . It is estimated that either Hanayu or Yuzu has allele R_2^+ . Since no clear difference in morphology and growth rate was found between albino seedlings as to al_1 and al_2 genes, their function to generate albino seedlings seems to be similar.

Conclusion

It seems that the genetic albinism of a given *Citrus* genotypes is a stable trait governed by one or more recessive nuclear genes. The albinism is not affected by environmental conditions and seedling age after seed germination, but recovered by green-restorers. Some *Citrus* accessions with relation to grapefruit and Hanayu carry these genes. These accessions will be very useful materials for producing markers in experiments such as micro-grafting, breeding and chloroplast research in *Citrus*.

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