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Artificial induction of autogamy in *Paramecium caudatum*

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SUMMARY

Three methods of artificial induction of autogamy in *Paramecium caudatum* were described: (1) treatment with KCl+papain, (2) treatment with KCl and then with KCl+papain and (3) ordinary mating reaction and then treatment with papain. As expected, one-to-one segregation ratios were obtained in the progeny from the parents heterozygous for the two loci: mating type and lactate dehydrogenase. A high rate of autogamy is induced by method (1), but its use is restricted to only a few clones. Autogamy is also induced at a high rate by method (2), by which the induction is more stable. Autogamy is induced at a lower rate by method (3), but this method can be widely applied to every species of *Paramecium* which has complementary mating types. Some exautogamous progeny become completely sterile through successive autogamy. The cause of this sterility is discussed.

1. INTRODUCTION

Autogamy is a sexual process occurring in single cells of *Paramecium.* It brings about complete homozygosity in the progeny and is very useful for genetic studies. Autogamy is known to occur naturally in species of the *Paramecium aurelia* complex, *P. jenningsi* and some other ciliates, but many species of *Paramecium* including *P. caudatum* lack autogamy. This is one of the reasons that most of the genetic studies in *Paramecium* are restricted to species of the *P. aurelia* complex.

Miyake (1968*a*) reported that nuclear reorganization in *P. multimicronucleatum* could be induced by treatment with KCl and ficin. However, he was unable to demonstrate if this reorganization was true autogamy since he lacked gene markers to prove homozygosity of progeny. We modified Miyake's method and succeeded in inducing autogamy in *P. caudatum* by three different methods. Recently, Mikami & Koizumi (1979) reported the induction of autogamy in *P. caudatum* by treatment with trypsin. One of the three methods to be reported here is essentially the same as their method, though we used papain instead of trypsin. To confirm the occurrence of autogamy, genes for mating type and lactate dehydrogenase isozymes were used as markers and 1:1 segregation ratios of those phenotypes were obtained in the exautogamous progeny. Though some problems of progeny sterility remain unsolved, the methods would be useful for genetic as well as other biological studies in *Paramecium* (Tsukii & Hiwatashi, 1978; Watanabe, 1978).

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2. MATERIALS AND METHODS

(i) *Stocks and culture methods*

Stocks used were Yt3, Em IV-3S 27a, and 16B205 all of which belong to mating type VI, and 27aG3 which belongs to mating type V in *P. caudatum* syngen 3. Yt3 is a natural stock collected by Yuuji Tsukii. 27aG3 is an exautogamous clone derived from the early mature mutant clone Em IV-3S 27a established by Myohara & Hiwatashi (1978). Stock 16B205 is a CNR (non-reversal mutant) heterozygote produced by two back-crosses of 16B101 (*cnrB/cnrB*, Takahashi, 1979) to 27aG3. Culture medium, in which cells for chemical induction of autogamy were grown, was Ca-poor fresh lettuce-juice medium inoculated with *Klebsiella pneumoniae* 1 day before use (Hiwatashi, 1968; Miyake, 1968*b*). Other techniques for culture and handling were those described by Sonneborn (1970).

(ii) *Determination of conjugation and autogamy ratios*

The percentage of conjugation was obtained by observation of living cells under a binocular microscope and recorded; $+++$ to indicate about 90% of conjugation, $++$ about 50%, $+$ about 10%, and $-$ 0%. Ranges of errors were usually 10% when checked by counting fixed samples.

To know the percentage of autogamy induced, cells were stained with aceto-orcein 1 day after initiation of the induction of autogamy, and those undergoing macronuclear fragmentation were counted. Samples were always checked to be sure there were no conjugating pairs throughout the experiments.

(iii) *Isolation of exautogamous cells*

Cells undergoing autogamy lose their ability to form food vacuoles. To discriminate cells undergoing autogamy from those not, indian ink was added 5 or 6 h after initiation of the induction. Cells which failed to enter the process of autogamy ingested indian ink into their food vacuoles and were seen as black cells. Cells undergoing autogamy were clearly distinguished as white cells about 1 h after the addition of indian ink and were easily isolated in depression slides. Procedures to eliminate non-conjugants and cells in macronuclear regeneration were essentially the same as those described by Sonneborn (1970).

(iv) *Electrophoresis of lactate dehydrogenase*

Electrophoresis of lactate dehydrogenase was done according to the method of Agatsuma & Tsukii (1979). Cells were harvested by centrifugation in a Kokusan H-210 centrifuge at approximately 200 ***g*** for 3 min. The cells were washed twice with distilled water and homogenized. The homogenate was centrifuged at 100 ***g*** for about 5 min and the supernatant was used for electrophoresis. Polyacrylamide gel electrophoresis was carried out essentially in the same manner as that used for detection of LDH in the housefly, *Musca domestica* (Agatsuma & Takeuchi, 1976). After electrophoresis, the gel was removed from the tray, trimmed, and placed in an incubation mixture for approximately 0·5 h at 37 °C.

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(v) *Chemicals*

Twice-crystallized papain (Sigma) was used in this work. Ficin (crude, Wako chemical) was partially purified by the method of Hammond & Gutfreund (1959) with slight modification. Methyl urea, Nonidet P-40, Tween 60, and Triton X-100 were obtained from Wako Chemicals Ltd.

3. RESULTS

(i) *Induction of autogamy by the solution containing KCl and papain (KCl+papain method) – (1)*

Our modification of Miyake's method of inducing autogamy in *Paramecium multimicronucleatum* replaces ficin with papain. Mating reactive cells of stock 27aG3 cultured in the Ca-poor medium were washed and suspended in the salt solution, K-PBS II (1·5 mM-NaCl, 0·01 mM $CaCl_2$, 0·1 mM-$MgCl_2$, 1·8 mM-KCl, 0·2 mM-K_2HPO_4, 1·8 mM-KH_2PO_4, pH 6·0). The cell suspension (2500–5000 cells/ml) was mixed in test tubes with an equal volume of the inducing medium for autogamy (12 mM-KCl, 100 mM methyl urea, $1{\cdot}0 \times 10^{-3}$% (v/v) Nonidet P-40 and crystallized papain (5–10 μg/ml) in K-PBS II). Methyl urea has promoting effects on both chemical induction of conjugation and autogamy (Miyake, 1968*a*). Nonidet P-40 was found to promote the induction of autogamy.

When cells were treated with the inducing medium with less than 3 μg/ml papain, ordinary conjugation was induced; pairs attached at anterior ends (hold-fast union) about 1 h after transferring the cells into the medium (KCl, methyl urea, and NP-40) and tight pairs (paroral union) formed about 2 h later. In proportion to the conjugation ratio, macronuclear fragmentation was observed in those samples the next day (Table 1). When macronuclear fragmentation was observed in the cells treated with medium containing more papain (4–10 μg/ml), its ratio was as high as those treated with the medium without papain, in spite of the fact that no conjugating pairs were observed during the experiment. Similar

Table 1. *Effect of Nonidet P-40 on the chemical induction of autogamy in* P. caudatum

		Concentrations of papain (μg/ml)					
		0	2	4	6	8	10
5×10^{-4}% NP-40	Conjugation*	+++	+++	++	–	–	–
	Macronuclear fragmentation†	89	96	93	83	82	90
No NP-40	Conjugation*	+++	+++	–	–	–	–
	Macronuclear fragmentation†	91	86	78	61	6	0

* Conjugation was observed 3 h after transferring cells into the conjugation-inducing chemicals. Grade of conjugation, +++ to –, see Materials and Methods.

† Percentage of cells which show macronuclear fragmentation one day after onset of the induction.

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data were obtained when ficin partially purified according to the method of Hammond & Gutfreund (1959) was used instead of crystallized papain. These data show that macronuclear fragmentation occurs without conjugating union and suggest the induction of autogamy by the chemicals.

Nonidet P-40 increased the percentages of cells entering autogamy. In the absence of NP-40, the proportion of autogamy induced at higher concentrations of papain was considerably low (Table 1). In this condition, half of the cells gradually died. Calcium ions released from the dead cells might cause the decrease in proportion of autogamy. Until 3 h after initiation of induction, cells were sensitive to very low concentrations of calcium ions, and about 40 μM-Ca ions completely inhibited the chemical induction of autogamy. Few cells died when the medium contained NP-40. When other detergents, Tween 40, Tween 60, or Triton X-100 were used instead of Nonidet P-40, a similar effect was not obtained.

This method, however, can be applied only to a limited numbers of stocks. Sometimes the method is undependable, and the proportion of the exautogamous cells often fluctuates. Stock 27aG3, which showed high survival after the treatment with the inducing medium, was one of the suitable stocks for this method. Some stocks which have low susceptibility to conjugation-inducing chemicals, however, did not undergo autogamy by this method, and some others were too sensitive to the treatment and died easily. Moreover, the proportion of autogamy induced sometimes fluctuated even in the presence of NP-40 as described below.

(ii) *Induction of autogamy by KCl and KCl + papain treatments (KCl – KCl + papain method) – (2)*

This method differs from method (1) in the time of addition of papain. When papain (5 μg/ml) was added to the conjugation-inducing medium (KCl, methyl

Table 2. *Degradation of conjugating pairs when papain was added at different times of incubation**

Time of addition of papain (h)	Time after transferring into the conjugation-inducing chemicals (h)								Macronuclear fragmentation† (%)
	0	0·5	1	2	3	4	6	8	
0	–	–	–	–	–	–	–	–	12
0·5	–	+	+ +	+	–	–	–	–	21
1·0	–	+	+ + +	+ +	–	–	–	–	35
1·5	–	+	+ + +	+ + +	+	–	–	–	46
2·0	–	+	+ + +	+ + +	+ +	–	–	–	62
2·5	–	+	+ + +	+ + +	+ + +	+	±	±	66
3·0	–	+	+ + +	+ + +	+ + +	+ +	+ +	+	69

* Conjugation was induced by the chemicals in test tubes. Volume of the incubation medium was 2 ml in each tube. Into each tube, 0·1 ml papain was added at different times to a final concentration of 5 μg/ml. Conjugation ratio (+ to + + +) was determined by observing cells under a binocular microscope. For details, see Materials and Methods.

† Percentage of cells which show macronuclear fragmentation, examined 1 day after initiation of the induction.

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urea, and NP-40) at various times from the start of the treatment, pairs once united were gradually disjoined (Table 2). When papain was added about 2 h from the start of the treatment, the ratio of autogamy induced was higher than when papain was present from the beginning. A small number of dead cells, which probably inhibits the induction of autogamy, sometimes occurs at higher concentrations of papain, even in the presence of Nonidet P-40. This shows the reason why the frequency of autogamy induced by the method (1) sometimes fluctuated. The concentration and the time of addition of papain were, therefore, critical for dependable induction of autogamy. The later is the time of addition of papain, the more dependable is the induction of autogamy. Though this method is a modification of method (1), the trigger of autogamy seems to be essentially different. In method (2), meiosis and subsequent nuclear events are most probably triggered by cell-to-cell union that occurs prior to the addition of papain, while in method (1), formation of tight cell union is inhibited from the beginning and meiosis and other nuclear events are probably triggered directly by the action of the conjugation-inducing chemicals.

(iii) *Induction of autogamy by treating natural conjugating pairs with papain (mating reaction – papain method) – (3)*

When cells of complementary mating types are mixed, they form conjugating pairs in about 2 h. When papain (up to 50 μg/ml) was added about 3 h after mixing, some conjugating pairs were gradually separated. Although the proportion of pair separation was not so high as in the case of method (2), cells in autogamy could be isolated from samples containing conjugating pairs by picking up white single cells using the indian ink method described in the Materials and Methods section. In this method, cells undergoing autogamy are a mixture of two different strains. In order to select exautogamous cells of a given strain, we can use behavioural mutants such as CNR (Takahashi & Naitoh, 1978) for one of the complementary mating types. Since cells of CNR mutants do not swim backward in a high K^+ solution in which wild-type cells swim backward, we can easily select cells of one mating type from the mixture.

(iv) *Segregation of marker genes in the artificially induced autogamy*

Stock Yt3, heterozygous both for the mating type (+/*mt*) and the lactate dehydrogenase (*Ldh F/S*) loci, was used to examine homozygosity after the artificial induction of autogamy. Table 3 shows that nearly 1:1 segregation occurs for each marker, indicating occurrence of autogamy. One-to-one segregation of the mating-type locus was also obtained when another stock, 16B205, was induced to autogamy.

As mentioned in Table 3, two exautogamous clones out of 130 from stock Yt3 remained heterozygous for the LDH locus. These exceptional segregants can be explained by one of the following alternatives. To obtain true exautogamous progeny, cells without ingested indian ink were isolated and grown to establish

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clones. Then, every clone was checked for mating ability. If some clones showed mating activity they were discarded as progeny of non-autogamous cells or of macronuclear regeneration. However, since the parental stock Yt3 undergoes selfing conjugation with low-frequency, heterozygous immature cells derived from selfing progeny may erroneously be picked up as exautogamous cells if the cells accidentally contain no indian ink. The other possibility may be disturbance of meiotic process by the autogamy-inducing chemicals. If two meiotic products instead of the usual one survived and fused to form a syncaryon, heterozygous progeny might occur. The former alternative is more probable because the indian ink method was not 100% exact for selecting autogamous cells.

Table 3. *Segregation of genetic markers after the chemical induction of autogamy*

Stocks	(Genotype)	Segregation of phenotypes: LDH* (F):(S)	Mating type VI:V
Yt3	(+/*mt*, *Ldh F*/*Ldh S*)	62:66 (64:64) $\chi^2 = 0{\cdot}0156$, $0{\cdot}95 > P > 0{\cdot}8$	72:52 (62:62) $\chi^2 = 1{\cdot}56$, $0{\cdot}5 > P > 0{\cdot}2$
16B205	(+/*mt*)		17:16 (16·5:16·5) $\chi^2 = 0{\cdot}015$, $0{\cdot}95 > P > 0{\cdot}8$

* Two unexpected FS heterozygotes were also obtained and are discussed in the text.

(v) *Difference of survival between selfing conjugation and autogamy*

Percentage of survival after autogamy was not always so high as that after selfing conjugation of the same clones (Table 4). Even if a given clone showed nearly 100% survival after selfing conjugation, the same clone showed lower survival after autogamy. The same phenomenon appeared in every generation (Table 5).

Both selfing conjugation and autogamy were induced chemically, but in the induction of autogamy papain is used in addition to the conjugation inducing chemicals. This may be one of the probable causes of the difference in survival. However, further study is necessary to answer this problem.

(vi) *Fertility of exautogamous progeny*

Fertility of an exautogamous progeny was examined by selfing conjugation and by crosses with other highly fertile stocks. Not all exautogamous progeny were fertile. Some were completely sterile and others showed various grades of fertility up to 100%. Strain 27aG3 showed the highest fertility among exautogamous F_1 progeny of the parental stock Em IV-3S 27a, when selfing conjugation was induced by chemicals. However, when the second and following generations were produced

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Table 4. *Isolation of exautogamous clones in* Paramecium caudatum

Stocks	Methods*	Number of cells examined† T	N.R.	M.R., N.C.	Survival ratios‡ (%)	Fertility of exautogamous clones§ Total	Fertile	Sterile
Em IV-3S 27a	K.M.F.	990	24	59	3	13	11	2
16B205	K.M.P.	960	54	—	6	—	—	—
Yt3	K.M.P.	696	133	55	35	20	12	8

* Methods of induction of autogamy; K.M.F. (KCl, methyl urea, and partially purified ficin), K.M.P. (KCl, methyl urea, and crystallized papain), both contained 5×10^{-4} % (v/v) Nonidet P-40. For detail, see Materials and Methods.

† T, Total number of cells isolated; N.R., true progeny cells grown to immature clones; M.R. and N.C., cells of macronuclear regeneration and of non-conjugants grown to mating reactive clones.

‡ 100 × (N.R.)/(T − M.R. and N.C.).

§ Fertility of exautogamous clones was tested on 15–30 pairs induced by chemicals, by sibling crosses, and by crosses with other highly fertile stocks. Sterile clones produced no viable descendants by selfing conjugation or crosses.

by autogamy, and fertility of exautogamous clones was tested by chemical induction of selfing conjugation, some progeny were again almost completely fertile but, quite unexpectedly, others were completely sterile. This characteristic pattern was maintained through successive autogamies (Table 5, Fig. 1). Similar patterns of segregation of fertility after autogamy were observed when other exautogamous clones which had different origin from 27aG3 were used.

If parental cells contain in heterozygous condition some recessive genes which are deleterious to autogamy or postautogamous processes but not to vegetative

Table 5. *Segregation of fertile and sterile clones in successive exautogamous generations derived from stock 27aG3*

Exautogamous generations	Methods for inducing autogamy*	No. of exautogamous cells isolated	Survival ratio (%)†	Fertility by selfing conjugation‡ No. of clones examined	Fertile	Sterile
F_1	K.M.F.	90	42	21	10	11
	K.P.	90	50	24	6	18
	Mr.P.	90	40	20	5	15
F_2	K.M.P.	210	44	40	6	34
F_3	K.M.P.	90	57	31	4	27
F_4	K.M.P.	90	20	8	2	6
F_5	K.M.P.	42	28	10	2	8

* K.M.F. and K.M.P. are the same as those in Table 4; Mr.P., the mating reaction – papain method.

† For detail, see Materials and Methods.

‡ Fertile clones showed nearly 100 % survival and sterile clones produced no viable progeny when conjugation was induced by the chemicals. One of the fertile clones was used to produce every next exautogamous generation.

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reproduction, some progeny would not survive the second autogamy. But once surviving this autogamy, clones of all the following generations would show high fertility. This was not the case. After every autogamy, some sterile clones were produced while others were nearly 100% fertile, when tested by chemical induction of selfing conjugation. Methyl urea, the promoting agent used for the chemical

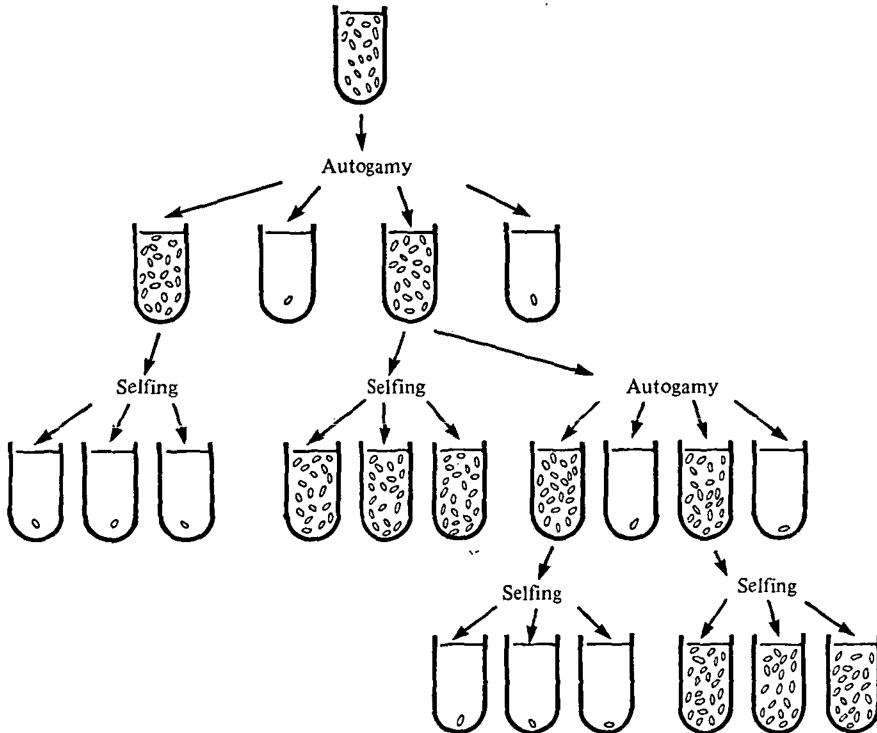

Fig. 1. Fertility of exautogamous progeny. Autogamy and selfing conjugation were induced chemically. Vials containing single cells indicate no growth or death after autogamy or selfing conjugation.

induction of conjugation, has nothing to do with this phenomenon, since a similar pattern of fertility was also obtained by autogamy without methyl urea (Table 5). KCl is probably not involved in this phenomenon, because the data for fertility of F_1 progeny produced by method (3) were quite the same as those produced by the method (1) (Table 5).

Another puzzling but interesting phenomenon is difference in the occurrence of macronuclear regeneration between clones of different fertility. When an exautogamous clone shows high survival by chemical induction of conjugation, 30–70% of the induced pairs show macronuclear regeneration. On the other hand, when an exautogamous clone shows poor or no survival by conjugation, occurrence of macronuclear regeneration was never observed. This phenomenon also needs further study.

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4. DISCUSSION

Three different methods for induction of autogamy in *P. caudatum* were described in this paper. The first method, KCl + papain method, is efficient in inducing high proportions of autogamy synchronously for some stocks but not for others. Proportions of autogamy induced often fluctuate. In the second method, KCl/KCl + papain method, ratios of autogamy induced do not fluctuate very much. As with method (1), this method is applicable to some stocks but not to others. Since both methods use the conjugation-inducing chemicals for the induction of autogamy, stocks less susceptible to the conjugation-inducing chemicals do not respond to the autogamy-inducing treatment. Thus, these methods are not applicable to *P. bursaria*, *P. calkinsi* and *P. polycaryum*, in which chemical induction of conjugation is unsuccessful (Miyake, 1968*b*). They are also inapplicable to kau mutants in *P. aurelia* complex (Cronkite, 1974, 1975) though natural autogamy would suffice for genetic study. The third method, the mating-reaction/papain method, which is essentially the same as the mating-reaction/trypsin method of Mikami & Koizumi (1979), can be used more generally in any species of *Paramecium* with complementary mating types. Though this method has not been as extensively studied as the former two methods, a shortcoming of this method is that the frequency of the induction of autogamy is not very high. Another shortcoming is that the cell population in which autogamy is induced is always a mixture of two different stocks. To obtain exautogamous clones from a certain stock, it is necessary to eliminate cells of the complementary mating type using some nuclear (e.g. amicronucleate clone as Mikami and Koizumi used) or cytoplasmic marker, or to use mating-reactive killed cells, detached cilia or membrane vesicles of the complementary mating type for inducing the mating reaction (Metz, 1954; Hiwatashi, 1969; Kitamura & Hiwatashi, 1976). The success of artificial induction of autogamy in *P. caudatum* offers many advantages for genetic studies in this species. Genetic studies in *P. caudatum* have long suffered from high mortality after conjugation. Highly fertile stocks have had to be sought among a large number of natural stocks or have been established after patient reiteration of crosses and selfing. By autogamy we can select highly fertile clones among those in the first exautogamous generation, and once such clones are selected, they continue to be highly fertile in the following generations if selfing conjugation is used to yield progeny.

Complete homozygosity is brought about by autogamy. This makes mass screening of recessive mutants possible. In screening recessive mutants from mutagenized clones in *P. caudatum*, selfing conjugation has been mainly used (Takahashi & Naitoh, 1978). In this method a large number of mutagenized cells has to be isolated to establish clones before selfing conjugations within each clone are induced. In the induction of autogamy, a whole mutagenized cell population can be screened following autogamy.

One problem to be solved in the artificial induction of autogamy is the repeated appearance of sterile exautogamous progeny. As mentioned in the Results, no

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macronuclear regeneration after selfing conjugation was observed in sterile clones. Mikami (unpublished) suggests that growth of the macronuclear anlage is necessary for macronuclear fragments to regenerate. In some sterile clones, lack of macronuclear anlagen was observed. Disturbance of the formation of the macronuclear anlage may be a possible cause of the sterility. Another possibility is that the sterile clones are haploid. Those abnormalities in the process of autogamy might occur by some unknown action of the chemicals used for the induction. As mentioned in the Results, KCl and methyl urea seem not to be the cause of the lethality after autogamy. Though use of papain seems one of the probable causes, this problem still remains to be pursued.

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