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NIAR MAFF
Ohmiya-machi, Naka-gun, Ibaraki-ken
Japan
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AND
MUTATION BREEDING

Report of Symposium

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Institute of Radiation Breeding
NIAR MAFF

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FOREWORD

Although mutation induction is the major practical method to obtain crops having newly modified genes, recent admirable progress in plant biotechnology enabled the production of plants from genetically manipulated cells in various crop species. Mutation breeding techniques should incorporate new biotechnology for their development, while the mechanism of somaclonal variation, control of which is required for the application of cell culture techniques to practical plant breeding and to plant propagation, should be discussed in comparison with the mutation induced by ionizing irradiation or chemical mutagens. From these requirements, the 30th Gamma Field Symposium was held with a title 'Biotechnology and Mutation Breeding'.

In the memorial lectures of the 30th Symposium, Dr. A. Micke reviewed history of mutation breeding and concluded his summary by the expectation for further advances in crop improvement with the combination of biotechnology and mutagenesis, and Dr. F. J. Novak gave the proposal of application of tissue culture techniques to mutation breeding. After these lectures, induced mutation and somaclonal variations in rice and soybean were compared and discussed in the molecular level. In addition, recent achievements in plant breeding using tissue and cell culture techniques with ionizing irradiation, in vitro selection, and genetic engineering were reviewed.

We wish to express our sincere thanks to Drs. A. Micke and F. J. Novak for their memorial lectures, and to the other lecturers, chairpersons and those who contributed for the Symposium. This Symposium was partially supported by Bio-oriented Technology Research Advancement Institution, Society for Techno-innovation of Agriculture, Forestry and Fisheries, Food and Agriculture Research and Development Association and nine companies.

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Memorial lectures
Chairperson : T. Kawai
Induced mutation for crop improvement .................................A. Micke
Chairperson : N. Murata
Mutation breeding using tissue culture techniques......................F. J. Novak

Session I
Chairperson : H. Yamagata
Mutation and DNA modification by ionizing irradiation in rice ........O. Yatou
Somaclonal variation in protoplast-derived rice plants .....................Y. SUKEKIYO

Session II
Chairperson : K. Harada
Spontaneous and induced mutations of seed proteins in soybean
(Glycine max L. Merrill) ..........................................................K. Kitamura
Somaclonal variation in soybean seed proteins ..............................T. Komatsuda

Session III
Chairperson : H. Kukimura
Plant breeding with the combination of chronic irradiation of
gamma-ray and tissue culture .......................................................S. Nagatomi

Session IV
Chairperson : Y. Futsuhara
Asymmetric protoplast fusion .....................................................T. Kumashiro

Session V
Chairperson : K. Nakajima
In vitro selection of disease resistant plants ................................H. Toyoda

Session VI
Chairperson : K. Hinata
Genetic engineering and mutation breeding ................................H. Uchimiya

Session VII
Chairperson : S. Tano
General discussion

Closing address : S. Tano
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INDUCED MUTATIONS FOR CROP IMPROVEMENT

A. Micke (Vienna, Austria)

Looking back 30 years—more questions than answers

Thirty Gamma Field Symposia mark 30 years of active plant mutation research in Japan. The past symposia seem to have discussed already all aspects of plant mutagenesis. The first ones discussed “radiosensitivity” and “somatic mutations”. Later ones looked at “quantitative traits”, or compared spontaneous with induced mutations, the effects of chemical mutagens with those of radioisotopes, the radiosensitivity under chronic and acute irradiation. Later on, breeding objectives became the focus of intense discussions, particularly grain protein improvement and the improvement of disease resistance, and various aspects of mutant screening. Mutagen induced chimerism as well as the use of induced mutants in plant research were also discussed. It would seem attractive to revisit all these topics now again and check, how the advancement of science has change our understanding and softened positions, that were forcefully defended 15 or 20 years ago. Perhaps one of the senior Japanese scientists would like to undertake this task, it can't be done within the frame of this lecture. The topic for the 30th symposium is “Biotechnology and Mutation Breeding” and it may be expected to look more into the future than to the past. But I am not a biotechnologist and fortunately not a prophet either. Therefore I suspect, that you want to hear from me the answers to the numerous questions that puzzled you during the past 29 symposia. It is true, that I worked in the area of plant mutagenesis ever since 1953, when I started my doctors-thesis, and this means during 38 years, including nearly 22 years as Head of the Plant Breeding and Genetics Section of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture in Vienna (Austria). I am afraid, I have to disappoint you: From all these experiences I feel now, that I know many more questions than answers. Perhaps we will find it useful, to compare our questions and eventually agree what should be done in future, to make more progress in plant through use of mutagenesis with or without “biotechnology”.
Early experiences

During my doctors thesis, dealing with x-ray mutation induction in Melilotus to remove a bitter and toxic substance (68), I was quite frustrated already after 2 years, because I had found a lot of mutants, but not the ones desired by the professor (70). I never found the answer, why it took 8 years and huge populations of screened M2 material before observing the monogenic-recessive mutation desired (71). This may be a consolation for those among you, who also searched in vain for a particularly desired mutant. But one thing I know is, that we used an inappropriate chemical screening test during the first 4 years (69), and this may be a lesson also to others. Anyhow, the various mutants we obtained were very interesting from botanical, genetic, evolutionary as well as agricultural viewpoint (72, 109). For example, we found many morphological mutants resembling those described by other legume researchers, this confirming Vavilov's law of homologous genetic variation (9). We also observed a striking mutant heterosis, in the sense that F1 plants derived from “back”-crossing mutants with their original strain exceeded the better parent in biomass by 20% to 100% (73, 74, 76). I have not yet found the satisfactory answer to the question about the cause of this heterosis, although there are of course a lot of hypotheses (28, 52, 64, 66, 121), mainly centering around “overdominance”.

Critical phases of the FAO/IAEA programme

When I took responsibility for the plant Breeding and Genetics Section of the Joint FAO/IAEA Division in 1966, I was confronted with an incredible criticism about this programme. It was the time, when the international agricultural research centres like CIMMYT and IRRI demonstrated to the public, that simply by proper cross breeding of the right parents one can create a kind of “green revolution”. Therefore people asked, why UN-Agencies spend money on such a sophisticated and so far rather useless plant breeding approach as mutation breeding. In particular, it was critizised that funds were used to train plant breeders from developing countries in this technology. Our reaction was, that either we prove the usefulness of the technology or stop working on it. Most of you are probably familiar with the further developments of the FAO/IAEA programme: In 1969, we organised the first international training course on mutation breeding for plant breeders and geneticists from 20 different developing countries in Italy, where at that time already a remarkable success had been achieved with productive mutant varieties of durum wheat. In the same year, an international symposium, reviewing mutation breeding worldwide was held in Pullman (USA), just after the Second International Barley Genetics Congress (21). The following year, the

**Fostering practical mutation breeding**

(a) **Closing a protein gap**

Up to this time, IAEA had mainly been interested in the radiobiological aspects of mutagenesis. Now, under the severe criticism from outside, the organisation found itself in a position to use its funds for proving the practical value of radiation and radio-isotope applications in agriculture. Number one objective for the plant breeding programme was actually defined by the UN-General Assembly in 1968, when it recognized a growing “protein gap” in the staple cereal crops (22). The efforts to close this gap were immense, even more so by international centres like IRRI and CIMMYT and national programmes (incl. USA, Japan, Sweden, India, USSR, Germany), than by FAO and IAEA. The results were quite promising, when “by a stroke of a pen” expert nutritionists reduced the human protein requirements and closed the “protein gap” (79). Nevertheless, the duration of the work was long enough, to have efficient screening techniques developed, which will be available, should the “proteín gap” show up again in future (23, 31).

(b) **Improving disease resistance**

The next topic taken up was commonly agreed as being relevant: the improvement of disease resistance of crop plants (25). Breeding for resistance had many successes, most of them however only of satisfactory impact during a very limited time period. The evolutionary capacity of the pathogens initially had not been recognized or was underestimated. Available genes for resistance, when incorporated into popular varieties, were becoming useless in defending the crop, as soon as the pathogen in question specialized on virulences against which the incorporated resistance genes were not effective. The first consequence was to search for other genes giving resistance, eventually in primitive varieties or wild relatives. But these genes succumbed to the same process, and when breeders began to run out of resistance resources, two solutions were envisaged: One was the idea, that there might also be genes that do not succumb to pathogens’ ability to evolve new virulences; and hence give long lasting, “durable” resistance. This idea became fashionable under the term “horizontal resistance”. But this epidemiological term, coined by Van der Planck, is often misused, e.g. by identifying it with polygenically inherited and only partially effective resistances. The resulting confusion lead many to the conclusion, that fully effective and monogenic resistances could not be durable. This is –as we know today– an unjustified
generalisation. The second option was to search for “new genes” for resistance in mutagen treated plant material. Some researchers felt, that this approach makes little sense, since mutagenesis was know to change only one gene at a time, leading usually from dominant to recessive alleles or to deficiencies, and could never produce a new gene in the strict sense. Nevertheless, because of high crop losses from diseases and pests not being controlled by other means, mutagenesis work was tried in many countries and on many plant species, sometimes out of scientific curiosity, still expecting only monogenic-recessive “vertical” and therefore non-durable resistances (25, 30, 34, 75, 103).

Many mutants with improved resistance have been reported in different crop species (30, 34, 60), some of which became rather useful (like for example a chickpea mutant with resistance to Ascochyla-blight in Pakistan (46), pearl millet mutants in India (61, 94) with resistance against Sclerospora graminicola or peppermint mutants resistant to Verticillium wilt (93) in the USA. It seems, however, that the majority of mutants found possessed alleles of resistance genes that were already known and such mutants, of course, were not so much appreciated. This however, was to be expected, since for a highly selected trait like resistance to a damaging pathogen, existing genes conferring some level of protection have most likely been uncovered during evolution and previous plant breeding. Therefore it is rather unlikely to discover new genes unless one deals with a situation, where the plant species is confronted with a particular pathogen for the first time in its evolutionary history (78, 85). However, I would like to draw your attention to the fact that some new ideas were born about the genetic regulation of host reactions to pathogens, based on induced mutants which showed alterations of pre-existing resistances (5, 30, 34, 55, 78, 96, 111, 112, 126). Perhaps also for the subject of improving disease resistance of crop plants one may consider the lessons learned being more important for future crop advancement than few more useful mutants found (34, 40, 77).

Assisting plant breeders

Other international projects of FAO/IAEA concerned specifically vegetatively propagated crops (26), grain legumes (38), root and tuber crops, oil seed crops (33) and bananas. We were concerned also about the genetic vulnerability of short culm cereals and succeeded to provide alternative genetic sources for culm shortening in rice, barley, wheat and oats (36, 65). In an interdisciplinary project one is looking specifically for possibilities to improve symbiotic nitrogen fixation of grain legumes by mutations in the host plant as well as by genetic manipulation of the bacteria (12, 47, 101).
All the work mentioned would fall into the category of technology adaptation and application. Unfortunately, due to financial constraints and the pressure from sponsoring governments to show marketable products, efforts on technology development had decreased steadily. One should not forget, that IAEA has also been providing substantial assistance in from of equipment, training and expert advice. All this assistance, however, was given only to "developing" countries and I consider it as very unfortunate, that plant breeders from "developed" countries had no access to our training courses. This certainly would have stimulated some cooperation on advancing the mutation breeding technology and would have helped its acceptance for commercial breeding programmes.

**More information needed**

I haven't mentioned yet the "Mutation Breeding Newsletter". Which over the years became a rather important part of the FAO/IAEA activities. Started by SIGURBJÖRNSON in 1972 as a means of communication among institutes actively working in plant mutagenesis, the Newsletter was met with increasing interest from plant breeders and even from biotechnology centres and libraries, so that more than 1000 copies were printed twice a year and distributed free of charge. The rising interest was certainly not only due to the listing of new mutant cultivars and the mutant germplasm mentioned, but also due to the informative research reports from all over the world.

Another publication series was called "Mutation Breeding Review" and intends to summarise for particular crop species or groups of crops information on procedures and results of mutation breeding (14, 50, 102, 114). Consultations were often arranged ad hoc with experts to identify problems, clarify objectives and make recommendations on the best approach. These consultations usually led to a publication on the subject e.g. "New Approaches to Breeding for Improved Plant Protein" (22), "Mutation Breeding for Disease Resistance" (25), "Induced Mutations in Vegetatively Propagated Plants" (26), "Tracer Techniques for Plant Breeding" (27), "Induced Mutations in Cross Breeding" (28), "Improvement of Oil-Seed and Industrial Crops by Induced Mutations" (33), "Selection in Mutation Breeding" (35), "Plant Domestication by Induced Mutation" (39). International symposia were organised in 1964, 1969, 1970, 1977, 1979, 1981, 1986 and 1990, the proceedings were all published by IAEA (20, 21, 23, 30, 31, 32, 37, 40). The last symposium tried primarily to compile the practical results achieved in the different countries. Therefore, the proceedings read perhaps like a tremendous success story, except that there were a few participants raising doubts about some of the claimed successes. This is the point, where I wish to come back to the questions that
I cannot answer.

**Why are there failures in mutation breeding?**

The question that bothers me most is “Why have some researchers not been successful, when so many others were successful in mutation breeding?” In general we tend to say that a lack of success in mutation breeding is due to
- too small populations
- not effective or too effective mutagen treatment
- false selection.

In many instances it was observed, that for other than visible traits, selection techniques were unsatisfactory (17, 35).

Among the successes, some are certainly false claims based upon improper starting material, outcrossing, unnoticed contamination, wrong interpretation of results, premature publication of unconfirmed observations (54). It certainly also happened, that good results were obtained from bad experiments simply by good luck. I think, there is no reason to question the validity of such results simply because their mode of origin does not comply with prescribed methodology. However, there is every reason to avoid a modification of prescribed methodology on the basis of such “good luck experiments”.

Absolutely non-acceptable to me is the accusation of false claims simply based on criteria such as
- the “mutant” is similar to variety “X”
- the “mutant” trait has been observed before in other germplasm
- the “mutant” carries several changes which are even independently inherited
- the “mutant” trait is dominantly inherited
- the “mutant” has never been obtained elsewhere again.

Everyone of us, who carried out mutation induction experiments with open eyes, had some unexpected results. Often, one was afraid to publish such observations and this is really very unfortunate.

**Where we may agree**

It may be good to summarize at this point from common experiences, where almost everybody seems to agree:

1. Mutagens break genetic stability and create mutations.
2. Phenotypically, mutations mostly lead to traits that appear to be recessive against the original trait. This is independent from the mutagen used and also applies for
newly observed “spontaneous” mutations as well for mutations caused by insertions (6).

3. Cytologically, mutations can be unnoticeable gene alterations, deletions or various forms of translocation.

4. At the molecular level, mutations could be due to base pair changes, recombination or transposition (11, 129).

5. There is no specificity in terms of mutagen action focussing on particular genes only or in terms of changing genes in the desired direction.

6. Mutagenesis produces undirected changes, which are not necessarily at random. The desired ones have to be selected from sufficiently large populations.

7. Mutagens differ in the “spectrum” of the mutations they produce (62), but this, so far, had little relevance for practical mutation breeding: Some breeders prefer a chemical mutagen, others use several different mutagens.

8. Varieties (genotypes) differ in their sensitivity to mutagens and in the spectrum of mutants that can be obtained (62).

9. Genes differ in their mutability and in the kinds of mutant alleles that can be obtained (63).

10. Very effective mutagen treatments (high doses) cause multiple mutations, which may render a selected mutant difficult to use or unusable.

Where we may disagree

It may also be good to try to summarize the points where many of us tend to disagree and to argue, even after 60 years of plant mutagenesis research:

1. What is the best mutagen?

2. What is the appropriate dose?

3. What is the best object of mutagen application (e.g. seeds, pollen, whole plants)?

4. What are the best treatment conditions (e.g. acute vs. chronic irradiation, concentration vs. duration, dry vs. presoaked seeds)

5. Should one favour “micromutations” (122)?

6. Should one avoid or favour “diploic selection”?

7. Is “chimerism” really causing problems (13, 114)?

8. Is “somaclonal variation” more promising than mutagen induced genetic variation (13) and if so, then also for vegetatively propagated plants?

9. Is it a waste of resources to look for other kinds of mutations than the ones that express visibly (17)?

10. If there is a choice—is it better to use a “natural” variant than to use an induced mutant (8, 59)?
No progress in technology during 25 years?

Some of you will recognize, that there are nearly the same arguments as in 1964 at the symposium sponsored by FAO and IAEA in Rome (20). Does this mean, that we have not made any progress in the methodology during 25 years, that we spent all efforts on applying an established technology and that still we have not been able to convince everybody about the usefulness of the approach (40, 60)?

In 1929, shortly after the International Genetics Congress in Berlin, where mutagenesis was the main theme, Stubbe addressed the plant breeders to make use of this new tool (118). But in 1942, Stubbe complained already, that mutagenesis has been known for 13 years, but breeding research pays little attention and practical breeders are reluctant to embark on mutation breeding (120).

So much has happened in genetics since then! We know so much more about genes, their number and size (92), their structure (2) and function (92), their regulation (100) and interaction (6), their replication and duplication, and even about mutation processes (1, 7, 11, 13, 18, 45, 51, 108) and repair (15, 41, 42, 48, 57, 128). We heard not only about structural genes and regulator genes (6), but also about housekeeping genes, peripheral genes, silent genes and redundant genes. Although induced mutations played a crucial role in these developments of genetics (7, 13, 64), there seems to be hardly any backfeeding via plant mutation research to the technology of mutation breeding. I described the reasons, why the FAO/IAEA programmes shifted away from basic technology development to technology transfer and practical application. Similar reasons may have affected the various national programmes. But now with 25 years of proven practical success (20, 40, 60) -is it too late to take up again the advancement of technology or is it just the right time? If it is the right time, what would be the tasks?

Why are certain mutations rare?

In the early sixties, people still worried, whether any useful mutants could be obtained (16, 20, 88, 117, 120). Now people worry about a particularly desirable mutant not being obtained. That is really a remarkable difference. Certain types of mutations are now almost taken for granted, e.g. giving earliness, lateness, short culm, more branching or tillering (17). Other mutations seem to be very hard or even impossible to get. An example may be the highly desired indehiscence of fruits of sesame (4). We anticipate from the common molecular structure of genes, that all genes can be mutated. But does this imply, that all desired mutations can be obtained? Apparently not. But even so, the practical results seem to be better than scientifically
based exceptions.

**What is the role of mutations in evolution vs. breeding?**

Remember, about 90 years ago Mendel and followers had just convincingly established, that the elements of heredity are stable and are passed on to subsequent generations unchanged. This provoked immediately De Vries (16) to postulate possibilities for breaking down this stability, otherwise one would have no proof of the mutations in evolution (43, 90, 91, 92, 122). One distinguished macro-evolution from micro-evolution (64) and when the means for mutation induction were found in “ionizing radiation”, there was great disappointment: One found “hopeless monsters” in macro-evolution and only detrimental variation in micro-evolution. It is well known, that Muller had been very optimistic about the potential of induced mutations to improve plants and animals, and even mankind (91). But Stadler’s experiments could not support this optimism (115, 116, 117). He felt, that only deficiencies were produced, at least by radiation, and therefore discouraged to expect anything useful from experimental mutagenesis. (It seems, that his results were particularly negative in maize. Neuffer (7) using another technique obtained quite positive results.)

**How can any mutation have a positive effect?**

At that time, genes were more or less thought to be beads on a string, that could be altered one by one or knocked out (90). Many experiments showed, that it is much easier to knock out a gene than to change it (119). Today we have a much better idea what a gene looks like (92): it may be made up by 1000-10000 base pairs, and codes for a complicated protein made up of hundreds or thousands of amino acids. This seems to open only two possibilities for mutagenesis: (a) an alteration that has a negligible effect on the protein—such a mutation will not be noticeable or (b) an alteration that changes the gene structure and the function of the gene product. No matter what was the cause of such an effective gene alteration, one should anticipate that the gene function will be impaired or even lost. Such an impaired or lost gene function has eventually multiple consequences due to gene interaction, their dimension depending upon the role of the particular gene in the metabolism of the cell or the plant.

**Dominance or recessivity?**

Such an impaired gene function should better not be confused with the phenomenon of “dominance” and “recessivity”. If such an impaired gene function requires
homozygosity to be noticed, we deal with a typical recessive mutation, but if the impaired function is already noticeable in heterozygous condition, we deal with a dominant or at least semidominant mutation. Freshly arising mutations start probably in heterozygous condition (with very rare exceptions). Those that express are immediately subject to selection. In most cases, the cell(s) carrying such a mutation will have a handicap and eventually be eliminated from the meristem by a kind of intrasomatic competition. On the other hand, detrimental mutations that require homozygosity to be expressed, can be tolerated by the heterozygous cell and can be carried along without problems through mitoses, as long as DNA replication is not stopping the mutation site. Is it then surprising, that at least in cross pollinating plants, dominant alleles provide vigor, when those that have a detrimental effect are eliminated by selection? The dominant alleles left will often show optimal functioning at the single dose level. This is very well in line with observations, that crosspollinating plants like maize tend to accumulate deleterious recessive alleles (thereby assuring a gene dose of 1 for dominant alleles) and derive a fitness advantage from this (52, 105, 107).

But in selfpollinating plants, where homozygosity is the normal stage, dominant and recessive alleles should have an equal chance to contribute to fitness as well as good performance as a crop plant (110). An observation, interesting in this context, was reported by Lundqvist: In a mutation experiment aimed at changing the number of rows in the barley spike, 11 loci mutated. 10 loci gave only recessive, one locus only dominant or semi-dominant mutations compared with the original two type (63).

I raise this point, because the prevailing recessivity of induced mutations is used again and again as a principle argument against the usefulness of induced mutations for crop improvement. Thereby it is often overlooked, that the same argument would be valid for “spontaneous” mutations (6) upon which -we assume- evolution ultimately is based. One should not neglect a vast amount of literature showing that dominant vs. recessive expression of a gene (allele) depends also on the “genetic background”.

**Do all genes mutate?**

At a symposium in honour of one of the pioneers of plant mutagenesis, Erwin Baur, in Gatersleben (Germany) some 25 years ago Marie from Feance posed the question “Do all genes mutate” (67)? At that time I would not have hesitated to answer “yes, of course”, in spite of the poor experience by Stadler and others (88, 117) that there was never a real gene mutation from radiation treatment. Looking, however, today at the work of Amano and Yatou (3, 131), I would have to say “no, of course not”! There can be several reasons why we don’t get a particular mutation:

-the gene may not mutate
the mutated gene may not express
the mutated gene may not be transmitted
the mutated gene may have a lethal effect on the plant
the mutated gene cannot be selected.

All genes are made up by DNA, why should some be exempt from mutation? Some differences in mutation rates could be explained by different gene sizes (number of base pairs), some by gene location or chromosome structure. But many genes (e.g. so-called house-keeping genes) occur in multiple copies. They may mutate but the mutation cannot be recognized phenotypically and exists only as cryptic genetic variation (to some extent comparable with the situation of recessive mutations in polyploids). The number of such duplicated genes seems to be substantial. A major part of the plants' genomes also seems to consist of (permanently or temporarily?) silent DNA, which nevertheless is meticulously copied for every cell division. What happens to mutational changes in this large part of the genome?

**Most mutations are repaired**

Another matter to be taken into consideration is repair of genetic damage. It has been estimated, that errors in DNA replication have a probability of 1 per 10 replicated genes. This potential “mutation rate” would prohibit any evolution, fortunately, however, it is reduced by post-replication repair to 1 per 100 000 000 (48). It is not know, whether repair conserves all genes equally, but it is certainly impressive that living organisms make such enormous efforts to avoid, prevent and repair genetic damage (15, 18, 41, 42, 128). Obviously, the basic principle of life must be conservatism, evolutionary progress appears to be based on exceptional events. On the other hand, Kiyosawa and Nomura presented at the Gamma Field Symposium 27 in 1988 a study according to which present mutation rates are much too low for explaining past evolution (58). Does this indicate, that conservative repair evolved only during more recent phases of natural history, when speciation slowed down?

For mutation breeding it would be important to know, whether all mutations derive from misreplication or misrepair, as now is claimed often for micro-organisms (108). Experimental interference with repair processes would then be very important for the outcome of mutagenesis. Repair capacity seems to be relative easily influenced by environmental conditions, cell metabolism, balanced availability of DNA precursors, imbalanced mineral nutrition, *in vitro* stress (15). Earlier experiments showing environmental influences upon mutagen effectiveness (119) may now be interpreted as effect of interference with repair. It should also be noted, that small mutagen doses were found to stimulate repair (128).
How realistic are breeding objectives?

The plant breeder wants heritable variation of a desired type, he does not care too much about the involved genes. But there must be biological limits for the breeders’ desires. Are breeders’ objectives always realizable? It may be good to remember Vavilov, who told us about parallel genetic variation in related species. This should be a useful guideline for the prospects as well as for the limitations of expectable genetic variation (19, 44, 56). Unfortunately Vavilov’s law cannot be taken as a guideline for disease resistance breeding, since pathogenicity of a particular pathogen is usually restricted to a particular host species and only rarely concerns several species (at least for obligatory parasites). Therefore Vavilov cannot help us much in predicting the existence of hidden genes controlling eventually compatibility or incompatibility with pathogens.

Hidden genetic variation

The area of disease resistance can also very well illustrate the situation that genes exist in the plant genome which cannot be recognized (7): A gene for resistance or susceptibility can only express in the presence of the matching pathogen; the matching pathogen, however, can only be recognized in the presence of the matching host. As a consequence one must conclude, that mutations affecting pathogen reaction genes are much more frequently induced than detected (and described in publications). It has been speculated, that pathogenic micro-organisms have higher mutation rates than crop plants. There is now convincing evidence from mutagenesis studies, that this is not the case (123, 125). Standard mutation rates are sufficient to explain the occurrence of “new” virulences. Virulences are commonly recessive, so it seems easy for the pathogen to accumulate some of them hidden till the time when they become useful for the pathogens’ survival.

Mutations affecting gene interactions

The pleiotropy of mutated genes (a phenomenon by no means limited to mutated genes) has been subject of concern in many studies. By influence of a different genetic background, pleiotropy can be modified or eliminated. This was shown for the mutant ml-o locus in barley, which had been detected in 1942 but was not used until 1986 because of pleiotropic necrotic spotting (59, 104).

There are other lessons to be learned from disease resistance, e.g. in recognizing the relativeness of dominance vs. recessivity. It has also been shown that monogenic
inheritance is not necessarily identical with "vertical" genetic interaction between hosts and pathotypes. Observations concerning the genetic control of disease resistance may easily us away from the concept of one structural gene-one trait and may force future breeders to think much more in terms of gene interaction and regulation of gene expression by other genes. I refer here to the work on barley powdery mildew by Röbbelen and co-workers in Germany (5, 103, 104, 127), by Jørgensen and coworkers in Denmark (55, 126), and to the work by Nakai and co-workers in Japan on bacterial leaf blight of rice (95, 96, 97). These should be taken as examples for complicated genetic interactions existing also for many other plant characteristics (4, 6, 12, 17, 43). Perhaps, mutation induction experiments will in future distinguish between effects upon essential ("structural")? genes that can hardly be tolerated by the cell/plant (unless the gene is duplicated), effects upon peripheral genes that possess no essential functions (e.g. colours), and effects upon "regulator" genes which only modify timing or location of activity of essential genes and therefore tolerate more variation (and have multiple alleles).

New interest in mutation breeding from biotechnology?

It seems that recently there is a kind of revival of interest in induced mutations. This comes from the observation, that under certain circumstances heritable changes occur in plant material during in-vitro culturing (10, 13, 106). Optimistic persons expect from this so-called somaclonal variation, that it fulfills the expectations not fulfilled so far from radiation or chemical induced mutations. Pessimists say that the percentage of useable "somaclonal variants" will be lower than of mutants obtained through classical mutation induction (89, 98). From the developments in biotechnology, optimists expect that much bigger numbers can be dealt with in mutation breeding experiments using in-vitro cultures than in normal ones using greenhouses or fields. The bigger numbers, however, would only be of advantage, if in-vitro selection can be practiced as well and that is not possible for most of the agriculturally relevant characteristics (10). Even for disease resistance, where selection by toxins was considered with great expectations, very limited prospects remain at present (49, 53). In any case, whatever would be selected under in-vitro conditions would have to confirm its performance in trials carried out under greenhouse and field conditions (89, 99).

Occasionally in the past there were reports about homozygous single gene mutations in MI. There were usually met with great scepticism and regarded as result of outcrossing or contamination. However, more recently such observations are made for mutants derived from in-vitro cultures, where putcrossing can be excluded (106). In
searching for an explanation, rather unorthodox ideas were developed which relate to the mechanisms of gene regulation (100). From experiments with *Antirrhinum* it is concluded, that the metabolic situation of a cell is not only able to switch genes on or off, but also to provoke "desired" mutations (51). In *E. coli* a high number of identical desired mutations was obtained simultaneously, probably by action of mobile elements (45).

Biotechnology will definitely make certain mutation breeding steps faster, simpler, eventually even cheaper by already established techniques such as clonal micropropagation or another culture for double-haploids, but the real progress beyond current possibilities will come from a combination of hybridization in a wider sense with mutagenesis in a wider sense at the molecular level, and here I mean gene engineering and "insertion mutagenesis" (113, 130). However, much costly research work focussing on real crop plants and agriculturally important genes will have to be done, before a tangible impact on crop improvement can be realized.

Mutation breeders now have almost 60 years of experience, which should be very useful also for biotechnologists. One of the most important experiences is, that the manipulation of individual genes can only have a limited impact upon the development of improved cultivars. Another important lesson biotechnologists could learn from mutation breeders is that chances of success for a new technology can be tremendously increased by application within the context of a good classical plant breeding programme.

**Summary**

Previous Gamma-Field Symposia have discussed already most aspects of mutation induction in plants and of induced mutants including their use for crop improvement. The topic of the 30th symposium "Biotechnology and Mutation Breeding" suggests, that more results in mutation breeding are expected from the development of "biotechnology".

From the beginning, mutation breeding had enthusiastic advocates and passionate opponents. Even now, after more than 30 years of success one can hear sceptics questioning the scientific soundness of reported practical results. At the same time there is an astonishing optimism about the prospects of "biotechnology" to solve once and forever the pending plant breeding problems.

Most of currently applied mutation breeding technology follows methods designed 25-30 years ago. Good results are obtained more or less empirically, advances in genetics and molecular biology are being ignored. Today, a much deeper and more comprehensive understanding of the principles of mutagenesis and the genetic implica-
tions of induced mutations should be possible than in 1977, when IAEA published the most recent edition of the “Manual on Mutation Breeding”.

The mutation breeder faces also other practical problems, particularly with regard to effective mutant screening techniques. Gaps of understanding the genetic control of plant metabolism impede the development of crop cultivars tolerant to stresses and more efficient in using inputs, but mutagenesis contributed already to a better understanding of the complex interactions between host plants and pathogens.

The primary biotechnology for crop improvement will be “gene engineering”, but many years of costly research focussing on crop plants are still needed, before a tangible impact can be expected. Presently available, more simple “biotechnologies” should be combined with mutagenesis and from such interaction, further advances in crop improvement could already be substantial.

References


INDUCED MUTATIONS FOR CROP IMPROVEMENT


129. WALKOT, V. (1988). Reactivation of the Mutator transposable element system following gamma irradi-


MUTATION BREEDING BY USING TISSUE CULTURE TECHNIQUES

F. J. Novak

Introduction

Existence of genetic diversity among plants in a population is a basic prerequisite of successful plant breeding. Special procedures have been developed to broaden the genetic variation before selection is applied. Hybridization as the basic breeding technique is practiced to combine the traits of distinct genotypes in order to achieve desirable recombinants in progenies. When genetic diversity is still not satisfactory in existing germplasm, the heritable variation may be broadened by the induction of mutations. Mutation induction by radiation or chemical mutagens is now a well established practical tool in plant breeding (Sigurbjörnsson and Micke 1974; Micke et al. 1987). More than 1500 varieties of crop plants have been released for cultivation (Maluszynski et al. 1991). The majority of the "mutant varieties" originated either as direct products of mutagenesis or as derivatives of subsequent cross-breeding with mutant parents in annual seed propagated crop plant species belonging to the self-pollinating cereals (e.g. rice, barley, oats) (Micke et al. 1985). However, the number of mutant cultivars of perennial and vegetatively propagated plant species is still rather small due to difficulties in their genetic manipulation and the little effort put in their breeding (Donini and Micke 1984).

More recently it has been discovered that in plant cell cultures spontaneous variation may exist. The variability displayed among the plants deriving from different types of tissue and cell cultures was named as "somaclonal variation" (Larkin and Scowcroft 1983). Some of the variants derived from somaclonal programmes have proved to be heritable changes and therefore useful for breeding purposes. Furthermore, it is possible to combine mutation induction techniques with in vitro culture (Novak and Micke 1988) and apply selection for desired traits to a large number of individuals (cells, organs, propagules) under controlled conditions in a small space (Dix 1990). In recent years, remarkable progress has been made in tissue culture technology and plant molecular biology. Although these research fields provide valuable contributions to plant breeding, biotechnology did not replace "classical" breeding methods. Still very few released varieties may be designated as a product of biotechnology.
(Micle 1991). These novel techniques offer complementary breeding strategies and their successful applications in the breeding process are very dependent on the plant species and the breeding objectives (Novak 1991).

**In vitro techniques in the M₁ generation**

Mutation techniques in plant cell culture are determined by the breeding system of the particular species. In crops with sexual propagation preferably seeds are subject to mutagenic treatment. However, only when the primordial cells forming the generative histological layer (L₂) derive from mutated initial cell of a seed embryo will the gametes contain mutated genes. After mutagenic treatment of seeds, vast numbers of somatic mutations (i.e. mutations in somatic cells) in layers L₁ and L₃ are lost because they don't participate in gamete formation.

Tissue and cell culture techniques may ensure the immediate incorporation of otherwise lost somatic mutations into the genetic make-up of individuals. The process is based on plant cell totipotency, i.e. the potential of a single cell to develop into a whole organism. In tissue culture conditions adventitious buds may develop directly on the "explants", i.e. cultured pieces from a leave, stem, flower part, etc. This process of adventitious organogenesis can be unicellular or multicellular, dependent upon whether a single cell or a group of cells (very often from the L₁ histological layer) form shoot meristems. In several plant species direct formation of somatic embryos of unicellular origin has been achieved in explant culture. Direct plant regeneration from somatic tissues of M₁ plants (usually sectorial chimeras) may avoid the main constraints of mutation breeding viz. detection of a mutation in chimerical somatic tissues and its stabilization in homohystont conditions (Fig. 1). A diagram of the mutation breeding scheme including plant regeneration from somatic tissues of M₁ plants is shown on Figure 2.

Haploid techniques may speed up the process of mutation breeding. When mutations are induced before in vitro culture and anther or microspore cultures are initiated from M₁ plants the haploid M₂ individuals provide an opportunity for direct selection of mutants (Szarejko et al. 1991). After chromosome doubling, homozygous mutant lines become available for the breeding process. Figure 3 shows the breeding scheme of introducing a double-haploids step into the mutation breeding process.

**In vitro mutation breeding of vegetatively propagated plants**

Vegetatively propagated crops are very often perennial plants with long generation times and complicated physiology (e.g. dormancy, seasonal cycles, specific condi-
Fig. 1. Direct regeneration (adventitious organogenesis or somatic embryogenesis) in plant tissue culture:
A - Formation of microtubers in axillary position of a potato plantlet;
B - Shoot regeneration from leaf segments of sugar cane;
C - Unicellular origin of a somatic embryo of cocoa;
D - Somatic embryo formation on a scutellum of maize.
Fig. 2. A diagram of the mutation breeding scheme including plant regeneration from somatic tissues of $M_1$ plants.
Fig. 3. A diagram of the mutation breeding scheme including a dihaploid step in M₁ population.
Fig. 4. A diagram of the mutation breeding scheme of vegetative propagated plants including plant regeneration from meristems and/or somatic tissues.
tions of flowering) as well as complex genetics (e.g. high degree of heterozygosity, clonal propagation due to sterility, apomixis, polyploidy). Most cultivars in this category of crop plants arose from spontaneous mutations ("sports"), but their frequency is too low, so that breeding progress is incidental and more extensive compared with seed propagated plants (Abbott and Atkin 1987). Logically, in such a situation, mutagens are being applied to obtain a higher frequency of somatic mutations from which useful characters may be selected (Broertjes and Van Harten 1988). The difficulties associated with the use of mutation induction techniques in vegetatively propagated crops may be overcome by using in vitro cultures. Thus, the number of vegetative parts available for the application of mutagens can be drastically increased by in vitro propagation of meristematic tissue. Axillary branching, enhanced by cytokinins in a nutrient medium, produces a great number of uniform, and developmentally synchronized units for mutagenic treatment (Novak et al. 1986). Mutagenesis applied to shoot tips gives rise to mericlinal (sectorial) chimeras. However, periclinal or homohostent shoots can be obtained after several subcultures on a medium inducing axillary branching. In vitro derived plantlets are commonly transplanted to the soil in the M_1V_1 generation.

When adventitious shoots or somatic embryos arise directly from the somatic tissue of explants (without callus phase) the chimerism in the M_1V_1 generation is essentially reduced. The adventitious bud technique accelerates the mutation breeding process, especially in species with good in vitro regeneration competence in explant cultures (e.g. potato, chrysanthemum, carnation, banana, strawberry). A diagram of the mutation breeding scheme of vegetatively propagated plants including direct plant regeneration from in vitro culture is shown on Figure 4.

Plant somatic cells grown in unorganized (callus, cell and protoplast suspension) cultures undergo extensive genetic changes (D'Amato 1990). In cell cultures of polysomatic species, the occurrence of polyploidy reflects the pre-existence of endoreduplicated cell nuclei in explants. In established cell cultures, polyploidy can originate through defective mitose or endoreduplication in the S-phase of the cell cycle. Nuclear fragmentation, multipolar mitosis, lagging chromosomes and other mitotic aberrations lead to aneuploidy and eventually to haploidy in cultured cells. Chromosomal structural changes and gene mutations occur frequently in undifferentiated tissue in in vitro culture.

All such heritable changes but also epigenetic factors generated through in vitro culture in somatic plant cells can express their influence on the phenotype of plants regenerated from them (Karp 1991).

Somaclonal variation has gained some importance for breeding. For example, positive variants have been found after screening for disease resistance and for
herbicide tolerance (Jones 1990; Smith and Chaleff 1990). However, it should be emphasized that the variation from *in vitro* culture will not replace the other sources of desirable variation, namely sexual recombination and induced mutagenesis (Novak 1991).

The main problem of somaclonal variation is the unpredictable and uncontrollable ways in which changes *in vitro* arise. In many cases the phenotypic alterations are non-heritable epigenetic changes or developmentally unstable mutations useless for practical breeding. Otherwise, the differences between somaclonal variation and mutated populations are not so relevant. There are several examples indicating that mutants originated during *in vitro* culture are identical to those induced by chemical or physical mutagens (Matthews *et al.* 1986; Bhatia and Matthews 1988). In specific cases the genetic variation in the selfed progenies of tissue culture regenerants (R<sub>2</sub> generation) may exceed that observed in the M<sub>2</sub> population, however, the spectrum of variants is similar (Novak *et al.* 1988). In comparing somaclonal and mutagen induced variation, Orton (1985) and Gavazzi *et al.* (1987) observed variants among tissue culture regenerants which have not been detected as mutants in a mutagen treated population. These types, however, were already described elsewhere as spontaneous or induced mutants, e.g. "potato leaf" in tomato, early flowering in maize and celery, etc.

More research effort is necessary to prevent the generation of variation in *in vitro*. The methods which yield a large number of uniform regenerated plants are to be preferred in mutation breeding programmes. Currently, the best procedure is to avoid an unorganized *in vitro* phase (callus, cell and protoplast culture) and perform a rapid passage through the meristematic stage (auxillary branching and direct formation of adventitious buds or somatic embryos). These stable cultures are not only important for micropropagation and germplasm *in vitro* storage but can also be useful for mutation breeding technique permitting treatment of meristems and facilitating mutant detection. Also for the analysis of induced mutants and their evaluation under field conditions, uniformly propagated plants would be very helpful.

**Conclusion**

Mutagenic radiation and chemical mutagenic agents together with *in vitro* culture can significantly raise the possibility of generating genetic variation for use in practical breeding. Effective incorporation of tissue culture into mutation breeding programmes is dependent on the reproductive system and breeding procedures of the species in question. In species with generative propagation, the chimeric M<sub>1</sub> plants may be used as a source of somatic tissue containing differently mutated cells that may be regenerated into heterozygous but homohistont (non-chimeric) plants. Obviously,
this process ensures the manifestation of a great number of mutations in the genetic make-up of individual plants. Haploids derived from mutated M₁ gametes provide the opportunity for direct detection of recessive as well as dominant mutations and through genome duplication for the production of homozygous mutant lines. In vitro mutation breeding however is particularly important for improvement of vegetatively propagated crops, i.e. plants with long generation time and with otherwise limited breeding possibilities. The potential lies mainly in establishing an effective methodology for mutagenic treatment of meristematic tissue, avoiding or eliminating chimerism and obtaining selectable homohistont mutants. Genetically stable morphogenetic in vitro cultures with reduced risk of somaclonal variation are best for the induction, detection and propagation of mutants and thus have the greatest potential for use in practical breeding.

Acknowledgements

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References


DNA MODIFICATION OF MUTANT GENES

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Introduction

A series of low amyllose rice mutants have been induced (Amano 1981). These mutants show continuous variation from amyllose-null mutants to near wild type mutants. These mutants have their own economic values depending on their amyllose contents. The mutant which have slightly lower amyllose content than wild type has been used as breeding material to improve cooking quality of Japanese rice. A rice cake, 'Mochi', with residual amount of amyllose which can be made from waxy mutant with lower amyllose content has its own special taste. After genetic analysis of these mutant, most of these mutants revealed to be mutants of \textit{waxy} locus. As shown in this locus, mutant genes of a locus do not have uniform phenotype. Then questions arose, that what phenotypes can be obtained in a locus by mutation, and that a specific, desired phenotype can be induced by controlling mutation induction procedure.

Historically crop breeder who concern in mutation induction to improve his crop have had hope to regulate mutation induction to induce any kind of mutations of their preference. In the early research on mutation, numerous research works were conducted to control spectrum of induced mutations by evaluating various mutagens and mutagen treatments. These research revealed that at least in chlorophyll mutation the spectrum of induced mutations was varied depending on mutagen applied. One of the most clear evidence on mutagen specificity in plants was obtained in a series of work in soybean (Vig, 1982). Three type of somatic mutation sectors on leaf of the soybean strain, which has heterozygous genotype at \textit{y}_{11} locus, showed different relative frequencies depending on mutagens treated. However chlorophyll mutations are less interested in mutation breeding because this mutations involve many chromosome reconstruction besides gene mutations. What we must know in crop breeding and gene research is the nature of spectrum of induced gene mutations.

The research on mutation spectrum of gene mutation in plants were limited

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because the research require a large number of independent mutants of specific loci and a large number of plants for their genetic analysis. Many early heading mutants in barley were isolated and genetic analysis was conducted (Ukai, 1983), and it was shown that mutagens has locus specificity at some degree in their mutation induction abilities. However most of the mutants of each locus showed similar phenotype. In Sweden, numerous barley mutants has been accumulated and they have been genetically analyzed, but again the phenotype of mutants in each locus were not much different (Lundqvist 1991).

Nelson tried to analyze genetic relationship between spontaneous and induced mutant genes of waxy locus in maize by observing pollen grain (Nelson 1976). In the same method, Amono analyzed waxy mutants of maize and rice (Amono 1981). Both of them constructed intra-genic genetic map of the loci. These work were completed because the mutation phenotypes were analyzed in pollen, while most of mutants induced in crop breeding and genetic research were left to be analyzed. Therefore information of mutagenic modification of gene has been limited. However recent advances in molecular genetics have offered new techniques to elucidate the molecular rearrangement of mutant genes.

**Genomic Southern blot analysis in waxy and nitrate reductase gene in rice**

Waxy gene and nitrate reductase (NR) gene were chosen in this study because many mutants have been obtained in the both locus and the both gene were cloned as DNA fragments. The cloned DNAs could be used as probes against mutant genes to investigate the mutational modification of their DNA sequences. Moreover because the phenotypic expressions of the mutants of the both locus have been identified, the comparison of DNA modification and phenotype might lead to understand their gene expression systems and their regulations.

Waxy locus have the sequence coding a amylose synthesis enzyme which catalyze the synthesis of amylose from NDP-glucose in endosperm. The phenotype of this locus was affected by several transacting loci. However, the locus is the only one which encodes the enzyme. This amylose synthesis system in endosperm is known to be common among cereal species. Because the mutants of the locus can be visually identified in endosperm and pollen and waxy character has economic importance, mutants in this locus have been extensively used in mutation research. Then numerous mutants have been accumulated. Moreover, the enzymatic reaction was identified and the enzyme coded in the locus was purified while the genetic analysis of the mutant was conducted. These situation gave us special advantage with the waxy gene in this study.
Table 1. Amylose content and waxy enzyme of cultivars and waxy mutants in rice. (Yatou and Amano 1991)

<table>
<thead>
<tr>
<th>Line</th>
<th>Original cultivar&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mutagen&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Waxy index&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Amylose content (%)</th>
<th>Waxy enzyme&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>Nihonmasari</td>
<td>W&lt;sub&gt;x&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.35</td>
<td>20</td>
<td>+</td>
<td></td>
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<tr>
<td>Akihikari</td>
<td>W&lt;sub&gt;x&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.20</td>
<td>14</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Norin 8</td>
<td>W&lt;sub&gt;x&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.60</td>
<td>14</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Norin 8</td>
<td>W&lt;sub&gt;x&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.60</td>
<td>14</td>
<td>+</td>
<td></td>
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<tr>
<td>Reimei</td>
<td>W&lt;sub&gt;x&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.89</td>
<td>13</td>
<td>+</td>
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<tr>
<td>Taichung 65</td>
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<td>++</td>
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<td>69.30</td>
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<td>KURwx4N1</td>
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<td>Gamma rays</td>
<td>51.64</td>
<td>4</td>
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<tr>
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<td>EI</td>
<td>60.60</td>
<td>0</td>
<td>+~</td>
</tr>
</tbody>
</table>

<sup>a</sup>N8 : Norin 8 ; R : Reimei ; NM : Nihonmasari.
<sup>b</sup>Mutagens were applied to the seeds. EMS : ethylmethanesulphonate ; EI : ethyleneimine.
<sup>c</sup>The waxy index was determined by a double spectra photometer after iodine staining :
less than 20 : non-waxy ; 20-65 : intermediate waxy ; more than 65 : complete waxy.
<sup>d</sup>The amount of Waxy enzyme determined by Sano (2) ; the amount of enzyme decreased in the order : ++ , + , + − and none, − −.

Low amylose mutations of rice analyzed here were isolated by Amano (Amano 1981 and his unpublished data). These mutants were identified by visual observation of endosperm of M<sub>2</sub> seeds and were classified by their color. Seeds of original cultivars were treated with gamma rays, thermal neutrons and EMS. In the following generations amylose contents in endosperm of these mutants were analyzed by KI-I<sub>2</sub> staining.
quick analysing system (Amano 1981). Amylose contents and the enzyme characters of waxy mutants analyzed in this study were shown in Table 1. The amylose contents of these mutants ranged from 0%, complete waxy, to the level of nearly wild type. By the genetic analysis of 29 mutants among these mutants, 19 mutants were identified as mutants of waxy locus (Amano unpublished). Molecular weights and protein amounts of the waxy enzyme in the waxy mutants were analyzed (Sano et. al. 1986 and unpublished data of Sano). In most of the mutants, the enzyme amount were correlated with amylose contents of the mutants, however there were mutants containing very few amount of amylose with the enzyme of the same amount as wild type and mutants have the enzyme of modified molecular weight and decreased content of amylose.

The complete DNA sequence of maize waxy gene was isolated by transposon tagging method (Shure et. al. 1983). This cloned maize gene could be used as a probe against rice waxy gene due to their sequence homology. In this study, 2.9kb fragment from the central portion of the maize DNA was made as a probe. Also 5.5kb fragment of cloned DNA from genomic library of rice cultivar 'Nipponbare' was used as probe. This genomic library was made by Dr. Matsuoka, National Institute of Agrobiological Resources, and the DNA was cloned with the 2.9kb fragment as a probe.

Genomic Southern analysis of rice using several restriction enzymes suggested that the sequence which shows high homology to the maize DNA is an unique sequence in rice genomic DNA. Also the high sequence homology between the rice DNA and the

![Image](image.png)

Fig. 1. Genomic Southern blot analysis of waxy gene in rice waxy mutants. (Yatou and Amano 1991)
1. 73wx1N1A  2. 74wx8N1A  3. 75wx1N1A  4. KURwx4N1  5. 83Gwx1  6. 78KURwx2  7. 82Gwx1  8. 79gwx2  9. 82REwx3  10. 84REwx3  11. 85REwx8  12. Wx1  13. wx19  14. Wx23.)
maize gene was shown while high homology in amino acid sequence is between waxy protein of rice and maize. These suggested that the sequence cloned here form rice genomic library was rice waxy gene.

In the analysis of 14 waxy mutants, only one mutant was found to have complete deletion of the whole DNA sequence of the gene. The other mutants was failed to be found their sequence modification. Fig. 1 shows the genomic Southern blot analysis. In the following analysis on the other 50 mutants among which mutants of unidentified locus were included, again no DNA modification was found. Throughout the analysis of 64 mutants only one mutant induced by thermal neutron irradiation was found to have DNA modification, which was a complete deletion of the whole gene. This mutant was a complete waxy mutant, 0% amylose, and completely lack the waxy enzyme protein in its endosperm.

Nitrate reductase in plants has its primary importance in nitrogen assimilation in plant. Plant root absorb the nitrate in soil, then enzymatic system reduce the ion to amino base. Nitrate reductase play the first roll of the reduction chain by reducing nitrate ions to nitrite ion.

Rice genes of nitrate reductase (NR) was isolated and cloned by using cloned cDNA of barley nitrate reductase gene (Hamat et. al. 1989). Two DNA sequences which showed homology to the cDNA were cloned from rice genomic library. One of these sequences corresponds to NADP-NR and the other to NADP (H)-NR. Though they have domains which show sequence homology, they were different in their restriction site maps. There is no evidence that the whole genes were included in the cloned DNAs, however 3' end of both genes were included in cloned DNAs and 1.8kb portion of each gene have homology to the barley cDNA and also to each other. In this experiment Mlu I /Bgl II fragment of this homologous fragment was used as a probe.

Four NR mutants were obtained among mutants maintained in Institute of Radiation Breeding (Hasegawa et. al. 1991). These NR mutants were progenies of irradiated plants in the gamma field of the institute. These mutants were selected at first on their morphological deviation from original cultivars, Norin 8, and were maintained in IRB as genetic stocks. Hasegawa et. al. conducted NR mutant selection in these mutants. The activity of nitrate reductase of a mutant with the lowest activity of the enzyme was as low as 10% of their wild type, however unexpectedly plant growth of these mutants were rather normal besides their morphological mutant phenotype.

Southern blot analysis of genomic DNA of these mutants was conducted with the probe (Fig. 2). However, no genetic modification was found. Though there still left possibilities where rather small DNA modifications could be involved in these mutant genes, the modification large enough to be detect with this method was not found.
In the genomic Southern blot analysis in mutant genes of waxy and nitrate reductase, any DNA modification large enough to be detected were found except the whole gene deletion in one waxy mutant. A mutation phenotype can be depened not only on mutation of the locus in interest but also on the mutation of genes which regulate the gene. In the genetic analysis of the low amylose mutants, at least several loci were involved in the phenotypic expression of waxy gene. However low amylose mutations analyzed here were the mutations of waxy locus according to the genetic analysis. Moreover some mutants among these mutants have modified enzyme protein of waxy locus. Therefore these waxy mutant genes were considered to have some DNA modification which is rather small to be detected by genomic Southern analysis.

Genetic analysis in the nitrate reductase mutants were not completed while these mutants have residual activities of NR gene. The existence of DNA modification can not be concluded in these mutants at this point.
DNA MODIFICATION OF MUTANT GENES

Only one mutant have large DNA rearrangement, though this deletion was that of complete sequence deletion of the gene. The deletion mutant was induced by thermal neutron irradiation whose LET is much higher than gamma rays irradiation. However such large modification was not found in the other two neutron mutants analyzed. Then, at this point, it was considered that large size modification may be rare events irrespective of mutagens applied.

**DNA modification by mutation in plants**

In spontaneous, induced and transposon-inserted waxy mutants of maize, intragenic modification were demonstrated by genetic analysis using pollen grain (Nelson 1976). To interpret this genetic map, genomic Southern analysis of these mutants was conducted (Wessler et. al. 1985). Among the mutants one of 3 gamma-ray-induced mutants have complete gene deletion, and any DNA modification was not found in the other 2 gamma-ray-induced mutants and a EMS mutant. In this analysis 12 spontaneous mutants have insertion or deletion. The other 5 spontaneous mutants were failed to be detected their modification. In rice, 10 waxy mutants were analyzed by genomic Southern analysis (Okagaki et. al. 1988), and one gamma-ray mutant was found to have 0.3kb insertion while any modification was not detected in the other mutants. In Neurospora, 81 his-3 mutants were analyzed by genomic Southern analysis (Dubins, et. al. 1989) and it was found that one gamma rays and one spontaneous mutant had new restriction site due to small sequence modification and one UV and two X-ray mutants had insertions. In the other 76 mutants, DNA modifications were not detected. These analysis suggested that most of mutational modification of DNA sequence, in general, might not be large enough to be detected by the genomic Southern analysis.

Mutational modification of APR'T' gene in hamster induced by gamma rays were investigated (Grosovskky et. al. 1986). Nine of 55 gamma-ray-induced mutants have detectable DNA modification in genomic Southern analysis while 2 of 67 spontaneous mutants have detectable modification. This is the indication that gamma ray tend to induced DNA deletion at higher frequency than spontaneous mutants. Two adh-gene mutants of Arabidopsis induced by EMS treatment were sequenced and found to have a base pair transition in both mutant (Dolferus et. al. 1990). Chemical mutagens have been considered, because of their specific effects on DNA, to induce small DNA modifications. However 30bp deletions were found in 2 waxy mutants of maize induced by EMS (Okagaki et. al. 1991).

As shown above, small DNA modifications, possibly less than 50bp, might be common in mutational modifications of genes. On the other hand, it was suggested that ion irradiation can have higher possibility of inducing large deletion or complete
deletion of DNA sequence of genes because of its higher LET (Yang 1979). In soybean
genetic marker strain, neutron irradiation induced yellow mutant sectors, most of
which may arise from deletions, at higher frequency than gamma rays irradiation (Itoh
et. al. 1991). The similar results with the soybean strain in ion irradiation experiment
was obtained (Yatou and Takehashi unpublished). Mouse HPRT mutant genes in-
duced with gamma rays, Ar, Ne irradiation were analyzed by multiplex PCR method
(Tsuboi 1991) and increased frequency of mutants with complete gene deletion with
increased LET of radiation was found. These results along with DNA analysis of rice
waxy gene shown above suggest that higher LET radiation likely induce relatively
large deletion at higher frequency.

To understand mutational modification of genes, further accumulation of muta-
tions might be required and also improvements of detection systems of DNA modification
is essential. With these study, a series of modified genes with modified phenotypes
will be in our hands, with which gene expression and genetic regulation system could
be elucidated. Moreover the complete deletion mutants as shown here in waxy gene
will be ideal material for genetic manipulation.

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突然変異による遺伝子 DNA の変異

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作物の突然変異育種を目的としてまた植物の遺伝研究を目的として従来から数多くの突然変異が誘発されてきた。これらの突然変異の一部は遺伝分析され遺伝子座の同定がされなかったが、これらの突然変異遺伝子自体の変異については解明されていなかった。わずかにトウモロコシやイネのモチ遺伝子座の突然変異について遺伝子座内の変異地図が遺伝分析によって作成されていたにすぎない。これは遺伝分析を花粉分析で行う事によって成功したが、他の遺伝子座ではこれは困難であった。そこで、近年急速な進歩を示している分子遺伝学の手法を用いて遺伝子内の DNA 変異の検出を試みた。イネのモチ突然変異遺伝子と硝酸還元酵素について検討したが、DNA の変異は 1 例を除き検出できなかった。DNA の変異が検出された唯一の突然変異は熱中性子の照射によって誘発されたモチ突然変異で、全遺伝子の完全な変異であった。当研究を含めこれまでに調査されてきた突然変異による DNA の変異の中にも、誘発原の種類にかかわらず、サイズの大きい変異は極めて稀であった。一方、大きなサイズの変異を誘発するためには熱中性子やイオン等の LET の高い放射線の照射が有効であると考えられた。
SOMATIC VARIATION IN PROTOPLAST- DERIVED RICE PLANTS

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Introduction

The utilization of tissue culture-derived variants (somatical variants) for the
development of new varieties has been proposed because of their wide phenotypic
changes observed in a number of crops.

In dicotyledonous species, Evans and Sharp (1983) obtained several single gene
mutations in tomato regenerated from tissue culture. Somatical variation in
protoplast-derived plants has been reported in tobacco (Lörz and Scowcroft, 1983),
potato (Shepard et al., 1980) and tomato (Shahin and Spivey, 1986).

In monocotyledonous species, although it was generally difficult to regenerate
plants from protoplasts, plant regeneration from rice protoplasts was described
previously (Fujimura et al. 1986), and Kyozuka et al. (1987) established a high frequency plant regeneration system from rice protoplasts by novel nurse culture
methods. Furthermore, Shimamoto et al. (1989) obtained fertile transgenic rice plants
from transformed protoplasts using direct DNA transfer technique. These advances
in genetic manipulation of rice at the cellular level are expected to be used for future
rice improvement.

In order to obtain transgenic rice plants, regeneration from protoplasts culture is
indispensable, thus it is necessary to investigate the nature of mutations (somatical
variation) occurring during protoplast culture and plant regeneration. But there are
only a few reports dealing with characteristics of somaclonal variants observed in
protoplast-derived plants.

Morphological changes in tissue culture-derived rice plants have been described
previously. Oono (1982) reported that 72% of 1121 rice plants regenerated from
seed-derived calli showed morphological mutations. Fukui (1983) also reported that
four mutations, early heading, albino, short culm length and sterility, were observed in
the progenies of rice plants regenerated from seed-derived calli.

As for protoplast-derived rice plants, Ogura et al. (1987) found that 19% of 126
plants regenerated from protoplasts showed phenotypic changes. Abdullah et al. (1989) investigated 13 agronomic traits in the progenies of plants regenerated from rice protoplasts and found that 10 of them were different from the original variety. Ogura et al. (1989a and 1989b) further investigated the yielding ability of the progenies derived from protoplasts of rice.

In this report we describe results of morphological investigation of protoplast-derived rice plants and of the possible relationship between phenotypic changes and changes in DNA. Examples in the application of protoplast-derived plants for rice breeding will be also described.

Morphological changes in the progenies from protoplasts-derived rice plants

Protoplasts culture and subsequent plant regeneration were performed as previously described (Kyoizuka et al., 1987). Regenerants from protoplasts (designated as R0 generation) were grown in greenhouse. Selfed seeds obtained from each R0 plant were used throughout the experiment. The R1 progeny lines derived from the protoplasts of 6 cultivars, Sasanishiki, Asahi, Hatsushimo, Norin 8, Norin 10 and Yamabiko, were first grown in greenhouse and transplanted in the paddy field as individual line culture. Several agronomic characters were investigated throughout the growth of the plants.

In R1 generation, mutations (somaclonal variation) were observed for several morphological characters. In addition to heterozygous mutations, such as dwarf, sterility and albino which were easy to be distinguished from normal plants, putative homozygous mutations were also observed in the R1 generation. Table 1 shows the

Table 1. Frequency of segregating morphological mutations in R1 lines (1989).

<table>
<thead>
<tr>
<th>Variety</th>
<th>No. of R1 lines examined</th>
<th>Morphological changes</th>
<th>Frequency(^) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sasanishiki</td>
<td>25</td>
<td>dwarf, sterility</td>
<td>8.0</td>
</tr>
<tr>
<td>Asahi</td>
<td>3</td>
<td>dwarf</td>
<td>33.3</td>
</tr>
<tr>
<td>Hatsushimo</td>
<td>9</td>
<td>short culm length, sterility, chlorina</td>
<td>88.9</td>
</tr>
<tr>
<td>Norin 8</td>
<td>23</td>
<td>short culm length, rolled leaf, narrow leaf</td>
<td>30.4</td>
</tr>
<tr>
<td>Norin 10</td>
<td>24</td>
<td>dwarf, small grain</td>
<td>12.5</td>
</tr>
<tr>
<td>Yamabiko</td>
<td>50</td>
<td>dwarf, sterility</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Average of frequencies                                        19.4

1) Frequency of the R1 lines which contained segregating mutants.
percentages of R₁ lines which exhibited the segregating mutants in certain morphological characters. Dwarf, sterility, short culm length, chlorina, rolled leaf, narrow leaf and small grain mutants were found among R₁ progenies. Short culm length and dwarf were most frequently observed.

Mutation frequencies were greatly different among the 6 cultivars examined. Based on the frequency of mutations, they can be divided into two types, one is the high variation type, the other the low variation type. The former contains Hatsushimo (the average mutation frequency is 88.9%) and the latter group contains Sasanishiki (8.0%), Norin 10 (12.5%) and Yamabiko (10.0%). The average mutation frequency of 6 cultivars was 19.4%. Similar frequencies of mutations were observed over three years (data not shown).

Homozygous mutations were also found in R₁ lines. Fig. 1 shows such homozygous mutations exhibiting short culm length and late or early heading detected in 11 R₁ progeny lines of Norin 14. Culm length and heading date were scored according to the conventional breeding method. Heading date was determined on the day when approximately 50% of panicles of a line emerged.

Based on the degree of changes in culm length, 11 lines were divided into two types; first type included 8 lines exhibiting similar culm length to the original variety and the second type included 3 lines exhibiting significantly shorter culm length than the

![Diagram showing variation in heading date and culm length in the R₁ lines of Norin 14 in 1987 (segregating lines were excluded). Heading date was determined when approximately 50% of panicles of a line emerged and culm length was classified in comparison with standard varieties; Koshihikari, 6; Nipponbare, 4. • indicates R₁ line and * indicates Norin 14 (heading date, Aug. 21st; culm length, 7).]
original cultivar. As for heading date, however, direction of change was variable; one line headed 6 days earlier and 2 lines 8 days later.

Fig. 2 summarized results of changes in the heading date among R1 lines derived from 13 cultivars in the 1987 to 1989 seasons. The R1 progenies from protoplasts of 4 cultivars were investigated in 1989. It was clear that the heading date of R1 lines changed to early as well as late as compared with the original cultivar. It became

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Fig. 2. Variation in heading date observed in R1 lines (segregating lines are excluded). See Table 2 for the actual data.
Table 2. Variations in heading data and culm length in R₁ lines of various varieties.

<table>
<thead>
<tr>
<th>Year</th>
<th>Variety</th>
<th>No. of R₁ lines examined</th>
<th>Heading date of variety⁽¹⁾</th>
<th>Culm length of variety⁽²⁾</th>
</tr>
</thead>
<tbody>
<tr>
<td>1987</td>
<td>Koshihikari</td>
<td>7 (0)⁽³⁾</td>
<td>Aug. 20th</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Norin 14</td>
<td>20 (4)</td>
<td>Aug. 21st</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Iwaimochi</td>
<td>12 (4)</td>
<td>Sep. 8th</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Nipponbare</td>
<td>33 (4)</td>
<td>Aug. 28th</td>
<td>4</td>
</tr>
<tr>
<td>1988</td>
<td>Tsukinohikari</td>
<td>49 (9)</td>
<td>Aug. 27th</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Aoisora</td>
<td>39 (7)</td>
<td>Aug. 23rd</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Norin 22</td>
<td>22 (4)</td>
<td>Aug. 30th</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Hatsushima</td>
<td>3 (0)</td>
<td>Sep. 5th</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Koshihikari</td>
<td>8 (3)</td>
<td>Aug. 23rd</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Sasanishiki</td>
<td>3 (0)</td>
<td>Aug. 16th</td>
<td>6</td>
</tr>
<tr>
<td>1989</td>
<td>Sasanishiki</td>
<td>25 (2)</td>
<td>Aug. 18th</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Asahi</td>
<td>3 (1)</td>
<td>Sep. 6th</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Hatsushima</td>
<td>9 (8)</td>
<td>Sep. 5th</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Norin 8</td>
<td>23 (7)</td>
<td>Sep. 2nd</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Norin 10</td>
<td>24 (3)</td>
<td>Aug. 24th</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Yamabiko</td>
<td>50 (5)</td>
<td>Aug. 27th</td>
<td>5</td>
</tr>
</tbody>
</table>

1) The number of lines which contained segregating mutants.
2) The day when 50% of panicles of a line emerged.
3) Culm length was classified in comparison with standard varieties.

apparent that the range of variation was different depending on the cultivar. The progenies of Norin 14 in 1987 and Aoisora in 1988 showed wide variation, and those of Norin 22 in 1988 and Norin 8 in 1989 showed little variation. However, the heading date of all the R₁ lines of Koshihikari examined in 1987 and that of Sasanishiki in 1988, were later than those of the original cultivars. On the contrary, the heading date of all the R₁ lines of Nipponbare in 1987 and Hatsushima in 1988 were earlier than that of corresponding parent cultivar. In general, the heading date of R₁ lines tends to be later than that of the original variety.

The culm length of R₁ lines also showed significant differences from original cultivars (Fig. 3). The culm length of all the R₁ lines of Koshihikari (1987 and 1988) and Sasanishiki (1988) was shorter than those of original varieties. Some R₁ lines of Iwaimochi (1987), Nipponbare (1987) and Yamabiko (1989) were taller than corresponding original varieties. The culm length of R₁ lines tends to be shorter than that of the original variety.
Fig. 3. Variation in culm length observed in R₁ generation (segregating lines are excluded). See Table 2 for the actual data.
Inheritance of somaclonal variants

To study mode of inheritance of some homozygous mutations, such as short culm length and late heading date, Hatsuyume was chosen and analyzed by crossing experiments.

The results were summarized in Fig. 4. The $F_1$ progeny (the average heading date, Aug. 15th, the range, Aug. 13th−17th) between Hatsuyume (the average heading date, Aug. 20th, the range, Aug. 16th−22nd) and Koshihikari (the average heading date, Aug. 13th, the range, Aug. 11th−22nd) did not show the characteristic late heading of Hatsuyume. In $F_2$ generation, not all of plants showed similar heading date as the parents did. Some of the $F_2$ progeny (13.8%) (heading date, Aug. 7th−10th) headed

Fig. 4. Frequency distribution of heading date in $F_2$ population of Hatsuyume×Koshihikari. This result was obtained in 1990 season. The number of examined plants ($n$), the average and the range of heading date were; Koshihikari, $n=175$, Aug. 13th, 11th−16th; Hatsuyume, $n=123$, Aug. 20th, 16th−22nd; $F_1$, $n=118$, Aug. 15th, 13th−17th. The number of $F_2$ progeny was 427. 13.8% of $F_2$ headed earlier than Koshihikari (Aug. 11th) and 7.6% of $F_2$ later than Hatsuyume (Aug. 22nd).
earlier than Koshihikari, and 7.6% (heading date, Aug. 23rd-25th) headed later than Hatsuymu. The late heading was not found in F₁ progeny. In F₂ progeny, plants heading as late as Hatsuymu were obtained (Fig. 4). However, it was doubtful that segregation in F₂ progeny fitted for the expected 3 : 1 Mendelian ratio, rather, the late heading trait was considered to be controlled by a few genes.

Another homozygous trait, short culm length, was not expressed in F₁ progeny between Hatsuymu and Koshihikari. The mean and the range of culm length of F₁ progeny were the same as those of Koshihikari. It was difficult to divide F₂ progeny

![Diagram](image)

**Fig. 5.** Frequency distribution of culm length in F₂ population of Hatsuymu×Koshihikari. This result was obtained in 1990 season. The number of examined plants (n), the average and the range of culm length were; Koshihikari, n=175, 86 cm, 78-94 cm; Hatsuymu, n=123, 78 cm, 70-86 cm; F₁, n=118, 86 cm, 77-94 cm. The number of F₂ progeny was 472. 8.9% of F₂ were shorter than Koshihikari (78 cm) and 4.8% of F₂ longer than Koshihikari (94 cm) in culm length.
into two groups, the short group like Hatsuyume, and the normal group like Koshihikari. Although it was not possible to reveal the mode of inheritance of the short culm trait, the F₁ and F₂ segregation patterns suggested that the short culm was controlled by several recessive genes.

These results indicated that patterns of two examined phenotypes segregation were not in agreement with the expected ratio of 3:1, therefore these mutations were not likely to be caused by single recessive gene. In F₂ generation we obtained fixed lines which headed earlier, Koshihikari type, Hatsuyume type and later type, and their genetic analysis of F₄ progeny are currently undertaken. Preliminary results indicated that the later heading trait of Hatsuyume was transmissible and that more than two mutations were involved in this mutant phenotype.

RFLP analysis of somaclonal variation

In order to reveal the relationship between observed morphological changes and alterations in DNA sequences, DNA polymorphisms in the plants regenerated from rice protoplasts were examined by restriction fragment length polymorphisms (RFLP) analysis by Dr. P.T.H. Brown at Max-Plank-Institute, Germany. Ten R₁ lines chosen at random among 26 lines derived from the protoplasts of Norin 10, were examined by RFLP analysis. The same R₁ lines used for the RFLP analysis were grown in the field of Plantech Research Institute, Japan, to investigate phenotypic changes at the plant level.

Fig. 6 and 7 summarized the results of the RFLP analysis. The detailed results have been described previously by Brown et al. (1990). Fig. 6 shows the results of the RFLP analysis of different gene domains. RFLP changes were detected in all the lines except 42 in 5' regulatory region. It was clear that there were great differences in the degree of DNA variation detected by RFLP analysis among the R₁ lines examined. In general, the 5' regulatory region showed higher level of DNA variation than the 3' noncoding region.

Fig. 7 compares the RFLP changes found in tissue specific genes and housekeeping genes. Although DNA variation was detected in both types of genes, the degree of DNA variation detected in tissue specific genes was generally higher than that in housekeeping genes.

Table 3 shows the relationship between DNA variation and phenotypic changes of the R₁ lines examined. Mean values of DNA variation were calculated based on the variation in coding region digested with Rsa I. In proportion to the increase of DNA variation, R₁ lines exhibited more mutations. Moreover, putative homozygous mutants, dwarf and short culm length mutants, were tended to occur when the
Fig. 6. RFLP changes found in different regions of gene sequences. Frequency was calculated from the results of the RFLP analysis by Brown et al. (1990).

Fig. 7. RFLP changes in different types of genes. Frequency was calculated from the results of the RFLP analysis by Brown et al. (1990).
Table 3. Phenotypic variations and rate of DNA variations in R1 lines of Norin 10.

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Mutation</th>
<th>Types of mutation</th>
<th>Rate of DNA variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td>Mutation</td>
<td>dwarf</td>
<td>2.5</td>
</tr>
<tr>
<td>32</td>
<td>Normal</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>31</td>
<td>Normal</td>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td>19</td>
<td>Normal</td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td></td>
<td>10.9</td>
</tr>
<tr>
<td>42</td>
<td>Mutation</td>
<td>chlorina (late maturity)</td>
<td>19.4</td>
</tr>
<tr>
<td>33</td>
<td>Normal</td>
<td></td>
<td>21.8</td>
</tr>
<tr>
<td>76</td>
<td>Mutation</td>
<td>short culm length (late maturity)</td>
<td>35.4</td>
</tr>
<tr>
<td>110</td>
<td>Mutation</td>
<td>short culm length</td>
<td>62.5</td>
</tr>
<tr>
<td>55</td>
<td>Mutation</td>
<td>short culm length</td>
<td>100.0</td>
</tr>
</tbody>
</table>

This experiment was carried out in 1989. The phenotypic variations were investigated at Plantech Research Institute and the rate of DNA variation was calculated from the results of the RFLP analysis by Brown et al. (1990).

The percentage of DNA variation reached higher than 20%. From these results it was concluded that there was a strong correlation between DNA variation and phenotypic variation.

**Application of somaclonal variation for breeding**

Breeding process based on somaclonal variation is shown in Fig. 8. Regenerants from protoplasts (R0) are individually grown in greenhouse. R1 progeny derived from each R0 individual are reared in the paddy field. Seeds of 3–5 plants in one R1 line which is superior to original variety in several agronomic traits are collected. We further examine transmission of these desirable traits to the following generations, and their stability within the family lines.

In addition to the line selection for superior traits, the preliminary performance trials and disease resistance tests to leaf blast and bacterial leaf blight were carried out.

Hatsuyume is the first variety developed by this procedure (Suikekiyo et al. 1989). Among the progeny derived from protoplasts of Koshihikari, one R1 line was found to be superior to original variety in several agronomic traits through R1 and R2 generation and selected. This line was registered as a new rice variety named Hatsuyume. The main characteristics of Hatsuyume are as follows; (1) Heading date of Hat-
Fig. 8. Schematic outline of protoplast breeding.
suyume is 5 days later than that of Koshihikari. Hatsuyume is considered as a medium variety similar to Nipponbare in Kanto area. (2) The culm length of Hatsuyume is about 10 cm shorter than that of Koshihikari, giving Hatsuyume improved lodging resistance. (3) As for another desirable agronomic trait, visual grain quality of Hatsuyume is superior to that of Koshihikari.

In addition to Hatsuyume, another line was selected among the progeny from protoplast-derived plants of Tsukinohikari. This line was also applied for resistration as a new variety with the name Yumekaori. Heading date of Yumekaori is similar to that of Tsukinohikari. The culm length of Yumekaori is significantly shorter than that of Tsukinohikari, and it is resistant to leaf blast, bacterial leaf blight and rice stripe virus.

These results indicate that somaclonal variants are useful for rice breeding. We propose to call this new breeding method “Protoplast Breeding”. The key features of this method are a short breeding term and quick fixation of the progeny giving several advantages over the conventional breeding method.

Discussion

The purpose of this work was to reveal the characteristics of somaclonal variations in protoplast-derived rice plants and to demonstrate a possibility of developing new rice varieties from protoplast-derived mutants.

Analysis of the progeny of protoplast-derived rice plants demonstrated the occurrence of frequent phenotypic changes caused by heterozygous and homozygous mutations. Oono (1985) reported that putative homozygous mutations causing short plant height were observed in the plants regenerated from rice callus. Evans and Sharp (1983) obtained a homozygous jointless pedicel mutant among tomato tissue culture-derived plants of tomato.

Somaclonal variations in agronomically important traits such as culm length and heading date, were observed in the progeny of plants regenerated from rice protoplasts. There are great differences in the degree of phenotypic changes observed. The frequency of mutation seems to be different depending on cultivars.

The results of the crossing experiments indicated that mutations appeared in protoplast culture are inheritable. Oono (1985) reported that although a putative homozygous dwarf mutant originated from tissue culture was stably transmitted at least for eight generations by self pollination, the mutant trait was not expressed in F2 plants after crossing mutants with the original plant. In our experiments, homozygous mutations for short culm length and late heading date were transmitted from F2 to F4 generation derived from the cross between mutant plants with the original plant.
Furthermore, this mutation was stably transmitted through five generations by selfed pollination (R₁₋₆). These two traits seem to be controlled by a few genes, however, their exact mode of inheritance is not yet understood.

Detailed analysis of the relationship between somaclonal variation and DNA changes in protoplast-derived rice plants was carried out by Brown et al. (1990). The results clearly demonstrated that DNA variations occur in all R₁ lines examined. The mean DNA variation rate differs greatly among the different genes, the different lines and the different gene domains. Evidence that methylation plays a major role in DNA polymorphism was not obtained, because levels of DNA polymorphism detected with methylation sensitive and insensitive restriction enzymes are not significantly different. Summation of all the variations detected in this analysis shows that 29% of plants contained DNA polymorphisms. Müller et al. (1990) demonstrated that 23% of plants regenerated from callus maintained for 67 days in culture, contained RFLP changes. The rate of RFLP changes found in protoplast-derived plants is similar to that in plants derived from callus, suggesting that culture itself is responsible for mutations. Though there existed a strong correlation between DNA and phenotypic changes in protoplast-derived plants (Table 3), phenotypic mutations were hardly detected in callus-derived plants (Müller et al., 1990). These data suggested that DNA changes detected in protoplast-derived plants could occur in the regions of DNA sequences that are related to morphological traits. More detailed analysis is necessary to understand the relationship between DNA and phenotypic variations.

Several authors suggested that somaclonal variation occurring during protoplast culture is a useful tool for crop improvement (Shepard et al. 1980, Evans and Sharp 1986, Ogura et al. 1987, Evans 1989). The mechanism of somaclonal variation is not, however, understood. Evans and Sharp (1983) postulated that a homozygous mutant found in tissue culture-derived tomato plant could arise by mutation followed by mitotic recombination. Lörz (1983) also suggested that genetic change occurring during cell culture were caused by enhanced rate of mitotic recombination.

The results available at present do not explain the nature of somaclonal variation. But it appears that R₁ lines derived from different panicles of a single R₀ plant were the same in morphological characteristics. One out of 5 R₄ plants derived from single protoplast-derived callus was heteroploid, but the rest of the Rₙ plants were diploids. Furthermore, among the R₁ lines derived from these four diploid R₄ plants, albino was segregated in one line at the seedling stage. The culm length of these 4 lines were all shorter than that of the parent, and no differences in culm length among the progeny lines were detected. These observations suggested that variation occurred throughout the culture and plant regeneration steps, and homozygous mutations took place at early stages during the culture. In protoplast-derived rice plants no homozygous
mutants were obtained except for short culm length and early or late heading date. One conjecture is, therefore, that regions of DNA which are related to culm length and heading date may be susceptible to mutation and to mitotic recombination. Whether this is true remains to be elucidated.

As a result of this work, Hatsuymue was registered as a new rice variety, and Yumekaori was applied for registration as a new variety. Development of these two commercial varieties indicated that protoplast breeding is a useful method for production of new rice varieties from protoplast-derived plants in rice.

Summary

Somaclonal variation is a useful tool for crop improvement. We investigated somaclonal variations detected among protoplast-derived rice plants, and obtained some results toward understanding somaclonal variations in rice.

In R1 lines derived from rice protoplasts, we observed the homozygous mutation causing variation in heading date and culm length as well as segregating heterozygous mutations. The results indicated that frequencies and degrees of such mutations were different among cultivars used, and phenotypic changes occurred in high frequency during protoplast culture and plant regeneration.

RFLP analysis of R1 lines revealed that the mean DNA variation rate differs among the different genes, the different lines and the different gene domains. The comparison of DNA variation with phenotypic changes suggested that R1 lines exhibited mutations in proportion to the increase of DNA variations.

To study mode of inheritance of some homozygous mutations, Hatsuymue, short culm and late heading mutant, was analyzed by crossing experiments. We concluded that these mutations were inherited to the following generations, however, their exact mode of inheritance is not yet understood.

Development of two commercial varieties, Hatsuymue and Yumekaori, proved that protoplast breeding is a useful method for producing new rice varieties from protoplast-derived rice plants.

Acknowledgment

We wish to express our thanks to Dr. R. Itoh and Dr T. Tanaka of Plantech Research Institute for the valuable guidance of rice breeding, Dr. H. Ogura of Okayama University for carrying out part of this work, Mr. Y. Yahiro, Mr. H. Kurihara and Mr. A. Horibata of Plantech Research Institute for cooperation in completing this work. We also wish to express our thanks to Dr. K. Shimamoto and
all the members of Plantech Research Institute for producing protoplast-derived plants, their advice and assistance.

References


イネのプロトプラスト再生系統における
ソマクローナル変異

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ソマクローナル変異は、多くの作物にみられる現象である。イネではプロトプラストからの再生系が確立し、ソマクローナル変異に由来する有用突然変異体が育種的に利用され、その成果が出始めている。

プロトプラスト再生後代（R₁世代）における突然変異の出現様式は、大きく二つに分けられる。ひとつは、突然変異個体がR₁系統内で分離して出現する場合であり、他のひとつは、系統全体が同一の変異を起こす場合である。しかし、このような変異を生じるメカニズムや分子レベルの研究。また、得られた突然変異体の遺伝学的研究など、プロトプラスト由来の変異に関する研究は、ほとんどなされていない。

植物工学研究所では、1986年イネのプロトプラスト培養に成功して以来、ソマクローナル変異の研究を行ってきた。以下に、その研究概要を述べる。

1．R₁系統の生育期間を通じて、突然変異体の検出及び育種的適観調査を行い、系統内で矮性個体や葉緑素突然変異個体など特徴的な突然変異体を分離する多くのR₁系統が得られた。このような分離系統の出現頻度は、プロトプラスト培養に用いた品種間で明らかに差がみられた。

また、系統全体が同一の変異を起こす現象として、出穂期の早晩化、稈長の短長化などがある。R₁系統の出穂期および稈長の変異は、一般に、出穂期は晩生化、稈長は短縮化する傾向が認められた。この場合の変異の幅についても品種間で大きな差異が認められた。これまでの植物体レベルの評価から、表現型に関する変異はかなり高頻度で起こっていることが推察された。

一方、RFLP解析に供試した農林10号R₁系統すべてにおいて、DNAレベルの変異が確認され、その変異の程度には系統間で大きな差異がみられた。また、遺伝子の部位別に比較すると、5′側上流域が3′側下流域より多くの変異を含んでいることが明らかになった。さらに、DNAレベルの変異と植物体レベルの変異を比較した場合、DNAレベルの変異が高くなるにしたがって、表現型に関する変異も起こり、しかも、系統全体が同一の変異を起こす傾向がみられた。この実験結果は、DNAレベルでの変異が表現型における変異に強く関係していることを示唆するとともに、ソマクローナル変異が広く起こっていることを裏付けた。

2．ソマクローナル変異の遺伝性を調べるためコシヒカリ由来の短稈、晩生育種「初夢」
と原品種との交配を行った。晩生形質に関して、F₁は、コシヒカリに近い出穂日を示した。F₂集団において晩生形質を単一劣性遺伝子と仮定した場合の分離は示されなかった。また、このF₂集団内にコシヒカリより早生個体が出現していることから、晩生形質には少なくとも2個以上の遺伝子が関与していると推察された。次に、F₁の稈長の分布はコシヒカリと重なり、短稈形質は劣性遺伝子によって支配されていることが予想された。しかし、F₂集団では、短稈型から長稈型まで連続的な分離を示し、短稈形質には、多数の遺伝子が関与していると考えられた。「初夢」の短稈、晩生形質の遺伝様式を明らかにするためにはさらに検討を必要とするが、両形質とも後代へ遺伝することは明らかになった。

3. ボマクローニアル変異と従来の育種法を組み合わせた技術（プロトプラスト育種）によってコシヒカリ由来の短稈、晩生品種「初夢」を育成した。「初夢」は1990年品種登録され、各県で評価試験を実施している。さらに、月の光由来の「夢かほり」を育成し、現在登録申請中である。「夢かほり」は栽培特性に優れ、しかも、イネの重要病害であるいち病、白葉枯病、繋葉枯病に強く、収量性、食味等が改善された新系統である。このようにプロトプラスト育種は、有用形質は維持しながら劣悪形質を改善し、より優れた諸特性を持つ品種を育成する場合に有用な手段であると考えられる。

以上、プロトプラスト再生後代にみられるボマクローニアル変異について、育種的利用を考慮しながら、その現象について検討を行った。
SPONTANEOUS AND INDUCED MUTATIONS
OF SEED PROTEINS IN SOYBEAN
(GLYCINE MAX L. MERRILL)

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Introduction

Soybeans contain the seed storage proteins which amount to about 70% of the total proteins and are responsible for contributing to the quality of soybean foods. In addition to the storage proteins, soybeans have long been known to contain several relatively abundant protein components with specific activities such as the proteinase inhibitors, the soybean agglutinin and the lipoxygenases, though their biological roles are unknown in the plant.

Mutation of the seed storage proteins

Although soybeans produce the highest seed protein yield and content among seed crops, its protein quality is limited by low contents of the sulfur-containing amino acids, methionine and cysteine. Seven S and 11S globulins are the two major proteins which amounts to about 70% of the total seed proteins. Sulfur-containing amino acid contents of the two globulins are quite different: 11S globulin contains 3 to 4 times more methionine and cysteine in unit protein than that of 7S globulin (Koshiyama, 1968). Furthermore, it was reported that the two globulins have very different functional properties in the soy-protein foods (Saio and Watanabe, 1978): the isolated 11S globulin fraction formed a much harder tofu-gel. These reports suggest that the increase of 11S globulin at the expense of 7S globulin would enhance the nutrition and improve the functionality of soybean proteins in food.

So far, the two types of mutant varieties (Fig. 1; 3 and 4) were identified by screening using SDS-polyacrylamide gel electrophoresis (PAGE) in the germ plasm varieties of soybean: Keburi and Mo-shi-dou (Gong 503) which were characterized by the absence of \( \alpha' \)-subunit and low levels of both \( \alpha \)- and \( \beta \)-subunits of 7S globulin, respectively (Kitamura and Kaizuma, 1981). The absence of the \( \alpha' \)-subunit is controlled by a single recessive allele and the reduction of the \( \alpha \)- and \( \beta \)-subunits by
respective independent single alleles (Tsukada et al. 1986).

By gathering the three variant alleles for the subunits of 7S globulin into one genotype, we obtained two types of 7S-low lines (A and E lines in Fig. 1) which are genetically fixed for the three alleles for the 7S globulin subunits and the allele producing or lacking the intermediate subunit of 11S globulin (Ogawa et al. 1989). Contents of 7S and 11S globulins of the 7S-low lines and the ordinary cultivars were estimated by single radial immunodiffusion analyses using anti-7S and anti-11S sera, respectively. Table 1 shows that the 7S-low lines have only half 7S content of those in the ordinary cultivars, on the contrary, about 15% higher 11S content than those of the ordinary ones.

Fig. 1. SDS-PAGE patterns of the total seed protein in soybean.
1: Suzuyutaka, 2: Williams, 3: Keburi (α'-null),
4: Mo-shi-dou Gong 503 (α- and β-low),
5: A-line (α'-null, α- and β-low, A5-type),
6: E-line (α'-null, α- and β-low, A4-type),
7: a mutant line lacking all group 1 subunits of 11S globulin.
Table 1. Total and fractional protein contents of ordinary varieties and 7S-low lines (Ogawa et al. 1989)

<table>
<thead>
<tr>
<th>Group of variety</th>
<th>No. of lines tested</th>
<th>Total protein %</th>
<th>% of total protein 7S</th>
<th>11S</th>
</tr>
</thead>
<tbody>
<tr>
<td>7S-low lines:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A lines (7S-low, A_5-type)</td>
<td>5</td>
<td>43.4 a&lt;sup&gt;)&lt;/sup&gt;</td>
<td>8.7 d&lt;sup&gt;)&lt;/sup&gt;</td>
<td>52.5 a&lt;sup&gt;ii&lt;/sup&gt;</td>
</tr>
<tr>
<td>E lines (7S-low, A_4-type)</td>
<td>5</td>
<td>44.4 a&lt;sup&gt;‘&lt;/sup&gt;</td>
<td>11.7 c</td>
<td>44.7 b</td>
</tr>
<tr>
<td>Ordinary variety:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A5-type variety (normal, A_5-type)</td>
<td>20</td>
<td>41.4 b</td>
<td>17.3 b</td>
<td>38.3 c</td>
</tr>
<tr>
<td>A4-type variety (normal, A_4-type)</td>
<td>20</td>
<td>41.1 b</td>
<td>19.5 a</td>
<td>31.2 d</td>
</tr>
<tr>
<td>Keburi (α'-null, A_5-type)</td>
<td>—</td>
<td>39.4</td>
<td>15.7</td>
<td>41.8</td>
</tr>
<tr>
<td>Moshi-dou Gong 503 (a-low, β-low, A_5-type)</td>
<td>—</td>
<td>42.9</td>
<td>9.9</td>
<td>45.7</td>
</tr>
</tbody>
</table>

<sup>)</sup> Different letters in a column indicate statistical significance of the differences among the mean values within a column at 5% level.

A highly negative correlation was found between the 7S and 11S globulin contents. The results suggest that 11S globulin might be overproduced to compensate for the reduction of 7S globulin keeping normal levels of the total seed protein content in the 7S-low lines. No deleterious effects were observed despite of the marked modification of protein composition in the lines. The mean value of sulfur-containing amino acid contents of the 7S-low lines was about 20% higher than that of the ordinary cultivars (Ogawa et al. 1989).

On the other hand, there exists genetic polymorphism of 11S globulin independent of that of 7S globulin. Eleven S globulin can be classified into two types, A5 or A4 types, according to the presence and absence of the intermediate subunit which is formed by the A5-subunit and the paired basic B4-subunit of 11S globulin (Fig. 2). The absence of the intermediate subunit is controlled by a single recessive allele (Harada et al. 1983). It was shown that the presence of the subunit is closely related to properties of gel formation of 11S globulin by heating (Nakamura et al. 1984) and of tofu-gel formation (Hara and Negishi 1988).

Kaizuma et al. (1990) identified an induced mutant soybean lacking all the group I intermediate subunits of 11S globulin by subjecting distal portion of individual M2
Fig. 2. SDS-PAGE patterns of soybean seed proteins (Ogawa et al. with partial changes).
1: the purified A5-type 11S globulin,
2: the purified A4-type 11S globulin,
3: the purified 7S globulin,
4: Suzuyutaka, 5: Raiden, 6: Nattoshoryu, 7: Bonminori

seeds to SDS-PAGE derived from the irradiated seeds of “Wasesuzunari” with 40 kR gamma-rays. The lacking characteristic has been shown to be controlled by a single recessive gene and to extremely decrease 11S globulin contents, on the contrary increasing 7S globulin contents in the seeds (Fig. 1; 7). It was shown that despite the marked reduction of 11S globulin, no deleterious effect was observed on the total protein contents as well as physiological aspects such as seed development and germination. Because 7S globulin has much superior food-functionality such as water-holding ability, adhesiveness and solubility at high temperature to those of 11S globulin, soybeans with extremely high 7S/11S ratios would be effectively used for special soy-protein ingredients in food industry.

Very recently, Kaizuma et al. (1990) identified an induced mutant soybean lacking both α- and β- subunits of 7S globulin with 40 kR gamma-ray irradiation to seeds of “Wasesuzunari” (Fig. 3; 1). Most of the mutant seeds germinated, but the plants died at seedling stage. Kitamura et al. (in preparation) identified an induced mutant soybean extremely lowering all the subunits (α-, α’- and β-) of 7S globulin with 40 kR
irradiation to seeds of "Kanto 101" (Fig. 3; 2). The mutant seeds normally germinate, but the plants abnormally grew and failed to produce seeds.

Eliminating seed lipoxygenases

Normal soybean seeds contain three lipoxygenase isozymes, called L-1, L-2 and L-3. These enzymes are responsible for the generation of grassy beany flavors and tastes which have limited the wide utilization of whole soybeans and soybean protein in certain food products. The three types of spontaneous mutants lacking L-1, L-2 and L-3, respectively, were detected in the early 1980's. Genetic studies have demonstrated that the absence of L-1 (Hildebrand and Hymowitz 1981), L-2 (Kitamura et al. 1985, Davies and Nielsen 1986), and L-3 (Kitamura et al. 1983) from the seeds is under the control of single recessive alleles, \( l_{\alpha 1} \), \( l_{\beta 2} \), and \( l_{\delta 3} \), respectively. By the use of these
recessive alleles, we have been attempting to breed new cultivars with low levels of the objectionable flavors.

Three to four backcrosses were made to the recurrent parent “Suzuyutaka”, a leading cultivar in Japan with selection of the absence of the isozymes to obtain near-isogenic lines lacking L-1, L-2 and L-3, respectively. From crosses among the near-isogenic lines, two productive lines lacking L-2 and L-3, and L-1 and L-3, respectively, having agricultural traits similar to those of “Suzuyutaka” were selected and named as “Kanto 101” and “Kanto 102”. The selected lines were tested to compare some agricultural traits in Tsukuba (latitude 36°N), and Morioka (latitude 40°N) in the summer of 1988. The results obtained in Tsukuba are shown in Table 2. No differences were observed in the traits examined among the lines and the cultivar. These results show that it is possible to develop commercial soybean cultivars lacking the two isozymes. Because L-2 is largely responsible for the generation of hexanal that is a major constituent of the flavor (Matoba et al. 1985), soybeans lacking L-2, and lacking both L-2 and L-3 would be acceptable by soy-food industries and consumers in the world.

However, neither double mutant seeds lacking both L-1 and L-2 nor triple mutant seeds lacking all the isozymes have been identified yet, presumably due to close linkage between L-1 and L-2. Very recently, however, Hajika et al. (1991) succeeded in inducing a soybean line lacking all the isozymes by gamma-ray irradiation (Fig. 4). Namely, about 1200 F2 seeds from crosses between “Kanto 101” × “Kanto 102” were irradiated with gamma-rays at a level of 15 kR. The treated seeds were planted to obtain M2 seeds. A total of 2,289 single M2 seeds were obtained from individual M2 plants. The presence or absence of the isozymes in individual M3 seeds was analyzed using distal portions of the seeds subjected to SDS-PAGE. A different induced mutant

Table 2. Performance and some characters of two leading varieties, Enrei and Suzuyutaka and the near-isogenic lines lacking L-2 and L-3 (Kanto 101), and L-1 and L-3 (Kanto 102) in Tsukuba in 1988.

<table>
<thead>
<tr>
<th>Variety line</th>
<th>Flowering time</th>
<th>Maturing time</th>
<th>Stem height cm</th>
<th>Seed yield kg/ha</th>
<th>100 seeds weight g</th>
<th>Protein %</th>
<th>Oil %</th>
<th>Soybean mosaic virus</th>
<th>Purple seed stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrei</td>
<td>Aug. 1</td>
<td>Oct. 10</td>
<td>43</td>
<td>24.0</td>
<td>30.8</td>
<td>45.3</td>
<td>20.3</td>
<td>Resistant</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Suzuyutaka</td>
<td>Aug. 3</td>
<td>Oct. 15</td>
<td>43</td>
<td>32.5</td>
<td>23.5</td>
<td>43.2</td>
<td>20.3</td>
<td>Resistant</td>
<td>Tolerant</td>
</tr>
<tr>
<td>Kanto 101</td>
<td>Aug. 3</td>
<td>Oct. 13</td>
<td>38</td>
<td>31.2</td>
<td>23.2</td>
<td>42.9</td>
<td>21.1</td>
<td>Resistant</td>
<td>Tolerant</td>
</tr>
<tr>
<td>Kanto 102</td>
<td>Aug. 4</td>
<td>Oct. 17</td>
<td>39</td>
<td>30.8</td>
<td>22.4</td>
<td>42.0</td>
<td>21.1</td>
<td>Resistant</td>
<td>Tolerant</td>
</tr>
</tbody>
</table>

* Averages of random 20 plants in two replicates. * Estimated by NIRS analyzer on a dry matter basis.
Fig. 4. Resolution of the lipoxygenase isozymes in soybean seeds by SDS-PAGE.
1 and 6: Suzuyutaka,
2: a line lacking all the isozymes,
3: a line (Kanto 101) lacking L-2 and L-3,
4: a line (Kanto 102) lacking L-1 and L-3,
5: a line lacking L-1 and L-2 obtained from a cross
   Suzuyutaka × a line lacking all the isozymes.

line lacking all the isozymes was identified in our laboratory by subjecting distal portion of individual M2 seeds to SDS-PAGE derived from the irradiated seeds of “Kanto 101” with 40 kR gamma-rays (unpublished data).

The triple mutant soybeans went through two generations and no physiological and agricultural problems were encountered, so far. In another experiment, it has been shown that there are no significant differences in activity levels of leaf lipoxygenase (Table 3), which seems to take important physiological roles (Hildebrand 1990) among the triple null lines, “Kanto 101” and “Kanto 102” lines and “Suzuyutaka”. This seems to explain why soybeans lacking the seed lipoxygenase isozymes are physiologically normal.
Table 3. Lipoygenase activities of soybean seeds and leaves (±SD).

<table>
<thead>
<tr>
<th>Plant type</th>
<th>Activity of seeds (ΔA234 nm/min, mg meal)</th>
<th>Activity of leaves (ΔA234 nm/min, mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6.5</td>
<td>pH 7.0</td>
</tr>
<tr>
<td>Suzuyutaka</td>
<td>4.76 ± 0.13</td>
<td>1.71 ± 0.14</td>
</tr>
<tr>
<td>Lacking L-2 and -3 (Kanto 101)</td>
<td>0.70 ± 0.05</td>
<td>1.62 ± 0.28</td>
</tr>
<tr>
<td>Lacking L-1 and -3 (Kanto 102)</td>
<td>3.35 ± 0.04</td>
<td>1.37 ± 0.17</td>
</tr>
<tr>
<td>Lacking L-1 and -2</td>
<td>0.21 ± 0.04</td>
<td>1.37 ± 0.17</td>
</tr>
<tr>
<td>Lacking L-1, -2 and -3</td>
<td>0.04 ± 0.01</td>
<td>1.89 ± 0.26</td>
</tr>
<tr>
<td>Lacking L-1, -2 and -3</td>
<td>0.04 ± 0.02</td>
<td>1.35 ± 0.30</td>
</tr>
</tbody>
</table>

Substrate: 2.5mM linoleic acid
1) Produced in Kyusyu National Agricultural Experiment Station
2) Produced in National Agricultural Research Center

Soybean cultivars lacking all the seed lipoygenases could be economically valuable on account of their utilization, and the storage stability of soybean would be enhanced since the effect of the enzymes which act as major oxidative factors associated with the deterioration of oil and protein in the seeds during storage would be eliminated.

Conclusion

Further, in addition to the mutant soybeans described here, more various spontaneous and induced mutants of the seed protein would be identified in future. It is considered that these mutants have important implications because they could be used not only as valuable genetic sources to develop newly modified soybeans for better human uses but also as gene markers in soybean genetics.

References

SOMACLONAL VARIATION IN SOYBEAN SEED PROTEINS

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Introduction

Somaclonal variation occurs through tissue culture in a wide range of qualitative and quantitative traits, so that tissue culture is an unexpectedly rich and novel source of genetic variability for plant improvement (as reviewed by Larkin and Scowcroft 1981). The somaclonal variation is also a problem, however, in in vitro vegetative propagation as well as in recombinant DNA techniques where specific genetic changes are desired in otherwise unaltered genomes (Karp 1990). Variation occurs in plants regenerated from both embryogenic and organogenic cultures (Karp 1990).

In soybean, plant regeneration system via organogenesis (Saka et al. 1980; Cheng et al. 1980; Kartha et al. 1981; Wright et al. 1987 a, b) and embryogenesis (Lippmann and Lippmann 1984; Ranch et al. 1985; Lazzeri et al. 1985; Barwale et al. 1986; Hammatt and Davey 1987; Komatsuda and Ohyama 1988; Hartweck et al. 1988) have been developed, making studies on somaclonal variation in soybean possible. Somaclonal variation in soybean has been reported for chlorophyllous and morphological traits (Barwale and Widholm, 1987; Freytag et al., 1987), yields (Graybosch et al., 1989), and herbicide tolerance (Wrather and Freytag 1991).

Soybean is an important protein source for human nutrition and animal feed. Therefore, novel genetic variation in seed proteins is desired for improving soybean protein quality and quantity. Somaclonal variation of biochemical traits such as seed storage proteins or oil compositions, however, has never been studied in soybean, although sometimes reported in wheat (Larkin and Scowcroft, 1984). The present study was undertaken to determine if somaclonal variation occurs in seed proteins of soybean plants regenerated via somatic embryogenesis.

Materials and Methods

1) Plant materials
G. gracilis Skvortz. is a weedy, intermediate form between G. max (L.) Merr (the cultigen) and G. soja Sieb. & Zucc. (the wild progenitor). G. gracilis strains had a higher ability of both somatic embryo production from immature embryos and germination than G. max (Komatsuda and Ohyama 1988). A total of 28 plants of G. gracilis so far regenerated (Komatsuda and Ohyama 1988) were grown to maturity (E₁ plants, Table 1). Self-pollination of the 28 E₁ plants made up 28 E₂ families (a total of 383 E₂ seeds), and self-pollination of the resulting 383 E₂ plants made up 383 E₃ families.

‘Masshokutou Kou 502’ appeared in Table 1 is commonly called ‘Moshidou Gong 502’, but appears in under the former name in the list of the Germ-plasm Seed Storage Center, NIAR, Tsukuba.

Table 1. Plant materials, code numbers, and number of E₁ and E₂ plants examined.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Code # of Variety</th>
<th>Code # of E₁ plants</th>
<th>No. of E₁ plants regenerated</th>
<th>No. of E₂ plants grown</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. gracilis</td>
<td>#2-</td>
<td>16 &amp; 17</td>
<td>2</td>
<td>54</td>
</tr>
<tr>
<td>G. gracilis T34</td>
<td>#3-</td>
<td>8, 50, 51, 53, 58, 58p, 58s, 61, 64, 65, 79, &amp; 0</td>
<td>12</td>
<td>182</td>
</tr>
<tr>
<td>G. gracilis T135-590</td>
<td>#6-</td>
<td>2 &amp; 6</td>
<td>2</td>
<td>51</td>
</tr>
<tr>
<td>Masshokutou Kou 502</td>
<td>#7-</td>
<td>3, 18, 21, 22, 24, V1, V15, V16, K1, K2, K3, &amp; K4</td>
<td>12</td>
<td>96</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>28</td>
<td>383</td>
</tr>
</tbody>
</table>

2) Nomenclature

In this paper, I use E₁, E₂, E₃⋯⋯ to term the embryo-derived regenerated plants, selfed progeny of E₁, selfed progeny of E₂⋯⋯⋯, to correspond with the M₁, M₂, M₃⋯⋯⋯ and F₁, F₂, F₃⋯⋯⋯ in mutation breeding and cross-breeding, respectively.

E₁ plants were self-pollinated to make up E₂ families. Up to 30 E₂ seeds chosen from each of E₂ families were planted in the field in NIAR, Tsukuba. Each of E₂ plants was self-pollinated to make up a E₃ family.

3) SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

A seed from each of E₃ families was screened for variations of storage proteins by the half seed method (Downey and Hervey, 1962). Two milligram samples of cotyledon
tissues were homogenized in 0.3 ml of SDS sample buffer containing 0.75 M Tris-HCl buffer (pH 8.0), 5M Urea, 0.4% SDS (sodium lauryl sulfate), and 1% mercaptoethanol, and centrifuged at 12,000rpm for 5 min. Twenty microliters of supernatant were loaded on 10% polyacrylamide gel and run according to the method of Laemmli (1970). The gel was stained with Coomassie Brilliant Blue R.

4) Inheritance studies

When some variations of SDS-PAGE profile were detected in an E₃ seed, 20 more seeds were chosen from each of E₃ families originated from single E₁ plant, and were analysed for storage proteins in order to determine if they were of variant or normal type. E₃ seeds of both variant and normal type were germinated on filter paper in a Petri dish, and then transferred to soil in pots in a greenhouse, and self-pollinated to make up E₄ seed families. All E₄ seeds were analyzed for seed proteins by SDS-PAGE. E₄ seeds of both variant and normal type were grown in a greenhouse and self-pollinated to make up E₅ families. Generation was progressed in the same manner until E₇ families.

5) Two-dimensional gel electrophoresis

Sixty microliters of the cotyledon extract were separated by isoelectric focusing using pH 3.5–10.0 ampholyte (Ampholine; Pharmacia LKB, Sweden) for 14 h at a constant voltage of 400 as described in Miller and Elgin (1974). The isoelectric focusing gels were equilibrated in the SDS sample buffer for 2 h. The gels were subjected to second dimensional electrophoresis on SDS-PAGE (10% acrylamide) as described above.

6) Glycoprotein staining

Ten microliters of the cotyledon extract were separated by SDS-PAGE (10% acrylamide) as described above. Proteins on a gel were electrophoretically transferred to nitrocellulose membrane using a semi-dry blotting apparatus (Sartorius) according to the method of Hirano (1987). Oligosaccharide chains of glycoprotein were stained by using lectin-peroxidase reagents as described in Kijimoto-Ochiai et al. (1985). Phytohemagglutinin (PHA, Phaseolus vulgaris agglutinin; Honen, Tokyo) and concanavalin A (Con A, Canavalia ensiformis agglutinin; Honen, Tokyo), coupled with peroxidase, were used.

7) Western-blotting experiment

Twenty microliter of the protein extracts were separated by SDS-PAGE (10% acrylamide) and were transferred to a PVDF membrane (Immobilon; Millipore, MA)
according to the method of Hirano (1987). The membrane was incubated in 1/2000
diluted antiserum raised against soybean basic 7S globulins heavy (HII) or light (LII)
chain subunits (Kagawa et al. 1987) obtained from Dr. H. Hirano, NIAR, Tsukuba, or
was incubated in 1/500 diluted antiserum raised against soybean β-conglycinin or
glycinin obtained from Dr. K. Harada, NIAR, Tsukuba. The bands that cross reacted
with the antibody were detected by alkaline phosphatase enzyme immunoassay (Bio-
Rad immunoblot assay kit, Bio-Rad, Richmond, CA).

Results

1) Variation producing a novel 26 kD seed protein

A novel protein (26 kDa) band was observed in E₂ seeds, which could not be
detected from the control seeds (Fig. 1). In the progeny of a E₁ plant #7-3 of
Masshokutou Kou 502 (Table 2), a E₂ plant (# 7-3-2) produced three mutant seeds
having the 26 kD protein, and another E₂ plant (#7-3-5) produced eight mutant seeds.
The other 24 E₂ plants from the E₁ plant (#7-3) did not produce any mutant seeds.

![Diagram](image-url)

Fig. 1. Analysis of E₂ seed proteins of soybean (*Glycine gracilis*
Skvortz). Protein extracts of cotyledon tissue were separat-
ed by 10% SDS-polyacrylamide gel electrophoresis, then the
gel was stained with Coomassie Brilliant Blue R. A novel 26
KDa protein is indicated by the arrow.
The same 26 kDa protein was observed in E$_3$ seeds originating from another E$_1$ plant coded #3-53 (Table 2).

Table 2. Segregation frequencies of the mutation having a 26 kDa protein in E$_3$ seeds.

<table>
<thead>
<tr>
<th>E$_3$ plant #</th>
<th>No. E$_3$ seeds tested</th>
<th>+26 kD</th>
<th>-26 kD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Masshokutou Kou 502 (#7-3)-derived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-3-2</td>
<td>12</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>7-3-5</td>
<td>21</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>7-3-2. (24 other E$_3$ plants, total)</td>
<td>260</td>
<td>0</td>
<td>260</td>
</tr>
<tr>
<td>G. gracilis T34 (#3-53)-derived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-53-25</td>
<td>11</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>3-53-29</td>
<td>21</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>3-53-2. (28 other E$_3$ plants, total)</td>
<td>318</td>
<td>0</td>
<td>318</td>
</tr>
</tbody>
</table>

2) Inheritance of the 26 kDa protein

The mutation having the 26 kDa protein was inherited from mutant E$_3$ plants to six out of 105 seeds (5.7%) (Table 3). In contrast of this, no mutant seeds were detected from 99 seeds derived from wild type E$_3$ plants. In the next generation, mutants were five out of 130 seeds (3.8%) (Table 4). No mutant seeds were observed in wild type plant-derived seeds. Furthermore, three out of 66 seeds derived from mutant E$_3$ plants were mutants (4.5%) (Table 5).

Table 3. Segregation frequencies of the 26 kDa protein mutation in E$_3$ seeds.

<table>
<thead>
<tr>
<th>E$_3$ mutant plant #</th>
<th>No. E$_3$ seeds tested</th>
<th>+26 kD</th>
<th>-26 kD</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-3-2-9</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>7-3-5-4</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>-10</td>
<td>22</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>-11</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>-13</td>
<td>16</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>-14</td>
<td>30</td>
<td>4</td>
<td>26</td>
</tr>
<tr>
<td>-18</td>
<td>13</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Total (7 mutants)</td>
<td>105</td>
<td>6</td>
<td>99</td>
</tr>
<tr>
<td>Control (9 wild types)</td>
<td>99</td>
<td>0</td>
<td>99</td>
</tr>
</tbody>
</table>
Table 4. Segregation frequencies of the 26 kDa protein mutation in $E_a$ seeds.

<table>
<thead>
<tr>
<th>$E_a$ mutant plant #</th>
<th>No. $E_a$ seeds tested</th>
<th>$+26$ kDa</th>
<th>$-26$ kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-3-5-14-3</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>-5</td>
<td>33</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>-8</td>
<td>25</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>-16</td>
<td>20</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>7-3-5-18-1</td>
<td>28</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td>-11</td>
<td>17</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td><strong>Total (6 mutants)</strong></td>
<td><strong>130</strong></td>
<td><strong>5</strong></td>
<td><strong>125</strong></td>
</tr>
<tr>
<td>Control (3 wild types)</td>
<td>40</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 5. Segregation frequencies of the 26 kDa protein mutation in $E_a$ seeds.

<table>
<thead>
<tr>
<th>$E_a$ mutant plant #</th>
<th>No. $E_a$ seeds tested</th>
<th>$+26$ kDa</th>
<th>$-26$ kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-3-5-14-8-15</td>
<td>14</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>-25</td>
<td>33</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td>7-3-5-18-1-22</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>-25</td>
<td>14</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td><strong>Total (4 mutants)</strong></td>
<td><strong>66</strong></td>
<td><strong>3</strong></td>
<td><strong>63</strong></td>
</tr>
</tbody>
</table>

Table 6. Segregation frequencies of the 26 kDa protein mutation in $E_a$ seeds.

<table>
<thead>
<tr>
<th>$E_a$ mutant plant #</th>
<th>No. $E_a$ seeds tested</th>
<th>$+26$ kDa</th>
<th>$-26$ kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-3-5-14-8-25-14</td>
<td>143</td>
<td>0</td>
<td>143</td>
</tr>
<tr>
<td>-29</td>
<td>162</td>
<td>0</td>
<td>162</td>
</tr>
<tr>
<td>7-3-5-18-1-25-11</td>
<td>82</td>
<td>0</td>
<td>82</td>
</tr>
<tr>
<td><strong>Total (3 mutants)</strong></td>
<td><strong>387</strong></td>
<td><strong>0</strong></td>
<td><strong>387</strong></td>
</tr>
<tr>
<td>Control (7 wild types)</td>
<td>460</td>
<td>0</td>
<td>460</td>
</tr>
</tbody>
</table>

However, the mutant $E_a$ plants did not segregate any mutant seeds, thus the mutation producing 26 kDa protein disappeared from the $E_a$ generation (Table 6).

The mutation appeared in #3-53 line did not transmit to the selfed progeny of the $E_a$ plants (data not shown). No further analyses were therefore conducted on the progenies of the $E_a$ plants.
3) Isoelectric point of the 26 kDa protein

The mutant seeds had additional two spots in the two-dimensional gel electrophoretic profile (Fig. 2, right), which were absent in normal seeds (Fig. 2, left). Both the two peptides had the molecular weight of 26 kDa, showing that they corresponded to the new 26 kDa band appeared in the SDS-PAGE (Fig. 1). The two spots however differed each other in their isoelectric points. One had a pI point almost the same with that of β-subunit of β-conglycinin, while another peptide had a very high pI point remaining beside the top of the isoelectric focusing gel (Fig. 2, right). The two peptides were connected by a long and faint 'tail', indicating the possibility that a certain modifications on the peptides changed their electric charge.

![Fig. 2. Two-dimensional gel electrophoresis of the 26 KDa protein. Total proteins extracted from normal (left) and mutant seed having a 26 kDa protein (right) were applied onto isoelectrophoretic gels (at the top left of each gel), and mobilized to the isoelectric points of the proteins. The isoelectrophoretic gel were equilibrated in the SDS-sample buffer and applied to 10% SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie Brilliant Blue R. Two major peptides corresponding to the 26 kDa protein band appeared in Fig. 1 were indicated by arrows (right).](image)

4) Oligosaccharide chains of the 26 kDa protein

Both PHA and Con A cross reacted with the 26 kDa protein band, showing the
protein has oligosaccharide chains (Fig. 3). This presumably causes the shifted isoelectric points of the 26 kDa peptides.

Fig. 3. Glycoprotein staining of the 26 kDa protein. Total protein extracts from normal (lane 1) and mutant seeds containing the 26 kDa protein (lane 2) were separated by 10% SDS-polyacrylamide gel electrophoresis, electro-transferred to a nitrocellulose paper. The proteins were reacted with peroxidase-coupled phytohemagglutinin (PHA, *Phaseolus vulgaris* agglutinin,) or concanavalin A (Con A, *Canavalia ensiformis* Agglutinin) and the bands that cross reacted with the lectins were detected by peroxdyase activity staining. The total proteins were stained with Coomassie Brilliant Blue R (CBBR).
5) Immunological homology between the 26 kDa protein and known soybean seed proteins

The 26 kDa band did not cross-reacted with the antibody raised against β-conglycinin and glycinin (Fig. 4, a). The negative results of the immuno-blotting experiment indicates that the 26 kDa protein is not a precursor or a derivative of major seed storage proteins. The antibody raised against basic 7S globulin H II and L II subunit cross-reacted with the H II (27 kDa) and L II (16 kDa) subunits, respectively, and also with a higher molecular weight band which probably is a dimer of basic 7S globulin H II and L II subunits or their precursor (Fig. 4, b). The antibodies, however, did not cross-react with the 26 kDa protein, indicating the 26 kDa protein have no immunological homology with the basic 7S globulin (Fig. 4, b).
Fig. 4. Western-blotting analysis of the 26 kDa protein. Total protein extracts from normal (lane 1) and mutant seeds (lane 2) were separated by 10% SDS-PAGE and were transferred to a PVDF membrane. The protein were cross reacted with antibody raised against soybean β-conglycinin or glycine (a), or with antibody against basic 7S globulin H II or L II subunits (b). The bands that cross reacted with the antibody were detected by alkaline phosphatase enzyme immunoassay.

Discussion

A somaclonal variation which produced a novel 26 kDa protein was observed in E3 seeds derived from regenerated soybean plants (E1). The 26 kDa protein has never been reported from wide screening studies (Kitamura and Kaizuma, 1981; Tsukada et al., 1986), in which seed proteins of thousands of soybeans and wild soybeans were analyzed by SDS-PAGE. The natural crossing of soybean is very rare. From these reasons, pollen transmission of the 26 kDa protein gene to our plant materials is not
likely to occur. Even if the gene coding the 26 kDa protein would be transmitted by
natural crossing, it should behave in a simpler genetic manner, because almost all of
the seed proteins are known to behave as simply inherited units coded by codominant
alleles (Kitamura and Kaizuma, 1981; Hildebrand and Hymowitz, 1982; Kitamura et
al., 1983; Kitamura et al., 1984; Kitamura et al., 1985; Tsukada et al., 1986).

The 26 kDa protein observed in this study is obviously due to a mutation that
occurred in a somatic cell of an immature embryo before or after initiation of somatic
embryogenesis. The somaclonal variations in storage proteins reported in wheat
(Larkin and Scowcroft 1984) included deletions of specific protein bands, appearance
of new bands and changes in intensity of protein bands. The occurrence of the new
protein in our study is in agreement with the variation detected in wheat.

The percentage of variants of the trait was calculated as 7.1% by dividing the
number of E1 plants that had mutant progeny (i.e., 2) by the number of E1 plants
regenerated (i.e., 28). The percentage (7.1%) looks higher than the nature of mutation,
but it is in agreement with the characteristics of somaclonal variation which shows a
high percentage of variation in both morphological (as reviewed by Karp, 1990) and
seed protein traits (Larkin and Scowcroft, 1984). The tissue culture may serve as a
new source of variation for mutation breeding of soybean storage proteins, com-
plementing the γ-ray irradiation method (Odanaka and Kaizuma, 1989; Kaizuma et
al., 1989).

The inheritance analysis of the selfed progeny indicated that the trait is heritable
and transmitted into a limited number (3.8–5.7%) of heterozygous progeny (E4, E5, and
E6). Therefore the occurrence of the 26 kDa protein is controlled by a dominant gene.
However, the homozygous mutants for the trait were never found. The reason why no
homozygote was segregated is not clear now. One of the possible reasons is a lethal
of the homozygote mutant embryos in the early stage of the development. However,
even if the lethal is evident, the percentage of the homozygote mutant is expected less
than 1% (0.05³ < 0.01). This little percentage of lethal embryo could not be distin-
guished from the natural occurrence of empty seeds in soybean pods. For determining
whether the homozygote mutant is lethal, doubled-haploid production from male or
female gametes will be a powerful technique. However, no reliable method for
obtaining haploids is available in soybean.

Another reason of the heterozygotic inheritance of the 26 kDa protein may be the
selective transmission of the dominant gene by the male or female gametes only. In
other word, the gene is eliminated from one of the gametes. To substantiate this
assumption, cross pollination with wild type plants would be necessary.

Preliminary studies on the 26 kDa protein characters indicated that the new
protein consisted of two major fractions of peptides; one has an isoelectro point of
nearly neutral and another of basic. The difference in the electric charge is presumably due to the modification by glycosylation of the peptides, because the 26 kDa protein had oligosaccharide chains. The molecular weight of 26 kDa and the glycosylation feature of the new 26 kDa proteins proposed the similarity with the basic 7S globulin H (heavy) subunits (27 kDa, glycosylated). However, the 26 kDa protein had no immunological relationships with the basic 7S globulin H subunit. Biological roles of the 26 kDa protein as well as its inheritance will be investigated in further studies.

Summary

Plants regenerated from soybean tissue culture via somatic embryogenesis were screened for variants of seed proteins. Extracts of E5 seeds derived from regenerated plants (E1) were tested by SDS-polyacrylamide gel electrophoresis, and a few mutants were detected. The mutant seeds produced a novel proteins (26 kDa) which accounts for about 6% of the total seed protein. The variant seeds were grown in the greenhouse and self pollinated. The novel band in E5 seeds were inherited in limited numbers (3.8–5.7%) of E4, E5 and E6 seeds, while homozygous mutants for the band have never been derived. The 26 kDa protein had a basic iso-electric point, and had oligosaccharide chains. The 26 kDa band did not cross reacted with the antibodies against conglycinin, glycinin, or basic 7S globulin subunits, indicating the protein is not the derivative from known seed storage proteins but is a novel proteins occurred by somaclonal variation.

Acknowledgements

The author wishes to thank Dr. H. Hirano and Dr. K. Harada for a generous gift of antisera. The author is indebted to Mrs. M. Kang for gel electrophoresis experiments. This work was supported in part by a grant 'Biotechnology' (1212–51, 1991) from the Ministry of Agriculture, Forestry and Fisheries.

References


組織培養によるダイズ種子タンパク質の変異

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305 つくば市観音台

組織培養の過程でタンパク質レベルの変異が生ずる現象は、これまでにコムギとトウモロコシでわずかながら認められている。これらのタンパク変異のタイプとしては、特定タンパク質に対応するバンドの欠失、新らしいバンドの出現、バンドの太さの変化等があげられる。さらに、一個のアミノ酸が別のアミノ酸に置換したことによる電気泳動的易動度の変化も報告されている。タンパク変異の遺伝様式としては、再分化培養で固定したと考えられるホモ常変異の場合と、次世代で分離を示すヘテロの突然変異の両方が知られている。しかし、このようなタンパク質変異の遺伝性を何世代にわたって追跡調査した例は無く、その遺伝様式に不明な点も多い。ダイズにおいては、組織培養によるタンパク質変異が報告された例はこれまで全くなく、形態的、葉緑素形質の、あるいは取量などの遺伝的変異のみ報告されている。本研究では不定胚形成によるダイズの再生植物体を用いて、それらの後代の種子タンパク質の変異を電気泳動によって分析した。まず半野生ダイズ系統（抹食豆文502）の未熟胚を2 mg/l 2,4-Dあるいは10 mg/l NAAを含むMS培地で培養した不定胚を誘導した。この不定胚から植物体（Eₐ）を再生して開花させ、自殖によって種子（Eₐ）を得た。この種子は分析母にそのまま植ええてEₐ植物体を育成し、個体別にEₐ種子を採った。各Eₐファミリーの種子の数粒について半粒法による電気泳動によってタンパク質変異をスクリーニングした。タンパク変異が見い出された時は、その遺伝性を確認する目的で全Eₐ種子を分析し、変異型及び正常型と判定された両方のEₐ種子を絞いて植物を育成し、その個体別にEₐ種子を分析した。同様の手順でEₐ植物まで分析を進めた。

Eₐ種子分析の結果、分子量約26 kDaの新しいタンパク質のバンドを持つ異変が見いだされた。このタンパク質が全種子タンパク質に占める割合は種子間で異なったが、多い時は約6％に達した。この26 kDaタンパク質の遺伝様式を調べたところ、このタンパクを持つ種子の後代の約5％の種子に同様の変異が伝わったが、26 kDaタンパクを持たない正常型種子の後代にあらたに変異型が生じることは無かった。このようにしてこの変異型質はEₐ世代まで遺伝した。しかし遺伝的に固定された変異体を得られなかった。つまりこのタンパク異変はヘテロで維持されていたものと推察した。このタンパク質バンドは細胞培養のサイクルを経ていない種子からは全く見いだされず、これまで多品種・系統を扱ったタンパク質スクリーニングの研究からも報告されていない。このことから、この26 kDaタンパク質の変異は、組織培養のいずれかの段階で生じた突然変異つまりソマクローナル変異によるものと考えた。
26 kDaタンパク質のおよその等電点を調べるため全タンパク質を二次元電気泳動で展開した。すると正常型種子には見られない2個のスポットが確認され、そのうちの一方は中性付近、他方はアルカリ側の等電点を持つことがわかった。しかしそれらのスポットの様子は、このタンパク質がなんらかの修飾を受けていることを示唆した。そこでレクチン・オキシダーゼ法で26 kDaを染色したところ、Con-AとPHAと反応したことから、26 kDaタンパク質は糖鎖による修飾を受けている事が明らかになった。この26 kDaタンパク質が既知のダイズ種子タンパク質と関係があるか否かを調べるために、いくつかのタンパク質の抗体を使ってウエスタンプロットをおこなった。その結果、この26 kDaタンパク質は、コングルリン抗体、グルリン抗体、塩基性7Sグロブリン抗体のいずれとも反応しなかったことから、これらの貯蔵タンパク質とは免疫的に別の物である事が明らかになった。不定胚形成によって生じた新しいタンパク質の生物的意義や、変異の遺伝様式は今後より詳細に検討すべき課題である。
ENLARGEMENT OF INDUCED VARIATIONS BY COMBINED METHOD OF CHRONIC IRRADIATIONS WITH CALLUS CULTURE IN SUGARCANE

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Introduction

In sugarcane, conventional cross breeding methods such as biparental crossing and back crossing are tedious and hard to add a desired trait to a cultivated variety, due to heterozygous and high polyploidy background of the crop. It takes more than ten years to obtain a desirable genotype for commercial production by selection from thousands of original seedlings. On the contrary, mutation breeding has great opportunity to sugarcane as any desirable mutants can be detected and easily multiplicated through vegetative seed canes in short period.

Number of works has been reported to utilize gamma ray as acute irradiation on vegetative propagules to induce desired mutants (Haq et al.³, Jagathesan & Ratnam⁸, Roach¹⁹, Siddiqui et al.²¹, Wu et al.²³), and as chronic irradiation on growing plants (Sankaranarayanan & Babu²⁹). However, only 6 induced mutation varieties have been registered in actual breeding of sugarcane in the world (Micke et al.¹⁸). The problems were pointed out that most of the induced mutants had chimeric constitution, instability of the mutants had occurred in vegetative propagation following irradiation of propagules, and the frequencies of favourable mutation generally were not high enough.

Recent advances in the techniques and applications of tissue and cell culture has opened new vistas in plant breeding (Krishnamurthy¹⁰). Heinz et al.⁵ have stated, in addition to sexual breeding techniques, the tissue and cell culture techniques provide new option for deriving desired genetic variability. Much attention have been focused on somaclonal variation obtaining from tissue and cell culture in sugarcane (Kresovich et al.⁹, Krishnamurthy & Tlaskal¹⁶, Liu & Chen¹³,¹⁴ Prasad & Krishnamurthy¹⁹). Number of useful mutants have been selected from regenerated subclones of tissue cultures¹⁶,¹⁴,¹⁹. On the contrary, Irvine⁷, Kresovich⁹ and Miller²⁷ suggested that
somaclonal variation may be of much less value than conventional techniques when considering frequencies of desired response.

The present study has been conducted to establish efficient method for mutation breeding to combine chronic irradiation with tissue culture techniques so as to obtain non-chimeric (solid) favourable mutants in high frequency. The experiments described in this paper had objectives of investigating the effects of mutation induction on callus generators which were derived from plants grown under chronic gamma ray irradiation. Also dealing with the magnitude of genetic variation of quantitative characters on callus derivatives, their stability through vegetative generations and its evaluation was studied and assessed in mutation breeding.

**Materials and methods**

**Materials and irradiation**

Six varieties of sugarcane, IRK 67-1, NCo 310, Ni 1, NiF 2, NiF 4 and POJ 2725 were used for experiments of chronic irradiation and tissue culture. Two plantlets developed from one-eye cuttings were planted on 40 cm diameter pots, and five pots were prepared each for 6 irradiation treatments including non-irradiated plants (control) which were grown on the experimental nursery outside an irradiation facility.

The irradiation facility used in this study was the gammafield of Institute of Radiation Breeding which was round field with 100 m radius surrounded by 8 m height bank for avoiding direct leak radiation. It had 88.8 TBq (2,400 Ci) of $^{60}$Co source which irradiated over the field for 20 hours per every day except Sundays and the National holidays.

The potted plantlets at 5 to 6 leaf stage placed in the gammafield on July 3 in 1985 were irradiated chronically at 50, 100, 200, 300 and 500 Gy in terms of total dose for 90 days until October 25. The distance from the source to the plantlets ranged from 29.0 m (Dose : 50 Gy, Dose rate : 0.56 Gy/day) to 11.1 m (500 Gy, 5.55 Gy/day).

During the irradiation, stalk height, number of expanded leaves, number of tillers and number of stalks with deficient spindle were recorded every half month for estimating the varietal radiosensitivity. When the irradiation scheme had terminated on October 25, 1985, four top portions with 2 expanded leaves of the plants in 0 (the control), 100 and 300 Gy plots were served as explant sources for tissue culture.

**Tissue culture**

Top portion of stalk was trashed up to upmost expanded leaf, trimmed to about 12 cm in length and sterilized in 70% ethanol in the laboratory. Under aseptic condition, one outside leaf was removed and then subapical meristem with rolled young
leaves was dissected for callus induction on a modified Murashige and Skoog (MS) medium as reported in Heinz\textsuperscript{9}. The basal MS medium was supplemented with 3 mg/l 2,4-D, 10% by volume coconut water and 100 mg/l myo-inositol. When callus was induced on the explant, those were subcultured to the same medium two times in every 5 to 6 weeks for callus proliferation. Then, callus were transferred to plant regeneration medium composed of the basal MS medium free from 2,4-D added with vitamins. Within 10–12 weeks, plantlets were differentiated and transferred to nursery flats filled with fumigated soil in the greenhouse. After the individuals were transplanted to pots, totally 1,895 plantlets had been developed for field experiment.

**Field experiments**

The regenerated plantlets (D\textsubscript{1}) in each treatment were planted with 30 cm space between plants in 125 cm rows on June 9, 1986, and developed according to the usual cultivation practices. In November, 1986, preliminary data were collected on agronomic characters such as stalk length, stalk diameter, number of stalks, leaf length, leaf width, weight per stool and some qualitative characters. Before the frost in November, three stalks of all plants were harvested and stored under the ground for overwintering.

The vegetatively propagated plants from each regenerated plant were established as a subclone (D\textsubscript{2}). These were germinated from one-eye cuttings in a greenhouse in February, 1987. Four plants per plot in each subclone were transferred to the experimental field arranged in randomized block design in 2 replications in May, 1987 and managed in the same practices. In November, 1987, all the plots were harvested and data were taken on 14 agronomic characters including brix by hand-refractometer and degree of infection of sheath rot disease.

In the successive clonal stages (D\textsubscript{2}–D\textsubscript{4}), 46 subclones chosen from 146 subclones in the D\textsubscript{2} generations and the donor variety, NiF 4 were vegetatively propagated to investigate repeatability of the mutated characters. Ten plants per plot of each subclone were planted in the same manner mentioned above on the experimental field arranged in a randomized block design in 2 replications. During each November in years 1988 (for D\textsubscript{3} generation), 1989 (D\textsubscript{4}), 1990 (D\textsubscript{5}), all the plots were harvested and records were taken on 14 characters.

The data were processed by the library program 7–3 (21) for analysis of variance and estimates of heritability by K. Kumagai\textsuperscript{11}, and by the library program 3–13 (188) for principal component analysis by S. Suzuki\textsuperscript{22} in the Computing Centre for Research in Agriculture, Forestry and Fisheries, Tsukuba.
Results

Varietal radiosensitivity

Sugarcane growing under the chronic irradiation exhibited response to radiation on stalk elongation, number of expanded leaves and deficiency of spindle leaves (Fig. 1). As the irradiation dose increased, stalk elongation was depressed, and expanded leaves decreased in number. Evident varietal difference was revealed on stalk height at 90 days after initiation of the irradiation. The variety, Ni 1 was the most sensitive to chronic irradiation, while NiN 2 showed the most tolerant.

Fig. 1. Varietal difference of irradiation effects on various characters under chronic irradiation in sugarcane.

The percentage of stalks with deficient spindle leaves had increased significantly as the radiation dose rose up to 300 Gy (dose rate : 3.33 Gy/day) and reached at 100% in some varieties at 500 Gy (dose rate : 5.55 Gy/day). Remarkable difference of
varietal radiosensitivity was observed on the percentage of stalks with deficient spindle leaves: the varieties, IRK 67-1, NiF 4 and Ni 1 were the most sensitive, NCo 310 followed and NiN 2 showed the most tolerant. The deficiency of spindle leaves resulted from injury of growing meristem of stalk by the irradiation. Therefore, rolled young leaves as cultured explants could have been obtained from stalk with undamaged meristem within the limits of 300 Gy irradiation.

The explants taken from the plants of 0, 100 and 300 Gy irradiations were cultured for callus induction. Some contamination and discoloration on cultured materials were observed in 300 Gy, whereas callus formation in 100 Gy was comparable to 0 Gy. Once the callus were induced, no difference was found on callus proliferation in subculture and the succeeding plant regeneration between the irradiations. Totally 1,895 regenerated plants were obtained from most of the varieties used except POJ 2725.

**Variation of characters**

Field experiments were conducted to investigate variations on agronomic characters in the populations of the callus derivatives from the control and the irradiations at the first (D₁) and the second (D₂) generations. The records were taken on the growth parameters in September and on the yield components in November of each year. In the present report, only the results of statistical analysis are shown in case of the variety NiF 4, since similar tendencies were obtained in the other varieties.

For major characters shown in Table 1, the means of the population were similar among irradiations in both generations. However, the coefficients of variation were greater in both generations as the irradiation dose increased. These results suggest that the regenerated populations from chronically irradiated plants showed rather wider variation than ones from non-irradiated. The ranges of variation extended not only in a negative but also in a positive direction.

Fig. 2 and 3 present scatter diagrams for stalk length and stalk diameter for the D₁ and D₂ generations of regenerated populations derived from the irradiated plants. No significant correlation was found in 0 Gy and 100 Gy populations, while highly positive correlations for stalk diameter (+0.866*** ) and stalk length (+0.585*** ) were revealed in 300 Gy. In addition, a significant positive correlation between two generations was found for stalk number per stool and cane weight per stool in any irradiations. Consequently, the results suggest that significant positive correlation could have arisen from the wider genetic variation among the subclones induced by gamma ray irradiation, and that such variation could be transmitted by clonal propagation.

**Variability of subclones**

Analyses of variance of the data for 14 characters in D₂ generation are given in
Table 1. Statistical data on quantitative characters of plants in D₁ generation from callus derivatives from chronic irradiated plants in sugarcane variety, NiF 4.
No. of plants n = 147 (0 Gy = 51; 100 Gy = 47; 300 Gy = 49)

<table>
<thead>
<tr>
<th>Character</th>
<th>Dose (Gy)</th>
<th>Mean</th>
<th>C.V. (%)</th>
<th>Skewness m³</th>
<th>Kurtosis a⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stalk length</td>
<td>0</td>
<td>117.4</td>
<td>10.96</td>
<td>-2.55</td>
<td>0.43</td>
</tr>
<tr>
<td>(cm)</td>
<td>100</td>
<td>124.1</td>
<td>12.67</td>
<td>-1.36</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>114.9</td>
<td>17.16</td>
<td>0.13</td>
<td>0.74</td>
</tr>
<tr>
<td>No. of stalks</td>
<td>0</td>
<td>6.08</td>
<td>25.45</td>
<td>0.20</td>
<td>0.56</td>
</tr>
<tr>
<td>/plant</td>
<td>100</td>
<td>5.87</td>
<td>29.84</td>
<td>0.15</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>5.84</td>
<td>46.96</td>
<td>0.84</td>
<td>-0.71</td>
</tr>
<tr>
<td>Stalk diameter</td>
<td>0</td>
<td>1.96</td>
<td>6.79</td>
<td>1.72</td>
<td>-1.05</td>
</tr>
<tr>
<td>(cm)</td>
<td>100</td>
<td>1.96</td>
<td>9.38</td>
<td>0.47</td>
<td>-0.33</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>1.84</td>
<td>13.11</td>
<td>-0.06</td>
<td>-0.46</td>
</tr>
<tr>
<td>No. of nodes</td>
<td>0</td>
<td>8.20</td>
<td>12.68</td>
<td>-1.90</td>
<td>0.74</td>
</tr>
<tr>
<td>/stalk</td>
<td>100</td>
<td>8.64</td>
<td>13.96</td>
<td>0.98</td>
<td>-0.89</td>
</tr>
<tr>
<td>(g)</td>
<td>300</td>
<td>8.69</td>
<td>18.22</td>
<td>-0.56</td>
<td>-0.66</td>
</tr>
<tr>
<td>Weight /stalk</td>
<td>0</td>
<td>453</td>
<td>19.52</td>
<td>0.22</td>
<td>-1.04</td>
</tr>
<tr>
<td>(g)</td>
<td>100</td>
<td>485</td>
<td>26.48</td>
<td>1.72</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>399</td>
<td>33.59</td>
<td>2.16</td>
<td>0.82</td>
</tr>
<tr>
<td>Cane wt. /plant</td>
<td>0</td>
<td>2770</td>
<td>34.09</td>
<td>2.14</td>
<td>3.12</td>
</tr>
<tr>
<td>(g)</td>
<td>100</td>
<td>2898</td>
<td>44.70</td>
<td>2.42</td>
<td>1.60</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>2396</td>
<td>64.88</td>
<td>4.81</td>
<td>6.86</td>
</tr>
</tbody>
</table>

Table 2, 3 and 4. F-values for block and subclone, coefficient of variation and heritability estimates in broad sense in callus derivatives from non-irradiated plants are given in Table 2. In this population, F-values for subclone reached at 1% significant level for leaf type, infection of sheath rot disease and brix in October, and at 5% significance for tiller length and number of stalks. In general, the mean squares for blocks were relatively much higher than for subclones, which resulted in lower heritability for most of the characters except leaf type.

In the 100 Gy population, F-value for subclones was significant at 1% level for nine characters, at 5% level for 2 characters and no significance for 3 characters. In yield components, F-value for subclone was significant for number of stalks, stalk diameter and weight per stalk. Heritability estimates were higher for leaf type, leaf color and tiller length.
Fig. 2. Scatter diagram for stalk length between the first and second vegetative generations on callus differentiated plants derived from chronic irradiated sugarcane. Variety: NiF 4.

Fig. 3. Scatter diagram for stalk diameter between the first and second vegetative generations on callus differentiated plants derived from chronic irradiated sugarcane. Variety: NiF 4.

In 300 Gy population, F-value for subclones was significant at the 1% level for all of the characters except brix. Heritability estimates reached 90% for infection of sheath rot disease, stalk diameter, weight per stalk, 80% for tiller length, leaf type, leaf color and stalk length, and 60% for number of tillers, leaf length, leaf width and
Table 2. Analysis of variance, coefficients of variation and heritability in broad sense of the subclones in D₀ generation of callus derivatives from chronic irradiated plants in sugarcane variety, NiF 4.

Dose : 0 Gy

<table>
<thead>
<tr>
<th>Character</th>
<th>F-value</th>
<th>C.V. (%)</th>
<th>Heritability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Block ((F_{30}^1))</td>
<td>Subclone ((F_{30}^0))</td>
<td></td>
</tr>
<tr>
<td>1 No. of tillers</td>
<td>7.08*</td>
<td>0.69</td>
<td>15.8</td>
</tr>
<tr>
<td>2 Tiller length</td>
<td>3.25</td>
<td>2.01*</td>
<td>6.1</td>
</tr>
<tr>
<td>3 Leaf length</td>
<td>6.26*</td>
<td>1.75</td>
<td>5.2</td>
</tr>
<tr>
<td>4 Leaf width</td>
<td>18.23**</td>
<td>1.50</td>
<td>8.0</td>
</tr>
<tr>
<td>5 Leaf type</td>
<td>9.88**</td>
<td>6.32**</td>
<td>4.8</td>
</tr>
<tr>
<td>6 Leaf color</td>
<td>0.00</td>
<td>1.85</td>
<td>5.5</td>
</tr>
<tr>
<td>7 Sheath rot disease</td>
<td>7.50*</td>
<td>2.58**</td>
<td>17.6</td>
</tr>
<tr>
<td>8 No. of stalks</td>
<td>10.56**</td>
<td>1.93*</td>
<td>13.3</td>
</tr>
<tr>
<td>9 Stalk length</td>
<td>1.78</td>
<td>1.82</td>
<td>6.3</td>
</tr>
<tr>
<td>10 Stalk diameter</td>
<td>6.75*</td>
<td>1.67</td>
<td>7.6</td>
</tr>
<tr>
<td>11 No. of internodes</td>
<td>2.27</td>
<td>1.67</td>
<td>4.5</td>
</tr>
<tr>
<td>12 Weight per stalk</td>
<td>0.03</td>
<td>1.55</td>
<td>11.0</td>
</tr>
<tr>
<td>13 Brix (October)</td>
<td>706.4**</td>
<td>1.65</td>
<td>5.0</td>
</tr>
<tr>
<td>14 Brix (November)</td>
<td>36.90**</td>
<td>1.52</td>
<td>5.8</td>
</tr>
</tbody>
</table>

Significance : * 5%, ** 1%

Table 3. Analysis of variance, coefficients of variation and heritability in broad sense of the subclones in D₀ generation of callus derivatives from chronic irradiated plants in sugarcane variety, NiF 4.

Dose : 100 Gy

<table>
<thead>
<tr>
<th>Character</th>
<th>F-value</th>
<th>C.V. (%)</th>
<th>Heritability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Block ((F_{30}^1))</td>
<td>Subclone ((F_{30}^0))</td>
<td></td>
</tr>
<tr>
<td>1 No. of tillers</td>
<td>4.43*</td>
<td>2.99**</td>
<td>14.2</td>
</tr>
<tr>
<td>2 Tiller length</td>
<td>21.33**</td>
<td>3.80**</td>
<td>7.1</td>
</tr>
<tr>
<td>3 Leaf length</td>
<td>2.00</td>
<td>2.03*</td>
<td>8.8</td>
</tr>
<tr>
<td>4 Leaf width</td>
<td>6.75*</td>
<td>0.89</td>
<td>9.5</td>
</tr>
<tr>
<td>5 Leaf type</td>
<td>0.66</td>
<td>6.91**</td>
<td>7.2</td>
</tr>
<tr>
<td>6 Leaf color</td>
<td>1.76</td>
<td>3.85**</td>
<td>7.2</td>
</tr>
<tr>
<td>7 Sheath rot disease</td>
<td>0.15</td>
<td>3.15**</td>
<td>49.0</td>
</tr>
<tr>
<td>8 No. of stalks</td>
<td>1.22</td>
<td>2.74**</td>
<td>18.0</td>
</tr>
<tr>
<td>9 Stalk length</td>
<td>2.22</td>
<td>1.72</td>
<td>7.7</td>
</tr>
<tr>
<td>10 Stalk diameter</td>
<td>23.73**</td>
<td>3.29**</td>
<td>4.4</td>
</tr>
<tr>
<td>11 No. of internodes</td>
<td>0.52</td>
<td>1.51</td>
<td>9.5</td>
</tr>
<tr>
<td>12 Weight per stalk</td>
<td>10.66**</td>
<td>2.47*</td>
<td>11.4</td>
</tr>
<tr>
<td>13 Brix (October)</td>
<td>364.3**</td>
<td>2.85**</td>
<td>4.9</td>
</tr>
<tr>
<td>14 Brix (November)</td>
<td>10.07**</td>
<td>2.47**</td>
<td>11.3</td>
</tr>
</tbody>
</table>

Significance : * 5%, ** 1%
Table 4. Analysis of variance, coefficients of variation and heritability in broad sense of the subclones in D2 generation of callus derivatives from chronic irradiated plants in sugarcane variety, NiF 4. Dose: 300 Gy

<table>
<thead>
<tr>
<th>Character</th>
<th>F-value</th>
<th>C.V. (%)</th>
<th>Heritability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Block</td>
<td>Subclone</td>
<td></td>
</tr>
<tr>
<td>1 No. of tillers</td>
<td>8.11**</td>
<td>3.15**</td>
<td>14.9</td>
</tr>
<tr>
<td>2 Tiller length</td>
<td>15.04**</td>
<td>8.41**</td>
<td>4.8</td>
</tr>
<tr>
<td>3 Leaf length</td>
<td>7.02*</td>
<td>2.89**</td>
<td>7.9</td>
</tr>
<tr>
<td>4 Leaf width</td>
<td>7.89**</td>
<td>3.38**</td>
<td>6.7</td>
</tr>
<tr>
<td>5 Leaf type</td>
<td>0.75</td>
<td>8.13**</td>
<td>8.1</td>
</tr>
<tr>
<td>6 Leaf color</td>
<td>1.00</td>
<td>7.48**</td>
<td>9.1</td>
</tr>
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<td>7 Sheath rot disease</td>
<td>0.02</td>
<td>10.11**</td>
<td>29.0</td>
</tr>
<tr>
<td>8 No. of stalks</td>
<td>4.52*</td>
<td>2.83**</td>
<td>16.4</td>
</tr>
<tr>
<td>9 Stalk length</td>
<td>3.49</td>
<td>8.12**</td>
<td>6.1</td>
</tr>
<tr>
<td>10 Stalk diameter</td>
<td>0.11</td>
<td>14.00**</td>
<td>4.2</td>
</tr>
<tr>
<td>11 No. of internodes</td>
<td>4.51*</td>
<td>2.40**</td>
<td>10.4</td>
</tr>
<tr>
<td>12 Weight per stalk</td>
<td>5.52*</td>
<td>11.58**</td>
<td>10.3</td>
</tr>
<tr>
<td>13 Brix (October)</td>
<td>108.1**</td>
<td>1.95</td>
<td>7.2</td>
</tr>
<tr>
<td>14 Brix (November)</td>
<td>10.89**</td>
<td>2.10*</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Significance: * 5%, ** 1%

number of stalks. From the above data, it was evident that variance of most agronomic characters among the subclones were markedly increased as the irradiation doses rose. It was also to be noted that the increased variances with higher heritability can be transmitted by clonal propagation and hence utilized as genetic sources in mutation breeding.

### Variability on synthesized characteristics

In order to elucidate variability of whole characteristics of the subclones, principal component analysis was computed based on a correlation matrix for 9 characters of 147 subclones from the variety NiF 4. Contribution for the first, second and third components was calculated as 44.4, 19.7 and 16.8% of the total variation, respectively. Cumulative contribution for these three components accounted for 80.9% (Table 5).

The biological meaning of the components was estimated by correlation between the components and the characters. The first component was negatively correlated
with cane yield \((-0.917)\), stalk weight \((-0.779)\), stalk length \((-0.708)\) and number of stalks \((-0.703)\). The first component represents low yield with short, thin and light stalks directing toward the positive side, and high yield with long, thick and heavy stalks toward the negative. The second component showed negative correlation with number of tillers \((-0.573)\), number of stalks \((-0.533)\) and internode length \((-0.421)\), and positive with number of nodes \((+0.580)\) and stalk weight \((+0.518)\), indicating whether the subclone was 'stalk weight type' or 'stalk number type'. The third component was correlated mainly with internode length \((+0.685)\) and number of nodes \((-0.675)\).

Table 5. Coefficients of correlation between principal components and characters of regenerated plants derived from callus of irradiated sugarcane.

<table>
<thead>
<tr>
<th>Character</th>
<th>Principal component 1</th>
<th>Principal component 2</th>
<th>Principal component 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Tiller length</td>
<td>-0.708</td>
<td>-0.223</td>
<td>-0.095</td>
</tr>
<tr>
<td>2 No. of tillers</td>
<td>-0.573</td>
<td>-0.552</td>
<td>-0.225</td>
</tr>
<tr>
<td>3 Stalk length</td>
<td>-0.708</td>
<td>0.303</td>
<td>-0.171</td>
</tr>
<tr>
<td>4 No. of stalks</td>
<td>-0.703</td>
<td>-0.533</td>
<td>-0.277</td>
</tr>
<tr>
<td>5 Stalk diameter</td>
<td>-0.547</td>
<td>0.464</td>
<td>0.549</td>
</tr>
<tr>
<td>6 No. of nodes</td>
<td>-0.438</td>
<td>0.580</td>
<td>-0.675</td>
</tr>
<tr>
<td>7 Internode length</td>
<td>-0.359</td>
<td>-0.421</td>
<td>0.685</td>
</tr>
<tr>
<td>8 Stalk weight</td>
<td>-0.779</td>
<td>0.518</td>
<td>0.335</td>
</tr>
<tr>
<td>9 Cane yield</td>
<td>-0.917</td>
<td>-0.102</td>
<td>0.031</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biological meaning (+)</th>
<th>Principal component (1)</th>
<th>Principal component (2)</th>
<th>Principal component (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short, thin &amp; light stalk; low yield</td>
<td>Many nodes, heavy, few stalks</td>
<td>Few &amp; long internodes</td>
<td></td>
</tr>
<tr>
<td>High, thick, heavy stalk; high yield</td>
<td>Few nodes, light, many stalks</td>
<td>Many &amp; short internodes</td>
<td></td>
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</tbody>
</table>

A scatter diagram between the first (Axis Z₁) and the second component (Axis Z₂) is reproduced in Fig. 4. This indicates that the subclones from the control (0 Gy) distributed in relatively limited region around the origin. The subclones from the 100 Gy and 300 Gy are scattered over a broad region. It is suggested that chronic irradiation, combined with tissue culture, broaden the variation of whole characteristics on the subclone populations. From the viewpoint of practical breeding, those
subclones from 300 and 100 Gy irradiations that lie in the $-Z_1, Z_2$ quadrant are of special interest, showing high yield with long, thick, and heavy stalks.

Fig. 4. Scatter diagram between the first and second principal components on regenerated plants from callus culture of sugarcane under chronic irradiation.
Variety: NiF 4, Year: 1st, 1986, (n=147).

Moreover, the same tendencies mentioned above were observed on the subclones from the other varieties studied. Fig. 5 represents one example of isolated subclones from 300 Gy irradiation in comparison with their donor variety, Ni 1 on the left side. These mutated subclones ranged from extremely low yield types with narrow leaf and thin stalk, to remarkably high yield types having broad leaf and heavy stalk. Fig. 6 also showed variation of stalk size and types extended from thin stalk with short internodes to thick with long ones.
Fig. 5. Variation of plant types on the subclones regenerated from callus culture of chronic irradiated plants at 300 Gy dose in sugarcane variety, Ni 1.
Left: the donor, Ni 1 The others: subclones

Fig. 6. Variation of stalk size appeared on the same subclones shown in Fig. 5.
The sixth on the left: the door The others: subclones
Repeatability of quantitative characters

Forty six subclones chosen from 146 subclones in the D\(_3\) generation and donor, NiF 4 were vegetatively propagated and grown by the same cultivation practices and in the field through the D\(_3\) to D\(_8\) generations. Repeatability of quantitative characters in mutated subclones could be estimated basing on coefficient of correlation and scatter diagrams of those between the vegetative generations, as shown in Fig. 7, 8, 9, 10, 11 and 12.

In stalk length (Fig. 7-A, B and C), coefficients of correlation showed +0.653, +0.568 and +0.435 between the D\(_3\) vs. D\(_1\), D\(_3\) vs. D\(_5\) and D\(_4\) vs. D\(_5\) generations, respectively. All of these indicating significantly positive at 1\% level, were fluctuating to some extent through 3 generations. The results suggested that repeatability of stalk length was moderate and the donor held a middle position in the distribution of the population.

In stalk diameter (Fig. 8-A, B and C), there were highly positive correlations, +0.927, +0.930 and +0.896 between the D\(_3\) vs. D\(_1\), D\(_3\) vs. D\(_5\) and D\(_4\) vs. D\(_5\) generations, respectively. It was evident that expression of stalk diameter of mutated subclone was stable through vegetative generations and its repeatability was evaluated high enough. The donor, NiF 4 located on the thickest group in the distribution of the population through 3 generations.

Coefficients of correlation for weight per stalk (Fig. 9-A, B and C) were +0.846, +0.693 and +0.623 between the D\(_3\) vs. D\(_1\), D\(_3\) vs. D\(_5\) and D\(_4\) vs. D\(_5\) generations, respectively, suggesting that its repeatability was moderately high and the donor located at the heavier group. In number of stalks (Fig. 10-A, B and C), coefficients of correlation gave +0.773, +0.773 and +0.795 between the generations mentioned above, showing that its repeatability was moderately high and the donor was in the fewer group.

Coefficients of correlation for cane weight per are (Fig. 11-A, B and C) were +0.628, +0.563 and +0.390 between the D\(_3\) vs. D\(_1\), D\(_3\) vs. D\(_5\) and D\(_4\) vs. D\(_5\) generations, respectively, indicating that its repeatability was moderate and the donor located at the middle position. Finally, coefficient of correlation for brix (Fig. 12-A, B and C) showed +0.379, +0.345 and +0.431 between the D\(_3\) vs. D\(_4\), D\(_3\) vs. D\(_5\) and D\(_4\) vs. D\(_5\) generations, respectively. The figures revealed that lower repeatability of brix should be due to immature stalk condition in November and the donor, NiF 4 was at the middle position of the subclones.

Stability of mutated subclones

Table 6 represents result of analyses of variance for 7 quantitative characters of 47 subclones and the donor over all generations from D\(_1\) to D\(_5\). This revealed that mean squares among the subclones were significant at 1\% level for all of the charac-
Fig. 7. Stalk length

Fig. 8. Stalk diameter
Fig. 9. Weight per stalk

Fig. 10. Number of stalks
Fig. 7～12. Scatter diagrams and coefficients of correlation between vegetative generation (D₃～D₅) for 6 major characters of 46 subclones regenerated from callus culture of chronic irradiated plants and their donor variety, NiF 4.

● : subclone, ■ : donor  A : D₃～D₅, B : D₃～D₅, C : D₃～D₅

Fig. 11. Cane yield per are
Fig. 12. Brix in November
ters. Year to year fluctuations showed significant at 1% level for all of the characters, but interactions between subclone and year were generally small relative to subclone differences. Estimates of heritability among all subclones ranged from 67.3 for brix to 96.5 for stalk diameter. These values reflect, in part, the extreme diversity of the mutated subclones and stability of expression for each character throughout the environments of different years.

Table 6. Analysis of variance for quantitative characters callus regenerated subclones from chronic irradiated plants over D1 to D4 generations in sugarcane variety, NiF 4.

<table>
<thead>
<tr>
<th>Character</th>
<th>Mean Square</th>
<th>C.V. (%)</th>
<th>Heritability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subclone (S)</td>
<td>Year (Y)</td>
<td>(S×Y) (D.f. = 92)</td>
</tr>
<tr>
<td>1 Stalk length</td>
<td>511.7**</td>
<td>25740.7**</td>
<td>145.1</td>
</tr>
<tr>
<td>2 Stalk diameter</td>
<td>0.2827**</td>
<td>0.8435**</td>
<td>0.0088</td>
</tr>
<tr>
<td>3 Weight per stalk</td>
<td>39232**</td>
<td>107325**</td>
<td>4213**</td>
</tr>
<tr>
<td>4 No. of stalks</td>
<td>363446**</td>
<td>419393**</td>
<td>31783</td>
</tr>
<tr>
<td>5 Cane weight</td>
<td>55536**</td>
<td>418841**</td>
<td>14155*</td>
</tr>
<tr>
<td>6 Brix (November)</td>
<td>1.987**</td>
<td>231.28**</td>
<td>0.6876</td>
</tr>
<tr>
<td>7 Cane brix ratio</td>
<td>766.2**</td>
<td>25541.9**</td>
<td>255.2</td>
</tr>
</tbody>
</table>

*, ** show significant difference by F-tests at 5% and 1% level, respectively.

Table 7 shows the difference of quantitative characters of 46 subclones by the means of 3 generations and their significant differences from the donor, NiF 4. The significant differences of each subclone indicate + in positive and − in negative comparing with the donor at 5% level.

Stalk length was significantly longer for 8 subclones and shorter for 7 ones. Three clones, A2-8, 9 and 10 derived from 300 Gy were notably the longest stalk among them. It was worth notice that stalk diameter and weight per stalk for most subclones shifted to negative side without a few exception. Only A2-51 was significantly heavier than the donor. On the contrary, number of stalks was significantly increased for 23 subclones and decreased only for 6 ones. In other word, callus derivatives in sugarcane generally seemed to shift from stalk weight type to stalk number. In higher irradiation, 300 Gy, number of stalks extended its variation to both positive and negative directions.

In yield components, cane weight per are was significantly higher for 2 subclones
Table 7. Means of quantitative characters of subclones over the D3, D4, and D5 generations derived from callus regeneration of chronic irradiated plants, and their significant difference from their donor variety, NIF 4.

<table>
<thead>
<tr>
<th>No. Subclone</th>
<th>Irradiation (Gy)</th>
<th>Stalk length (cm)</th>
<th>Stalk diameter (cm)</th>
<th>Weight per stalk (g)</th>
<th>No. of stalks per are (a)</th>
<th>Cane weight per are (kg)</th>
<th>Brix</th>
<th>Cane brix ratio</th>
<th>Sig.*</th>
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<tbody>
<tr>
<td>1</td>
<td>300</td>
<td>110</td>
<td>2.64</td>
<td>569</td>
<td>1223</td>
<td>696</td>
<td>10.6</td>
<td>75.9</td>
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</tr>
<tr>
<td>2</td>
<td>4</td>
<td>117</td>
<td>1.77</td>
<td>332</td>
<td>1504</td>
<td>504</td>
<td>11.1</td>
<td>57.0</td>
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</tr>
<tr>
<td>3</td>
<td>7</td>
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<td>112</td>
<td>130</td>
<td>2.27</td>
<td>520</td>
<td>1131</td>
<td>589</td>
<td>11.9</td>
<td>71.7</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>113</td>
<td>132</td>
<td>2.28</td>
<td>544</td>
<td>1126</td>
<td>607</td>
<td>12.5</td>
<td>77.1</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>118</td>
<td>112</td>
<td>2.28</td>
<td>475</td>
<td>1211</td>
<td>584</td>
<td>11.1</td>
<td>66.9</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>123</td>
<td>122</td>
<td>2.33</td>
<td>528</td>
<td>1135</td>
<td>603</td>
<td>12.0</td>
<td>74.4</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>124</td>
<td>103</td>
<td>2.21</td>
<td>403</td>
<td>844</td>
<td>352</td>
<td>12.2</td>
<td>44.7</td>
<td></td>
</tr>
</tbody>
</table>

* Sig. + and − indicate positive and negative significant difference from the donor, NIF 4 by F-test at 5% level, respectively.
and lower for 11 ones. Brix was increased for 6 subclones and decreased for 7 ones. Cane brix ratio equivalent to available sugar yield, was obviously higher for 2 subclones and lower for 1. These result suggested that no remarkable inclination in yield components was found on subclones derived from irradiation and callus culture.

From the standpoint of actual mutation breeding, two subclones, A2–51 and A2–132 were remarkably higher in cane weight over the donor through 3 generations. In addition, 5 clones, A2–1, 7, 11, 38 and 46 from 300 Gy, 3 ones, A2–66, 73 and 82 from 100 Gy, and A2–145 from 0 Gy had some potential for improved cane yield under the commercial cane belts. It is much interesting from the viewpoint of increasing sugar content that 4 subclones, A2–8, 9, 48 and 49 from 300 Gy, 3 ones, A2–73, 90 and 96 from 100 Gy and 3 ones, A2–113, 123 and 124 from 0 Gy were markedly higher than the donor.

Discussion

Tissue culture versus irradiation-tissue culture combination

First of all, the subject to be discussed is variability of callus derived subclones induced by the process of tissue culture and by mutagenic treatment. In the present study, some extent of somaclonal variation was observed on the subclones by tissue culture without using irradiation (Table 2). None of the quantitative characters such as stalk size and weight of cane showed significant variability. Also, no correlation was observed for stalk size and yield components between the first and second generation. Only some qualitative characters like infection of sheath rot disease and leaf type were significant in non-irradiated control. It is concluded that only minor variations had occurred among tissue culture subclones.

There are reports in the literature of successful selection of useful somaclones from tissue and cell culture including resistances to eye spot disease, Fiji disease, and downy mildew, high yield, early maturity, tolerance to salinity and so on. On the contrary, Kresovich et al. reported that useful variability observed in plants regenerated from callus culture was very limited. Irvine and Miller suggested that somaclonal variation may be of much less value than conventional techniques when considering the frequencies of desirable mutants.

The author considers that variations of callus derived subclones are not comparable to populations from conventional cross breeding, but has some potential from the viewpoint of mutation breeding that one or two important defects can be improved without disturbing the favourable agronomic genetic background of an existing cultivar. In such a case, induction and recovery of somaclonal variation with limited change of faults in commercial use would be more favourable than a wider spectrum of variation. These findings are in agreement with those of Heinz et al., who
suggested that the most promising use of sugarcane cell cultures for crop improvement was in the development of subclones of commercial clones which were deficient in one or two desirable characters, especially disease resistance.

On the other hand, the chronic irradiation of growing plants from which tissue culture explants were derived induced major variations on quantitative character such as stalk size and yield of cane in their regenerated subclones (Tables 2, 3 and 4). Such variations in the D2 generation extend toward not only the negative but positive extreme as the irradiation doses increased. These results suggest that chronic irradiation in the gammafield combined with tissue culture possessed great potential to induce useful genetic variation in mutation breeding.

**Stability of variation**

Mean squares among the subclones totalized from D3 to D6 generations were highly significant for all of the characters and also estimates of heritability among all subclones were generally high enough to evaluate desirable genotypes (Table 7). In fact, not only a number of subclones showed higher yield than the donor, but many subclones exhibited significantly higher sugar content than the donor. It is notable that these promising subclones were selected from few population, only 147 regenerated plants, compared with population size of cross breeding work in other stations. These results indicated that the subclones possessed extreme diversity of the variations.

Highly significant positive correlations were generally recognized for most of the quantitative characters between D3, D4 and D6 generations (Fig. 7-12). Therefore, such variations could be transmitted stably by vegetative generations. One of the reasons for wide differences of coefficient of correlation between the generations might be the range of genetic diversity in each character of subclones. The other is that a plot size on the present experimental scale was not large enough to evaluate stable expression in such characters as number of millable stalks and cane weight per are.

Stability of expression for each character was dependent on interaction between genotype and environmental conditions. It is generally accepted that heritability estimates are relatively high in brix, stalk diameter and stalk length, intermediate in weight per stalk, and low in number of stalks, cane weight and available sugar yield. In this study, relatively low heritability in brix and stalk length were, in part, due to the fact that the experimental location at the latitude on North 36° was unfavourable site for sugarcane cultivation. The productivity tests of the elite subclones has been carried out in large scale in the cane belts in Okinawa Prefecture.

Since mutagenic treatments of tissue and cell cultures had been attempted by Heinz et al., little other work has been reported. From the present study, it is evident that gamma ray irradiation plus tissue culture is a method of enlarging variation and
increasing mutation frequency. Such technique may be profitable to sugarcane breeders who expect wider somaclonal variations comparable to crossed seed populations.

**Acute irradiation versus chronic irradiation**

Acute gamma irradiations have been commonly adopted in sugarcane mutation breeding\(^1\), but rarely has chronic irradiation been used except one case reported in India\(^6\). Optimum acute radiation doses to seedcane were reported for 2–4 kR\(^1\) (20–40 Gy), 3.5 kR\(^2\) (35 Gy), 3–5 kR\(^3\) (3–5 Gy), 3.5 kR\(^4\) (3.5 Gy), and 2–3 kR\(^5\) (20–30 Gy). In the present report, 300 Gy was the highest chronic irradiation dose for survival of a viable meristem from which in vitro explants were taken and, later, seed pieces. The chronic irradiation method adopted in the study accumulated a relatively high dosage but with reduced radiation damage prejudicial to proliferation and differentiation of the callus. In other words, every cell in the growing plants under chronic irradiation is irradiated during these cell division process which is generally the most sensitive and critical phase for mutagenesis. Higher mutation frequencies among the subclones may be assumed to originate from one or few cells in which mutation have occurred during the cell division due to long chronic irradiation.

It is concluded that the tissue culture technique combined with chronic irradiation is an effective method of widening mutation spectrum and increasing mutation frequency in regenerated plants. In addition, this method is valid to improve any crop species which can regenerate plants through callus culture.

**Summary**

The present study was conducted to elucidate the effects of gamma ray irradiation and callus culture upon induced variation of the regeneratives. The populations regenerated from young leaf tissue of chronic irradiated plants grown under a gammafield receiving a total dose of 300 and 100 Gy, showed rather wider variation on quantitative characters than plants from populations of the non-irradiated. This variation extended in both negative and positive directions. Analysis of variance also revealed that variation and heritability in broad sense of most agronomic characters increased significantly among the subclones as the irradiation dose rose.

Principal component analysis also indicated that the subclones from the irradiated population were more variable than the non-irradiated. Such variation with higher heritability could be transmitted to the following generations by clonal propagation and utilized as genetic sources in mutation breeding. The combined method with chronic irradiation followed by tissue culture is evaluated as an effective method of
widening mutation spectrum and increasing mutation frequency in regenerated plants. In addition, this method is valid to improve any crop species which can regenerate plants through callus culture.

References


サトウキビの緩照射とカルス培養の
複合法による誘発変異の拡大

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ガンマ線縫照射とカルス培養との複合法によって得たサトウキビの再分化個体に発現する誘発変異の解析を行った。ガンマーフィールドの縫照射条件下で総線量300および100Gyの照射を受けて生育した植物体、ならびに無照射の植物体から幼葉を外植片としてカルス培養を行って得た再分化個体群では、照射量が高まるにつれて量的形質の変異幅が拡大した。照射による個体群の変異は、負の方向のみならず正の方向にも拡大した。各再分化個体由来の栄養系統について分散分析を行なった結果、高線量区では大多数の農業形質で系統間に顕著な有意差を認め、その広義の遺伝力も増大した。

各系統の総合的形質の変異を解析するために、主成分分析を行ないその散布図から判定すると、照射区の系統群は無照射区に比べてより広い範囲に分布し、育種的に有用な基質型・多収性系統の出現頻度が高まった。原品種に比べて有意に高い高糖性や多収性の優良系統が比較的少数の系統図から検出された。そのような変異形質の遺伝力も高く栄養繁殖によって次代によく伝達され、突然変異育種の遺伝資源として利用されうることが明らかになった。

緩照射とカルス培養との複合法は、照射による変異細胞を再分化した突然変異体として拡大できる有効な突然変異育種法として評価できる。本法はサトウキビに限らず、カルス培養から再分化個体が得られる植物種であれば、改良手段として広く適用することができ
ASYMMETRIC PROTOPLAST FUSION

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Introduction

The technique of protoplast fusion has opened up numerous possibilities for the parasexual manipulation of plants. Since the first report of somatic hybrids obtained from fusions between Nicotiana glauca and N. langsdorffii (Carlson et al. 1972), numerous examples have been described (for review, see Bajaj 1989). Somatic hybridization combines the entire genomes of two parents, and the resultant hybrids can be used as new amphidiploid plant varieties. From the perspective of plant breeders, however, complete somatic hybrids derived from protoplast fusion are not the final product in many cases. Rather, the aim of many breeding experiments is often the integration of a few unique and useful traits from a species that is cross-incompatible with the plant species of interest. Therefore, even if complete somatic hybrids are obtained, such somatic hybrids must often be improved by conventional methods of breeding to produce a novel addition and substitution line.

Transfer of maternally inherited traits has another significance in plant breeding. Among such traits, cytoplasmic male sterility (CMS) is important in the field of hybrid-seed production. Conventionally, this trait has been introduced into a target variety by repeated backcrosses, a procedure that requires a considerable amount of time.

To reduce the time required for the transfer of the trait of interest, a modified version of protoplast fusion has been developed. To facilitate the preferential loss of chromosomes from one of the fusion partners, the technique of inactivation of the nucleus by irradiation has frequently been applied (Zelcer et al. 1978). When the effects of the radiation are not too severe, the fusion products contain part of the nuclear genome from the irradiated partner together with the complete genome of the other. These products are known as asymmetric nuclear hybrids. However, if the effects of the radiation are very severe, the entire nucleus is inactivated, with resultant generation of cybrid plants that possess the complete genome of one species and a mixture
of cytoplasms from both parents.

Although Negrutiu et al. (1989) used the terms “asymmetric hybrid” to describe the nuclear constitution of fusion products, in the present report the term “asymmetric protoplast fusion” is used to describe the mode of protoplast fusion (Figure 1). Thus, asymmetric fusion yields not only an asymmetric nuclear hybrid plant that is asymmetric with respect to the nuclear genomes of the parents, but also cybrid plants that possess one type of nucleus and a mixture of cytoplasms.

This paper describes 1) the outcome of our experiments on the transfer of CMS to commercial varieties of tobacco by asymmetric fusion; 2) a molecular approach designed to identify fragments of mitochondrial DNA associated with CMS using fusion-derived CMS plants; and 3) transfer of part of the nuclear genome by asymmetric fusion, as well as a review of recent progress by others in these areas.

![Diagram of symmetric and asymmetric fusion](image)

**Fig. 1.** Asymmetric fusion and symmetric fusion.
Production of cytoplasmic male sterile tobacco by asymmetric fusion

Cytoplasmic male sterility (CMS) occurs in *Nicotiana tabacum* when the cytoplasm of particular wild species is substituted for that of *N. tabacum*. This phenomenon was found in the course of backcrosses that were intended to introduce potentially useful traits, such as disease resistance, from wild species. Among some 60 species of *Nicotiana*, 14 species have been found to induce CMS in combination with the nucleus of *N. tabacum* (see review Gerstel 1980; Kubo 1985). Although CMS is not a favorable trait when certain traits under control of nuclear genes are to be transferred, CMS offers a major advantage in the production of hybrid seeds. As in the case of other major crops, *F₁* hybrid varieties have been evaluated in tobacco, and *F₁* hybrid varieties have been cultivated.

After deciding on a particular *F₁* hybrid combination, one has to introduce male sterility into the female line. Since, when conventional methods are used, nucleus-cytoplasm exchange is achieved only after several generations of repeated backcrosses, it obviously takes a long time for the transfer of CMS to a given variety.

In efforts to shorten the time required for the transfer of cytoplasm, we have evaluated a system of asymmetric protoplast fusion as an alternative technology. The emphasis of our work has been placed on the following issues: 1) the efficiency of production of CMS regenerants; 2) the stability of CMS in subsequent generations; and 3) the extent of untoward changes among CMS regenerants and their progeny.

1) Procedure for donor-recipient protoplast fusion

Leaf mesophyll protoplasts isolated from nuclear donor and cytoplasm donor species were used for fusion experiments which were conducted by the previously described protocol (Kumashiro and Kubo 1986a). As nuclear donor species, we used *Nicotiana tabacum*, cv. Consolation 402, Tsukuba 1, F114, and Burley 21, and as cytoplasm donor species we used *N. debneyi*, *N. repanda*, *N. megalosiphon*, *N. africana*, and *N. tabacum* cv. MS Burley 21. MS Burley 21 is a cytoplasmic male sterile tobacco variety with the cytoplasm of *N. suaveolens*, (suv) Burley 21.

Before protoplast fusion, the nuclear functions of protoplasts from the cytoplasm donor species must be appropriately inactivated. For this purpose, we have routinely used X-irradiation, the dose of which differs from species to species. A suitable dose can be determined by monitoring the inhibition of cell division or colony formation (Figure 2). Since plating efficiency is expressed in terms of the frequency of colony formation from protoplasts and can vary depending on culture conditions or plant materials, such observations do not always provide a reliable indication of the appropriate dose. Alternatively, the dose can be set from an estimate of the DNA content.
per nucleus, which has been reported to be inversely correlated with sensitivity to irradiation (Sparrow and Miksche 1961). The amount of DNA per nucleus has been reported for a large numbers of plant species (Bennett and Smith 1976) and can be determined precisely by flow cytometry using isolated nuclei and subsequent staining with a fluorescent dye (Galbraith et al. 1983). Details of a protocol involving the use of propidium iodide are described elsewhere (Ishida et al. 1989). For example, estimated DNA contents of the plant species used in our studies relative to that of *N. tabacum* are as follows: *N. debneyi*, 0.99; *N. repanda*, 1.07; and *N. megalosiphon*, 0.59. In *N. tabacum* and *N. debneyi*, 100 grays (Gy) were found to be sufficient for complete inactivation of nuclear functions. However, in the case of *N. megalosiphon*, the DNA content of which is about a half that of *N. tabacum*, irradiation at 100 Gy were insufficient for complete elimination of nuclear functions (see below). Furthermore, in the case of rice, which has about 10% of the DNA of *N. tabacum*, the optimum dose has been reported to be around 1200 Gy (Akagi et al. 1989). These examples confirm the validity of using the DNA content per nucleus as an indicator of the dose of irradiation for a given species of plant.

In our experiments, protoplast fusion was accomplished by the method that involves polyethylene glycol (PEG) and details of the procedure can be found elsewhere (Kumashiro and Kubo 1986a). Unless specified, no selection for heterokaryons was performed after fusion treatment. For each combination used in a fusion, about 300 colonies of 8-10 weeks of age were randomly transferred to a medium for induction of
shoots. One regenerant per independent colony was transferred to soil in a pot and grown in a greenhouse. Sterile regenerants, in which normal setting of seeds was observed only upon pollination with normal fertile pollen from *N. tabacum*, were regarded as exhibiting CMS.

2) Efficiency of formation of cybrids

Table 1 summarizes various examples of the production of CMS regenerants. The frequency of CMS regenerants varied from experiment to experiment. Since there was no selection for heterokaryons, the observed frequencies seemed quite high. Recently, an improved method for donor-recipient fusion has been widely used, in which protoplasts of the nuclear donor species are treated with iodoacetamide (Nehls 1978) or iodoacetate (Medgyesy et al. 1980), permitting only heterokaryons to grow. The examples presented in Table 1 indicate that without any treatment of nucleus donor protoplasts, transfer of cytoplasm would take place at a rather high frequency.

*N. repanda* and *N. africana* are cross-incompatible with *N. tabacum*. It was unknown that cytoplasm of *N. africana* induces CMS in the combination with the nucleus of *N. tabacum*. Even in these cross-incompatible combinations, CMS plants were obtained through transfer of cytoplasm by fusion. Furthermore, intergeneric transfer of chloroplasts was demonstrated in a combination of *N. tabacum* and *Petunia hybrida* (Glimelius and Bonnett 1986; Pental et al. 1986). These findings together suggest that transfer of mitochondria and/or chloroplasts can be accomplished by protoplast fusion beyond the limitations of cross compatibility.

3) Cytoplasmic male sterility in fusion-derived plants

CMS regenerants, in general, exhibited morphology similar to that of diploid *N. tabacum*. In several cases, however, regenerants showing aberrant morphology, such as thick leaves and swollen corollas, were observed. These plants were largely tetraploid (Table 1). In addition, aneuploid regenerants were found in the case of fusion of *N. tabacum* and *N. repanda*. Alterations in chromosome number were probably induced during tissue culture or arose from products of multiple fusions. The finding that the majority of CMS regenerants had a normal diploid complement of chromosomes indicates that the procedures used for tissue culture and protoplast fusion in these studies are less likely to induce cytological changes.

CMS tobacco plants, which are developed by repeated back crosses, exhibit their own distinct flower morphology which depends on the species of the cytoplasm donor (Gerstel 1980). Such distinct flower morphology was evident among CMS plants derived from fusions between *N. repanda* and *N. tabacum* (Kumashiro et al. 1989). In a number of cases, however, CMS regenerants exhibited a particularly novel flower
Table 1. Efficiency of transfer of CMS trait by protoplast fusion.

<table>
<thead>
<tr>
<th>Combination of fusion (cytoplasm + nucleus)</th>
<th>Total number of regenerants</th>
<th>Number of CMS regenerants</th>
<th>Diploid</th>
<th>Tetraploid</th>
<th>Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. debneyi</em> + Consolation 402</td>
<td>318</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>(sue)</em> Burley 21 + Tsukuba 1</td>
<td>207</td>
<td>84</td>
<td>23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>(sue)</em> Burley 21 + F114</td>
<td>117</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>N. repanda</em> + Consolation 402</td>
<td>150</td>
<td>8</td>
<td>0</td>
<td>7 (46, 47)</td>
<td>0</td>
</tr>
<tr>
<td><em>N. africana</em> + Burley 21</td>
<td>300</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>N. megalosiphon</em> + Consolation 402</td>
<td>130</td>
<td>0</td>
<td>0</td>
<td>5 (60-71)</td>
<td></td>
</tr>
</tbody>
</table>

In part ploidy was estimated by their morphological traits.
Number in parenthesis indicates somatic chromosome number of aneuploid plants.

<table>
<thead>
<tr>
<th>Type</th>
<th>N</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anther</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Dehiscence</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Pollen Grain</td>
<td>Few</td>
<td>Trace</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Fig. 3. Illustration of different flower morphology observed among CMS regenerants derived from fusion between *N. debneyi* and *N. tabacum*.

morphology. For example, as shown in Figure 3, among CMS regenerants derived from a fusion between *N. debneyi* and *N. tabacum*, three intermediate types were observed in addition to type C, which is identical to that obtained by repeated backcrosses. Furthermore, among CMS plants derived from fusions between *N. suaveolens* and *N. tabacum*, morphological differences were evident mainly in the shape
of the pistils (Kubo et al. 1988). Since not only CMS but also such novel flower morphology were stably inherited by the progeny obtained by pollination with pollen from *N. tabacum*, such novel morphology might reflect recombinational events between two different mitochondrial genomes, as will be discussed below.

4) The chloroplast genome

The chloroplast genome in fusion-derived CMS plants was characterized by examining one of following parameters: electrophoretic mobility of the large subunit of ribulosebisphosphate carboxylase (Rubisco); reaction to tentoxin (Burk and Durbin 1978); and the restriction pattern of chloroplast DNA (cpDNA). In most cases, CMS regenerants possessed chloroplasts of only one or the other parental type (Table 2), a

<table>
<thead>
<tr>
<th>Combination of fusion (cytoplasm+nucleus)</th>
<th>Chloroplast type*</th>
<th>Method of determination</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. debneyi</em> + Consolation 402</td>
<td>15 11 0</td>
<td>Rubisco, cpDNA</td>
</tr>
<tr>
<td><em>(sus)</em> Burley 21 + Tsukuba 1</td>
<td>34 5 4</td>
<td>Tentoxin</td>
</tr>
<tr>
<td><em>(sus)</em> Burley 21 + F114</td>
<td>8 7 1</td>
<td>Rubisco, Tentoxin</td>
</tr>
<tr>
<td><em>N. repanda</em> + Consolation 402</td>
<td>7 1 0</td>
<td>Rubisco</td>
</tr>
<tr>
<td><em>N. africana</em> + Burley 21</td>
<td>0 1 0</td>
<td>Rubisco, cpDNA</td>
</tr>
<tr>
<td><em>N. megalosiphon</em> + Consolation 402</td>
<td>5 0 0</td>
<td>Rubisco</td>
</tr>
</tbody>
</table>


result that is in accordance with many observations reported in the literature (Kumar and Cocking 1987). When sensitivity to tentoxin is used to characterize the chloroplast genome, regenerants of the mixed type were found in a few cases. In the tentoxin test, the nature of the chloroplasts of a regenerant was determined by evaluating a large number of progeny in the BC1 generation. Thus, it was possible to identify the mixed type even when the ratio of one type to the other was uneven. By contrast, estimations by restriction patterns of cpDNA or by measurements of the electrophoretic mobility of the large subunit of Rubisco were performed on a single-plant basis. If the ratio of one type and the other is too far from one to one it is likely to result in failure of detection of the mixed nature. Therefore, there may appear to be more regenerants with a mixed population of chloroplasts among regenerants when cpDNA or Rubisco are examined than what are observed.
5) Field performances of CMS progeny

As reviewed by Larkin and Scowcroft (1981), plant tissue-culture systems themselves tend to generate various forms of genetic variability. Since fusion-derived cytoplasmic male sterile plants have been exposed to conditions in vitro for a long time, they might be expected to express both favorable and unfavorable genetic variations. Before the asymmetric fusion method is used as an alternative to a conventional backcross method, it is essential to assess whether fusion-derived CMS plants are similar to CMS plants produced by repeated backcrosses in terms of stability of CMS as well as overall agronomic performances.

Progeny of fusion-derived CMS plants of different generations were evaluated in the field. Three different groups of fusion-derived CMS lines: 1) *N. debneyi* + *N. tabacum* cv. Consolation 402; 2) (swu) Burley 21 + *N. tabacum* cv. Tsukuba 1; and 3) (swu) Burley 21 + *N. tabacum* cv. F114, together with the selfed counterparts, were grown with a fashion of randomized block design or split-plot design with at least two replications.

In the case of CMS lines obtained by protoplast fusions between *N. debneyi* and Consolation 402, the majority of lines in the BC₁ generation exhibited field performances similar to those of the selfed counterparts, although a few lines showed some altered characteristics, such as late maturity and reduced plant height (Table 3). These altered traits disappeared in the subsequent backcross generation (Kumashiro and Kubo 1986b). Furthermore, these lines with altered traits did not carry a particular type of chloroplast genome. These findings indicate that the altered quantitative traits observed in the BC₁ generation were controlled by recessive nuclear genes, which were

<table>
<thead>
<tr>
<th>Fusion combination</th>
<th>Number of CMS lines evaluated</th>
<th>Number of altered lines*</th>
<th>Total***</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. debneyi</em> + Consolation 402</td>
<td>26</td>
<td>2 3 3 3 2 0</td>
<td>4</td>
</tr>
<tr>
<td>(swu) Burley 21 + Tsukuba 1</td>
<td>22</td>
<td>2 0 2 0 2 0</td>
<td>4</td>
</tr>
<tr>
<td>(swu) Burley 21 + F114</td>
<td>10</td>
<td>1 1 4 0 1 —</td>
<td>5</td>
</tr>
</tbody>
</table>

* : significantly different from the nucleus-donor variety at the 5% probability level.
** : DF, days to flowering; PH, plant height; LN, number of leaves; LL, leaf length; LW, leaf width, YD, yield
*** : total of lines judged to have deviation at least one of the traits.
probably induced during the tissue-culture phase of growth.

Similarly, stable performances were observed in CMS lines derived from fusions between (suv) Burley 21 and Tsukuba 1. From these lines, one line with overall characteristics that were judged to be identical to those of the nucleus donor variety was selected and released as a commercial CMS variety (Kubo et al. 1988).

Among the CMS lines obtained by fusion between (suv) Burley 21 and F114, lines with altered characteristics were found at a higher frequency than in the two cases mentioned above. In this fusion, there were also more regenerants with morphological aberrations among the non-CMS regenerants, which presumably carried various changes in chromosome number. The rather high frequency of altered lines appears to be due to a prolonged tissue-culture phase in this particular experiment.

Field traits of CMS lines derived from the three fusion experiments suggest together that 1) the majority of CMS lines was similar to the parental inbred line with respect to quantitative traits, 2) a few lines with altered traits appeared to have changes in the nuclear genome, 3) the extent of such changes can be minimized by limiting the duration of the tissue-culture phase, and 4) the chloroplast genome had no influence on the agronomic performance of the CMS lines.

6) Scheme for production of CMS varieties of tobacco

The agronomic performance of CMS lines of tobacco obtained by asymmetric fusion was comparable to that of inbred counterparts. Thus, the protoplast fusion method can be used for the production of commercial CMS varieties of tobacco.

About 6 months from the time of protoplast fusion are necessary for confirmation of CMS of regenerants in a greenhouse. Then the progeny obtained by pollination with the nucleus donor cultivar should be grown in a field. This evaluation step is important because there are a few lines that differ from the parental variety as a result of somaclonal variation, which may occur during the tissue-culture phase. If a CMS line exhibits an agronomic performance identical to that of the parental line, the line can be used as the female parent in production of an F₁ hybrid variety. Therefore, in the case of the asymmetric protoplast fusion method, the total time needed to introduce cytoplasmic male sterility should be less than two years. Thus, relative speed is clearly one of advantages of protoplast fusion.

The mitochondrial genome in CMS regenerants

The mitochondria of the fusion partners are initially mixed together in the cytoplasm of the heterokaryons that are formed after protoplast fusion. With respect to the fate of mixed mitochondrial genomes, the great majority of the reports in the
literature suggests that extensive rearrangements take place through inter- or intramolecular recombinations (for review, see Pelletier 1986; Ichikawa et al. 1989). This phenomenon was first observed in cybrid plants of *N. tabacum* by Belliard et al. (1979). The mtDNA of cybrids gave novel restriction patterns that were different both from those of the parental mtDNA and from the combination of both parental patterns. Belliard et al. (1979) attributed the novel restriction fragments to intermolecular recombination between two mitochondrial genomes. By contrast, there are a few reports that suggest the sorting out to homogeneity of parental mitochondria in cybrid or somatic hybrid plants (Yarrow et al. 1986; Nagy et al. 1983; Kothari et al. 1986). In order to examine whether such rearrangements occurred during formation of our CMS regenerants by fusion and to determine whether the CMS regenerants carry part of the mitochondrial genome from the cytoplasm donor species, we performed restriction analyses of mtDNA.

1) Novel restriction fragments of mtDNA in CMS regenerants

Restriction patterns of mtDNA from five different CMS regenerants obtained by fusions between *N. debneyi* and *N. tabacum* cv. Consolation 402 were compared with one another and with those of the parental species (Asahi et al. 1988). Each of the five CMS regenerants gave different restriction patterns (Figure 4). Furthermore, there were several restriction fragments that were not seen in the parental species. Novel restriction fragments were also detected in CMS plants derived from fusions between *N. africana* and *N. tabacum* (Kumashiro et al. 1988) and between *N. repanda* and *N. tabacum* (Kumashiro et al. 1989).

It appears from these findings, as well as an overwhelming number of reports in the literature, that recombination between two different mitochondrial DNAs is a very common, but not universal, event in the heterokaryons formed after protoplast fusion.

2) Identification of CMS-specific fragments of mtDNA

Comparison of restriction patterns of CMS regenerants obtained by a given asymmetric fusion makes it possible to identify those fragments of mtDNA, if any, that might confer cytoplasmic male sterility on the nuclear donor species. Close investigation of the restriction patterns in Figure 4 reveals that four BamHI fragments of mtDNA, of 11.2, 9.4, 6.4, and 3.4 kb, respectively, were generated by five CMS regenerants as well as by the cytoplasm donor species, *N. debneyi*. Among the four fragments, the fragments of 6.4 and 3.4 kb were used to examine the mtDNA of other CMS regenerants derived from the same fusion combination by Southern hybridization using each mtDNA fragment as probe. The 6.4 kb fragment was missing in the case of only one regenerant out of 26, while the 3.4 kb fragment was detected in the analysis
Fig. 4. Restriction patterns of mtDNA of CMS lines derived from fusion of *N. debneyi* and *N. tabacum*. (for the picture, see Asahi et al. 1988)

of all of the CMS regenerants.

The prevalence of the 3.4 kb BamHI fragment was examined in other species of *Nicotiana*, the cytoplasms of which are known to cause CMS in combination with a nucleus from *N. tabacum*. The species evaluated were *N. suaveolens*, *N. repanda*, *N. megalosiphon*, *N. gossei*, *N. rustica*, *N. glauca*, *N. undulata* and *N. plumbaginifolia*. In
an analysis by Southern blotting, positive signals corresponding to fragments of the same molecular size were observed in the case of *N. suaveolens* and *N. megalosiphon*. *N. gossiei* gave a positive signal corresponding to a fragment of a different size (ca. 6.0 kb), and the other species gave no signal.

CMS regenerants obtained from fusion between (*sus*) Burley 21 and Tsukuba 1 were analyzed for the occurrence of the 3.4 kb BamHI fragment. Each of 30 regenerants generated this particular fragment. Furthermore, five CMS regenerants derived from a fusion between *N. megalosiphon* and Consolation 402 gave a positive signal when hybridized with the 3.4 kb BamHI fragment. These data indicate that the 3.4 kb BamHI fragment of *N. debneyi* is closely associated with cytoplasmic male sterility in *Nicotiana*.

3) Molecular characteristics of the 3.4 kb BamHI fragment

The entire 3.4 kb BamHI fragment was sequenced. Open reading frames (ORFs) were located within the fragment (3413 bp) and compared with those in a data base. Even the longest ORF, with a length of 411 bp, exhibited no homology to reported sequences.

Northern hybridization was performed using a labeled 3.4 kb BamHI fragment and mtRNAs prepared from suspension cultures of *N. tabacum*, *N. debneyi*, and a fusion-derived CMS line. The probe hybridized with 1.4 kb transcripts. One strong and one slightly weaker signal were detected in the case of *N. debneyi* and the male sterile line, respectively (Figure 5), whereas almost no signal was found in the case of the fertile *N. tabacum*. There were no such clear differences in the intensities after northern hybridizations with other mitochondrion-specific probes, such as the *cox II* and *atpA* genes of pea. Thus, it appears that the observed variations in intensities in the above experiment were specific to the 3.4 kb fragment. Further northern hybridization using different parts of the 3.4 kb fragment indicated that only a fragment from one end hybridized to the transcripts from *N. debneyi* and the male sterile line.

Sequence analysis of the fragment adjacent to the particular end of the 3.4 kb fragment revealed a region that was completely homologous to the *atp9* gene (234 bp). We found that the 1.4 kb transcript spans the end of the 3.4 kb fragment and the *atp9*, and the *atp9* gene has another transcript of 0.7 kb. Experiments are in progress to investigate the significance of the differences in the amount of 1.4 kb transcript between normal fertile tobacco and CMS tobacco.

**Asymmetric nuclear hybrids**

Even without any treatment to accelerate the preferential loss of part of one
Fig. 5. Northern blot hybridization using 3.4 kb BamHI fragment as a probe.
CMS *N. tabacum* is a CMS regenerant derived from fusion between *N. debneyi* and *N. tabacum*.

genome, asymmetric nuclear hybrids can be generated spontaneously, in particular from combinations of remote species. In such cases, however, the direction as well as the extent of the loss of genomes in the final fusion product is largely unpredictable. Irradiation of one partner is, therefore, effective in controlling at least the direction of elimination of nuclear material.

Induced asymmetric fusion was first described by Dudits et al. (1980): carrot
plants regenerated from fusion products between irradiated parsley and untreated carrot protoplasts contained a normal complement of carrot chromosomes together with an extra chromosome.

A procedure for the introduction of a limited amount of nuclear genetic material is essentially the same as that used to obtain cybrids, although the dose of radiation for nucleus donor protoplasts may differ from that used in formation of cybrids. We tried to incorporate a part of a genome using *N. megalosiphon* as a model species since the DNA content per nucleus of this species is about half of that of *N. tabacum*. In the first experiment, mesophyll protoplasts of *N. megalosiphon* were irradiated with X-rays at a dose of 100 Gy and fused with protoplasts of *N. tabacum*. A total of 130 regenerants were obtained from independent colonies. Among them, five plants had dark-green leaves. Since the recipient species has yellow leaves, with the color controlled by a recessive gene, these dark-green plants can be considered to be hybrids nature, as was confirmed by the presence of the two types of small subunit of Rubisco that were

**Table 4. Asymmetric nuclear hybrids obtained by fusion of *N. tabacum* and X-ray (100 Gy) irradiated *N. megalosiphon* protoplasts.**

<table>
<thead>
<tr>
<th>Regenerant</th>
<th>Chromosome number (2n=)</th>
<th>Petal morphology</th>
<th>Anther morphology</th>
<th>Rubisco*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgT 10</td>
<td>60-63</td>
<td>Split</td>
<td>Feminized</td>
<td>meg</td>
</tr>
<tr>
<td>MgT 28</td>
<td>61-63</td>
<td>Semi-split</td>
<td>Normal</td>
<td>meg</td>
</tr>
<tr>
<td>MgT 50</td>
<td>70-71</td>
<td>Semi-split</td>
<td>Filament-like</td>
<td>meg</td>
</tr>
<tr>
<td>MgT 57</td>
<td>64-67</td>
<td>Semi-split</td>
<td>Normal</td>
<td>meg</td>
</tr>
<tr>
<td>MgT 78</td>
<td>64-68</td>
<td>Split</td>
<td>Feminized</td>
<td>meg</td>
</tr>
</tbody>
</table>

* : large and small denote subunits of Rubisco.

**Table 5. Relationship between radiation dose and formation of asymmetric hybrids.**

<table>
<thead>
<tr>
<th>X ray dose (Gy)</th>
<th>NF <em>tabacum</em></th>
<th>Regenerants classified by their morphological traits and amount of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>megalosiphon</em></td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>4 (22.2)</td>
</tr>
<tr>
<td>100</td>
<td>26</td>
<td>7 (13.2)</td>
</tr>
<tr>
<td>250</td>
<td>3</td>
<td>1 (3.8)</td>
</tr>
</tbody>
</table>

Number in parenthesis indicates percent of each class of regenerants presumably involved in a fusion event.
Fig. 6. Somatic chromosome of a regenerant from asymmetric nuclear fusion of *N. megalosiphon* and *N. tabacum*. Arrow indicates fragments of chromosomes.

specific to the respective spices. The morphology of flowers differed among hybrids but all exhibited cytoplasmic male sterility. From the somatic chromosome number and karyotype analysis, these regenerants were judged to be asymmetric hybrids (Table 4). Since the diploid species of *N. megalosiphon* has 40 chromosomes, approximately a half of its genome was present in these regenerants. Furthermore, in these asymmetric hybrids, a few tiny chromosomes that were not present in the parental species were detected; they may have been produced as a result of the irradiation (Figure 6).

Another experiment was performed to determine the relationship between the dose of X-rays and the amount of the introduced genome. In this experiment, mesophyll protoplasts of *N. megalosiphon* were exposed to three different doses (25, 100, 250 Gy) of irradiation and were then fused with protoplasts of *N. tabacum* that had been treated with iodoacetamide. Regenerants from independent colonies were first evaluated in terms of their morphological characteristics, then the DNA content per nucleus was determined by flow cytometry. Asymmetric hybrid plants that had a DNA content intermediate between that of *N. tabacum* and that of the symmetric somatic hybrid appeared at all doses tested (Table 5). The proportion of asymmetric
hybrids among fusion-derived regenerants was higher at the lowest dose (25 Gy), although there was no direct correlation between dosage and this proportion. With respect to the amount of the introduced genome in the asymmetric hybrids, no direct relationship was observed either; at 25 Gy, it was 13% to 48%, at 100 Gy, 8% to 72%, and at 250 Gy, 11% to 36%.

Discussion

Asymmetric nuclear hybrids

Asymmetric fusion makes it possible to introduce part of a genome from irradiated protoplasts into a fusion product, as observed in our experiments. Thus, asymmetric protoplast fusion provides a shortcut when the final goal of protoplast fusion is the incorporation of a specific trait. To achieve this goal, we need, however, a method that allows us to control or minimize the amount of genetic material introduced from the donor species.

No clear relationship between the amount of the introduced genome and the dose of X-irradiation was observed in our experiments. This negative result implies that there is no appropriate way to control the amount of the genome that is transferred. With respect to the relationship between doses of irradiation and the extent of loss of the genome, no established rule can be extrapolated from the literature. Menczel et al. (1982) showed that a decreased frequency of somatic hybrids and an increased frequency of cybrids occurred with increased doses of irradiation in the range between 50 and 300 Gy. By contrast, limited elimination of chromosomes, together with the absence of a dosage effect, was observed in asymmetric somatic hybrids of various species. For example, retention of the donor chromosomes ranged from 11% to 90% in N. plumbaginifolia (recipient) + Atropa belladonna (donor) hybrids (100–1000 Gy; Gleba et al. 1988), from 8% to 75% in N. plumbaginifolia + N. sylvestris hybrids (100–1000 Gy; Famaelaer et al. 1989), from 25% to 100% in Brassica oleracea + B. campestris hybrids; Yamashita et al. 1989), and from 21% to 250% in Lycopersicon peruvianum + L. esculentum (Wijbrandi et al. 1990).

Transfer of a limited amount of nuclear genome via asymmetric fusion has been reported in several cases, namely, parsley (Petroselinum hortense) + an albino mutant of carrot (Dudits et al. 1980), Physalis minima + an albino mutant of carrot (Gupta et al. 1984), kanamycin-resistant N. plumbaginifolia + N. tabacum (Bates et al. 1987), and N. plumbaginifolia + kanamycin-resistant Petunia hybrida (Hinnisdaels et al. 1991). It should be emphasized that, in these cases, fusion products were selected on the basis of complementation or by use of a nucleus-encoded selectable marker. These examples suggest that a selection force aimed at a marker gene may stimulate transfer of
a limited amount of genetic material. If such stimulation does indeed occur, the use of a selectable marker may facilitate selectable transfer of chromosomes of interest. Therefore, it is important to obtain a donor strain into which such a selectable marker gene has been introduced by appropriate transformation (Komari et al. 1989). For preference, the marker gene should be on the same chromosome as that on which the gene for the trait of interest is located. With appropriate techniques, the recovery should be possible of asymmetric hybrid plants that carry only one chromosome that carries the gene for a practical trait of interest.

**Rearrangement of mtDNA in fusion-derived CMS plants**

Restriction patterns of mtDNA from fusion-derived CMS regenerants differed from one another, with donor- and recipient-specific as well as novel fragments. Such extensive rearrangement of mtDNA in cybrids has been reported in many instances. Even in a normal situation, recombination seems to be a significant cause of mutation of mitochondrial genes, as indicated by the detection of chimeric genes in naturally occurring CMS plants, which are probably produced by intramolecular recombination events that involve repeated sequence in the genome (Fragoso et al. 1989). Therefore, the extensive rearrangement of mtDNA observed in cybrids, which is a result not only of intramolecular but also of intermolecular recombination, appears to be very common.

The phenomenon of extensive rearrangement of mtDNA is of significant utility in cases where an attempt is made to identify fragments of mtDNA that are closely associated with CMS, as demonstrated by Booshore et al. (1985) in somatic hybrids of petunia. Using a similar approach, we identified four fragments which were detected in analyses of all cybrids tested, as well as in the cytoplasm donor species. Although most part of the 3.4 kb BamHI fragment was not transcribed, our study revealed that one end of this fragment corresponded to a part of a 1.4 kb transcript. In the study by Bland et al. (1986), the northern blot analysis of tobacco mtRNA using the *atp9* gene as a probe revealed a very complex pattern of transcription, indicating the presence of a primary transcript that is extensively processed. In our study, when the *atp9* gene was used as a probe, two transcripts of 0.7 kb and 1.4 kb, respectively, were detected; the former can be regarded as the mature processed transcript of the *atp9* gene. Almost no difference was detected between the amounts of 0.7 kb transcript in CMS and fertile lines, whereas there was a difference in the amount of 1.4 kb transcript between them. Hakansson and Glimelius (1991) compared patterns of transcription in many alloplasmic male sterile lines of tobacco with those in the corresponding fertile lines, and they observed an identical pattern of transcription with all cytoplasmic and nuclear backgrounds when the *atp9* gene was used as a probe. Their observations are
in harmony with our failure to detect differences in the levels of transcripts, as far as the mature transcripts were concerned.

There are many examples of rearrangements of mtDNA and altered patterns of transcription and translation associated with CMS plants. For instance, the mitochondrial genome of the CMS cytoplasm of *Brassica* is highly rearranged relative to its parental counterpart, and rearrangements of mtDNA have been associated with altered patterns of transcripts (Makaroff and Palmer 1987). Altered patterns of transcripts have been identified for genes such as *atpA*, *atp6* and *cox I* (Makaroff *et al.* 1990). In rice, a novel gene, which was presumably the fused product of the 5′ region of the *atp6* gene and an unidentified sequence, was detected only in a CMS plant that carried cytoplasm from Chisurah Boro II (Kadowaki *et al.* 1990). In a CMS sunflower, rearrangements in mtDNA were found either within or near the *atpA* gene, and the transcripts of the *atpA* gene differed between the fertile and the CMS lines (Siculella and Palmer 1988, Köhler *et al.* 1991). Furthermore, in petunia, the CMS phenotype has been shown to be associated with specific fragments of the mitochondrial genome (Boeshore *et al.* 1985). This region of mtDNA contains atypical sequences of the *atp9* and *cox II* genes together with an unidentified open reading frame (Young and Hanson 1987).

Although the evidence is still indirect, our findings imply some correlation between a transcript of the *atp9* gene and the CMS phenotype. The examples from the many reports mentioned above suggest that this hypothesis is worth examining in detail. Future efforts should be focused particularly on elucidation of the role of the 1.4 kb transcripts.

**Transfer of CMS by asymmetric fusion**

An asymmetric fusion system focusing on the production of CMS varieties of tobacco meets all the requirements cited at the beginning of this paper: 1) the CMS trait can be transferred to the variety of interest within less than two years, 2) the CMS cultivar thus obtained stably exhibits cytoplasmic male sterility; and 3) fusion-derived CMS varieties are similar to CMS cultivars obtained by a conventional backcross in terms of agronomic performance. Encouraged by these findings, we have been using this asymmetric fusion system to produce a number of practical CMS varieties of tobacco.

Asymmetric fusion has also been used to produce CMS varieties of *Brassica*. By fusing protoplasts of a CMS line of *B. napus* with protoplasts of an atrazine-resistant line of *B. campestris*, an atrazine-resistant CMS line was obtained (Pelletier *et al.* 1983). These lines are now being incorporated into *Brassica* breeding programs (Pelletier 1990). CMS traits have also been incorporated by protoplast fusion into carrot
(Tanno-Suenage et al. 1988) and rice (Akagi et al. 1989; Kyozuka et al. 1989). Evaluation of the field performance of these CMS lines will contribute to generalize the practical value of asymmetric fusion as a method for production of CMS varieties in a wider range of crops.

Are there any limitations to the application of asymmetric protoplast fusion systems to a wide range of crop species? First of all, a suitable donor of cytoplasm that confers cytoplasmic male sterility on the species of interest is required. Also, for the crop species in which the reproductive organs are utilized, a corresponding fertility-restoration system must be available. Apart from these biological factors, the greatest limiting factor appears to be the availability of an established protoplast-regeneration system, since steady progress has been made in fusion methods, as well as in methods for selection of heterokaryons. A protoplast culture system used for this purpose should not only be efficient enough in terms of plant regeneration, but it also should be as free as possible from complications due to cytological changes and genetic mutations.

Reference


31. KUMASHIRO T. and KUBO T. (1986b) Stability of agronomic traits in cytoplasmic male sterile tobacco


非対称細胞融合

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日本たばこ産業株式会社 遺伝育種研究所

細胞融合は交雑不可能な植物種の有する有用形質の導入を可能にした。しかし、完全な体細胞雑種植物は多くの場合そのままでは実用品種として利用されることは少なく、体細胞雑種から大部分の不要な染色体を除くという煩雑で時間をかかる操作が必要となる。また細胞質因子に支配される雄性不稔性は特にF1雑種品種を育成する上で重要であるが、その育成は通常交配法によっており、細胞質雄性不稔性を目的とする品種に導入するためにはなり長い時間を必要としている。これらの点を回避するため、細胞融合時にプロトプラストに放射線照射を施し、ゲノムを不活化あるいは分断された後に目的種のプロトプラストと融合する方法が試みられている（非対称融合法）。この報告では、非対称細胞融合法による細胞質雄性不稔性系の作出、および核の部分ゲノムの導入に関するタバコでの我々の知見を中心に紹介する。

細胞質供与種のプロトプラストにX線を照射し、無処理の核供与種のプロトプラストと融合させることによって、目的とする細胞質雄性不稔性の再分化個体が比較的高頻度で得られた。倍数体、異数体などの変異体の出現頻度は総じて低かった。細胞質雄性不稔性を示す再生個体の大多数は二親のいずれか一方の葉緑体タイプを示したが、トントキシン耐性をマーカーとした場合には、低頻度で、両タイプを含む数個体が認められた。これらのが細胞質雄性不稔性個体に正常な核供与種の花粉を交配して得られたBC1系を圃場に栽培して核供与種と生育特性能を比較したことより、一部に雌性化、小型化した系統が見られたものの大部分の雄性不稔性系統と同等の生育特性を示した。変異が認められた系統は、戻し交配世代が進むにつれて核供与種と同等の特性を示すようになり、一部では雌性化、小型化した系統が見られたものの大部分の雄性不稔性系統と同等の生育特性を示した。変異が認められた系統は、戻し交配世代が進むにつれて核供与種と同等の特性を示すようになり、一部では雌性化、小型化した系統が見られたものの大部分の雄性不稔性系統と同等の生育特性を示した。変異が認められた系統は、戻し交配世代が進むにつれて核供与種と同等の特性を示すようになり、一部では雌性化、小型化した系統が見られたものの大部分の雄性不稔性系統と同等の生育特性を示した。変異が認められた系統は、戻し交配世代が進むにつれて核供与種と同等の特性を示すようになり、一部では雌性化、小型化した系統が見られたものであることを示している。融合由来の雄性不稔性系統は、戻し交配の後代においてもすべて安定して雄性不稔性を示した。

*N. debneyi*と*N. tabacum*との細胞融合によって作出した雄性不稔性個体のミトコンドリアDNAを制限分析したところ、その制限断片には両親に特異的な断片の一部とともに新しい断片が認められ、さらに雌性不稔個体間でも異なったパターンを示した。これは細胞体において2種類のミトコンドリアが複雑に組換えを起こした結果であると考えられる。この現象を利用して、細胞質雄性不稔性に関連するミトコンドリアDNAの断片を検索した。3.4 kb BamHI断片は*N. debneyi*とすべての融合由来の雄性不稔個体に共通して認められた。この断片の塩基配列を決定したがその中には報告されている配列と相同なもの
はなかった。この断片はN. debneyiと雄性不稔個体では1.4 kbの転写産物と反応したが、
N. tabacumとはほとんど反応しなかった。3.4 kb BamHIの近傍領域の塩基配列からこの
1.4 kbの翻訳産物はatg9遺伝子の未成熟産物である可能性が示唆された。1.4 kbとRNAと
tabaccoにおける細胞質雄性不稔性との関係をさらに検討するため解析を進めている。
非対称融合法による核の一部のゲノム導入をN. megalosiphonを材料として検討した。一部
の染色体が導入された再生個体は種々の線量区で認められたが、X線の照射量と導入ゲ
ノム量との間には明瞭な関係が見られなかった。今後、この方法を実用的な育種に応用す
るためには、できるだけ少数の染色体の移行、さらに目的とする染色体の移行を可能とす
る方法が必要となると考えられる。
SOMATIC VARIATION FOR BREEDING OF DISEASE RESISTANT PLANTS

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Introduction

A prominent feature of plant tissue cultures is that genetic variations are frequently induced by spontaneous or mutagen-induced mutations or chromosomal abnormalities (Evans and Sharp 1983; Sunderland 1977). These callus tissues are genetically chimeric with respect to newly induced characters and therefore could be useful gene sources for effectively isolating variant cells. Such a variations induced in callus tissues has been designated as somaclonal variation and has provided us with a new tool for improving crop plants (Larking and Scowcroft 1981).

In the present paper, the author presents some examples of an efficient selection for disease resistance and discuss a possible application of somaclonal variation to the breeding of disease resistant plants. Plants and pathogens used for selection were listed in Table 1.

Table 1. Plants and pathogens used for selection of disease resistance.

<table>
<thead>
<tr>
<th>Diseases</th>
<th>Plants</th>
<th>Pathogens</th>
<th>Selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>fungal wilting</td>
<td>tomato</td>
<td><em>F. oxysporum</em></td>
<td>fusaric acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>f. sp. <em>lycopersici</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>strawberry</td>
<td><em>F. oxysporum</em></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>f. sp. <em>fragariae</em></td>
<td></td>
</tr>
<tr>
<td>bacterial</td>
<td>tomato</td>
<td><em>P. solanacearum</em></td>
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<tr>
<td>wilting</td>
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</tr>
<tr>
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<td>viral disease</td>
<td>tobacco</td>
<td>tobacco mosaic virus</td>
<td>mosaic symptom</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>appearance</td>
</tr>
</tbody>
</table>
Selection for fungal-wilt resistance

One of the most effective application of this technique to the field of plant pathology is to select toxin resistant cells by culturing plant cells in the presence of toxic compounds. In fact, the in vitro selection for disease resistance was first conducted by Carlson (1973), who isolated tobacco cultures resistant to methionine sulfoximine, a model toxin of tabtoxin produced by Pseudomonas syringae pv. tabaci and produced a resistant line against this disease. Similar selections have been effectively made in some diseases caused by pathogens which produce host-specific toxins (Brettell and Ingram 1979). Since these toxins are determinants of pathogenicity, the toxin-resistant regenerants were expected to be actually resistant to diseases caused by these toxin producers.

There are some plant diseases in which the pathogens produce non-specific toxins to extend the disease symptoms. Fungal wilting caused by Fusarium species is a typical disease included in this class. Low-molecular-weight toxin has been found in culture filtrates of some Fusarium species and implicated as wilting agents in the tomato. It is a pyridine derivative, fusaric acid (5-n-butylicolinic acid), and has been detected in much higher concentrations in plants infected by virulent strains than those inoculated with avirulent strains. In our previous works (Toyoda et al. 1988a; Ouchi et al. 1989), bacteria capable of detoxifying fusaric acid were isolated and tested for their ability to protect tomato plants from the wilting disease caused by the pathogen. The bacteria used were fusaric acid-resistant mutants derived from an avirulent strain of P. solanacearum. Tomato plants were protected from wilt when they were pretreated with fusaric acid-detoxifying bacteria before inoculation with the pathogenic fungus. The role of fusaric acid in symptom development of wilting has been well established. Thus, it was expected that an isolation of fusaric acid resistant lines would contribute to the production of disease tolerant plants. Fusaric acid-resistant cells were isolated from tomato leaf protoplasts (Shahin and Spivey 1986) and leaf-derived callus tissues (Toyoda et al. 1988b) and the regenerants from these tissue cultures were indeed enhanced in disease tolerance.

As pointed out earlier, an in vitro system made it possible to effectively select variants resistant to host-specific toxins and produce resistant lines to the diseases caused by fungal toxin producers. However, it has not been easy to apply such a system to the selection for resistance in diseases caused by soil-borne pathogens, where the mechanisms for pathogenicity or host resistance have not been clarified. Recently, Heath-Pagliuso et al. (1988) reported the successful isolation of plants resistant to soil-borne diseases caused by Fusarium oxysporum f. sp. apii by directly inoculating regenerants with the pathogens. Also in strawberry, the disease caused by the soil-
borne pathogen, *F. oxysporum* f. sp. *fragariae* is a serious problem and the production of resistant lines has been an urgent matter. In a previous paper (Toyoda *et al.* 1990), the author reported an efficient system for plant regeneration from leaf-derived callus tissues of strawberry and a system to select resistant somaclonal variants in this plant. In this section, therefore, the selection of disease resistant strawberry was actullay attempted by transplanting regenerated plants to a pathogen-infested soil (Toyoda *et al.* 1991).

Callus induction and plant regeneration of strawberry (*Fragaria × ananassa* cv. Hoko-Wase, susceptible to the wilt-inducing pathogen, *F. oxysporum* f. sp. *fragariae*) were carried out by the method previously described (Toyoda *et al.* 1990). Namely, young leaves of daughter plants newly developed from runners were surface-sterilized and cultured on Murashige-Skoog (MS) (1962) medium supplemented with 0.1 µg/ml 2, 4-D and 1.0 µg/ml 6-benzylaminopurine for callus induction and subsequent plant regeneration. Three-week-old cultures of *F. oxysporum* f. sp. *fragariae* grown on potato sucrose agar were macerated in water and used as the inoculum. A pathogen-infested soil was produced by mixing the inoculum with soil in a bed (1×2 m, 30 cm–depth). Such a treatment was repeated five times at a one-week interval. For examining the infectivity of the pathogen applied to soil, control strawberry plants from which callus tissues were originally induced were planted in this bed, and the appearance of disease symptoms was examined. In order to ensure the active infection by the pathogen, all of the experiments were carried out in a greenhouse during summer months when the temperature of soil was between 27 and 32°C (Winks and William 1965).

In this study, the experiments were designed to take into account the following two factors; i) establishment of a highly efficient system for plant regeneration from callus tissues, and ii) production of a heavily infested soil with the pathogen for an efficient selection system. The former was useful for supplying numerous plants, and the latter for eliminating misleading plant selection due to infection escape by plants. An efficient regeneration of strawberry callus tissues has been established in our laboratory (Toyoda *et al.* 1990), and therefore the present study focused on an efficient selection of resistant lines using the pathogen-infested soil. For this purpose, it was important to examine the infection condition under which all of susceptible plants were infected. The pathogen was isolated from the test soil using the Komada's selective medium for *F. oxysporum* (Komada 1975). Soil (1 g) was suspended in sterilized water, diluted, and spread on Komada’s selective medium. The populations of the pathogen were between 5 × 10^6 and 10^8 cfu (colony forming units)/g soil, indicating that the levels of the pathogen in the present soil were high enough to induce the infection in plants.

In the pathogen-infested soil prepared in the present study, approximately 80% of
Table 2. Selection of resistant strawberry lines to *F. oxysporum* f. sp. *fragariae* using plants regenerated from leaf-derived callus tissues.

<table>
<thead>
<tr>
<th>Selection1)</th>
<th>No. of Plants tested</th>
<th>No. of Plants</th>
<th>No. of plants forming daughter plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>wilting</td>
<td>forming daughter plants</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>wilting resistant</td>
</tr>
<tr>
<td>1st selection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>38</td>
<td>12</td>
</tr>
<tr>
<td>Regenerants</td>
<td>1225</td>
<td>1042</td>
<td>183</td>
</tr>
<tr>
<td>2nd selection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Isolated lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KHRF-1126</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>KHRF-0228</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

1) Regenerated and control strawberry plants were transplanted to a pathogen-infested soil, and normally growing plants which could produce daughter plants through runner formation were selected as putative resistant lines in the first selection. In the second selection, daughter plants of both isolated lines (third generation since the original regenerants) were directly inoculated with this pathogen and then transplanted to the pathogen-infested soil.

2) Generation of daughter plants in which wilting symptoms were detected.

3) Generation of daughter plants vegetatively produced from plants transplanted to a pathogen-infested soil.

The control plants tested showed typical primary symptoms of the disease (wilting of leaves, reddish brown vascular discoloration) within 2–3 weeks and plants withered one month after planting. The remaining plants which escaped infection developed runners and produced secondary daughter plants (Table 2). In these daughter plants, however, when they were transplanted to infested soil, symptoms appeared more quickly and all of the plants wilted within 2 weeks. These results clearly indicated that the present method would enable us to obtain resistant lines by isolating non-diseased plants normally multiplied in this soil over two generations of daughter plants obtained through runner formation. Using the present isolation system, we attempted to select resistant lines from regenerated plants. Plant regenerants obtained from culture were grown for two weeks in soil (non-infested with the pathogen) in pots in a greenhouse and then the plants were transplanted to the test soil. Two regenerated plants developed runners and produced daughter plants throughout three generation (Table 2). Thus, the authors successfully isolated two putative resistant lines (KHRF-1126 and -0228) from 1,225 regenerants. In order to further confirm the successful propagation of resistance acquired in the original plant regenerants (KHRF-1126 and -0228), the daughter plants (third generation since original regenerants) were tested in
the summer season of the next year. In this experiment, the roots of plants were
dipped in a spore suspension (10^6 spores/ml) of the pathogen and then transplanted to
a pathogen-infested soil. With this inoculation, control plants (50 plants) were infected
and all of the plants died within 3 weeks after planting. These results indicated that
the present inoculation was effective enough to eliminate the infection plant escapes.
With this method, the disease resistance of the selected strawberry lines was
examined. The data showed that the present lines grew normally and developed
runners even after direct inoculation and produced daughter plants through four
generations in a pathogen-infested soil (Table 2). Thus, our present study demonstrat-
ed that the plant disease resistance acquired through tissue culture could be stable
propagated to daughter plants vegetatively, though the mechanisms for disease resis-
tance acquired is not obvious in this study.

Selection for bacterial wilt-resistance

Bacterial wilt disease by *P. solanacearum* is a typical soil-borne-disease of major
crop plants, and causing tremendous damage to crops all over the world. Unfortunate-
ly, few effective resistant lines of tomato have been produced in Japan by convetional
breeding techniques. It is thus urgent to establish the efficient selection and production
of resistant plants. In this section, the author describes the selection for bacterial wilt
resistance in *in vitro* culture of tomato treated with culture filtates of *P. solanacearum,*
and in regenerated plants inoculated with the pathogen (Toyoda *et al.* 1989a).

Tomato callus tissues induced from leaf explants (Toyoda *et al.* 1985c) were at
first exposed to diluted culture filtrates of both the virulent and avirulent strains of *P.
solanacearum*. The toxic effect was detected only when the tissues were treated with
the culture filtrate (VF) of virulent strain, suggesting that some toxic substances were
specifically produced by the virulent bacteria. VF-resistant plants were regenerated
from VF-treated callus tissues. In this experiment, about 2000 callus clumps were
transferred to a selective medium and cultured using the liquid-on-agar method. The
method was our conventional method for examining the toxic effects of applied
substances on callus, because the substances penetrate into the tissues homogeneously
and effectively (Toyoda *et al.* 1984a; 1984b). Most callus clumps became brown within
10–12 days after transfer and the subsequent growth of calli had completely ceased.
However, some of browned clumps proliferated fresh callus clumps (42 of 2021 clumps).
Callus tissues growing in this medium were transferred to the medium for shoot
formation, and then to the medium for root formation. Thus, finally 25 VF-resistant
regenerants (R_1-lines) were successfully obtained from VF-treated callus tissues and
used for subsequent inoculation with a virulent isolate. Fig. 1 shows the time of
Fig. 1. Time course of disease symptom appearance in susceptible tomato plants (A) and bacterial culture filtrate-resistant regenerants (Rf-lines) (B) inoculated with virulent strain (U-10) of *P. solanacearum*.

Results are averages of 25 inoculated plants. Open, slant-lined, and black columns represent leaf yellowing, partial wilting, and complete wilting in inoculated plants, respectively.
appearance of disease symptoms in R₁ and control tomato seedlings inoculated with virulent strain of the pathogen. Inoculated control plants showed a yellowing in partial or whole portions of the lower leaves 4-6 days inoculation and subsequently partial wilting in the upper leaves of seedlings. Consequently, all inoculated plants were completely wilted within 17 days after inoculation (Fig. 1-A). On the other hand, regenerants from VF-resistant calli did not show such symptoms at this early stage of infection, and normally grew to form the first and second fruit clusters 30-40 days after inoculation. However, the plants suddenly showed partial wilting at the later stages of growth and rapidly withered (Fig. 1-B). Fruits formed in R₁-lines were immature and no viable seeds were obtained. The present results suggest that virulent bacteria produce toxic substances at the early stage of infection and cause damage to host plants. Detectable damage was a rapid yellowing of leaves probably due to chlorophyll disintegration by the toxic substances. In non-inoculated control plants, such a phenomenon was not observed at this early stage, though at much later stages (30-50 days after planting) some of these plants showed a leaf yellowing in lower leaves due to senescence.

VF-resistant regenerants were resistant to the attack by virulent bacterial strain U-10, suggesting that the same toxic substances as secreted into the culture medium are produced in planta during the infection process by the virulent strain. Although the detailed functions of these toxic substances remained to be elucidated, the resistance of regenerants to these substances is certainly effective in suppressing or delaying the growth of invaded bacteria.

Virulent strains of this bacteria produce an abundant extrapoly saccharides (EPS) by which vessels of host plants are plugged to cause the wilting (Akiyama et al. 1986). In the present study we did not succeed in separating these toxic substances from EPS, because both were high molecular weight compounds which precipitated with EtOH, eluted in the void volume during Sephadex G-50 gel filtration, and were not dialyzable. Further isolation and characterization of these toxic fractions of VF is under way.

Since in vitro selection by the bacterial culture filtrate was not enough to obtain completely resistant clones against inoculation with virulent bacteria, we attempted to directly isolate disease-resistant plants among inoculated progeny of the regenerants. For this purpose, self-pollinated progeny (R₂-lines) of regenerants derived from non-selected callus tissues were used for inoculation, because the resistance would be detected even if it was recessive. Table 3 shows the segregations of the phenotypes in R₂ lines. Although phenotypical segregations in each group of R₂-lines were too diverse to know how many genes were involved in the expression of complete resistance to bacterial wilting, these results suggest that resistance is controlled by more than one gene.
Table 3. Symptom expression of self-pollinated progenies (R2-lines) of un-selected regenerants derived from leaf callus tissues of tomato after inoculation with virulent strain (U 10) of *P. solanacearum*.

<table>
<thead>
<tr>
<th>R2 lines</th>
<th>No. of inoculated plants</th>
<th>No. of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>LNSR-3</td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>- 8</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>- 9</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>-10</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>-14</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>-16</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>-17</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>-19</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>-21</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>-22</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>- 1</td>
<td>65</td>
<td>58</td>
</tr>
<tr>
<td>- 4</td>
<td>51</td>
<td>45</td>
</tr>
<tr>
<td>-11</td>
<td>31</td>
<td>28</td>
</tr>
<tr>
<td>-13</td>
<td>54</td>
<td>48</td>
</tr>
<tr>
<td>- 5</td>
<td>58</td>
<td>12</td>
</tr>
<tr>
<td>- 6</td>
<td>43</td>
<td>22</td>
</tr>
<tr>
<td>-12</td>
<td>48</td>
<td>16</td>
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<td>35</td>
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<tr>
<td>-20</td>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td>- 2</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>- 7</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>-18</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>control ²</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

¹ Symptoms of inoculated plants were classified into three types; S, rapid wilting within 15 days after inoculation; MR, delayed appearance of wilting 30–40 days after inoculation; HR, no wilting throughout the experiment (3–4 months).

² Susceptible tomato plants from which callus tissues were originally induced.

In the present study, highly resistant plants (LNSR-7) were obtained by inoculating R2 lines with virulent isolate U-10. Therefore, the reliability of the results strongly depend on whether inoculation was successful or not. For confirming the validity of inoculation, we attempted to isolate the bacteria from inoculated LNSR-7. The densities of bacteria detected in LSNR-7 were $2 \times 10^3$, $3 \times 10^4$, and $6 \times 10^9$ cells per gram.
fresh weight of tissue 1, 2, and 3 months after inoculation, respectively. These values were considerably lower comparing with those \((10^2-10^9 \text{ cells/g. fr. wt.)}\) of wilted plants. These results clearly indicate that non-wilting of LNSR-7 was due to the suppression of bacterial growth in xylem tissues of inoculated plants, but not to the failure of inoculation.

**Selection for tobacco mosaic virus resistance**

Resistant plants to viral disease were at first isolated by Murakishi and Carlson (1982), who induced callus tissues from mutagenized haploid tobacco plants and selected tobacco mosaic virus (TMV)-resistant tobacco by inoculating the regenerants with this virus. In viral diseases of plants, the isolation of resistant variants are rather difficult because addition of some toxins to the medium would not enhance the selection pressure. For the practical use of breeding, therefore, it is convenient to establish a system for *in vitro* isolation for viral disease resistant clones. Moreover it has been known that somaclonal variation can be frequently induced in plant tissue cultures even when mutagens are not used (Shepard *et al.* 1980; Evans and Sharp 1983). Actually, we succeeded in isolating bacterial wilt-resistant tomato from non-mutagenized tomato callus cultures. From this point of view, the author will describe in this section an efficient system for isolating tobacco lines resistant to tobacco mosaic virus (Toyoda *et al.* 1989b).

In our laboratory, various callus lines have been isolated from axillary buds of TMV-infected and healthy tobacco plants. One of the callus lines derived from TMV-infected tobacco plants was CMT callus line in which higher level of TMV amounts were constantly maintained during subculturing (Toyoda *et al.* 1985b). CMT callus line was friable and cell-aggregates were easily released by gently shaking the tissues with liquid medium. A microscopic observation of the line showed the frequent formation of inclusion body of TMV in the aggregates, indicating that TMV multiplied and translocated in frolferated callus cells. In this callus line, shoots were effectively differentiated by changing plant hormones added to the medium and differentiated shoots developed small leaflets with the typical mosaic symptom of TMV. The results indicated that TMV was stably multiplied in callus tissues and efficiently translocated to leaflets regenerated from the tissues (Toyoda *et al.* 1985b). These results suggested that if TMV resistance was induced in callus cells, shoots differentiated from these cells would develop healthy leaflets, and that TMV-resistant plants could be easily and effectively isolated by selecting those healthy shoots. In the present experiments, CMT-1 callus tissues were subcultured for several passages in order to enrich the efficiency of somaclonal variation and to enhance multiplication of the variant cells.
We selected healthy shoots (first step for selection) and then transplanted to soil. After 30 days of cultivation, symptomless, healthy regenerants were selected as putative TMV-resistant plants (second step for selection). To confirm TMV resistance of these plants, they were inoculated with TMV (third step for selection). Table 4 shows the numbers of plants selected in each step. In this selection, finally 3 highly resistant (no symptom) (CMT-1R) and 30 moderately resistant (delayed symptom) regenerants were successfully obtained from 105 shoots selected as primary candidates. Chromosome number in root tip cells of TMV-resistant regenerants (CMT-1R03) as assessed at the metaphase was normal, giving rise to $2n = 48$.

For evaluating the effectiveness of this selection, we examined whether TMV resistance acquired in the regenerants would be passed to their progeny. In this experiment, selfed progeny (R2 plants) of highly resistant regenerants (CMT-1R03) were inoculated with TMV and the segregation of resistance and susceptibility was determined. Table 5 shows the numbers of resistant and susceptible plants determined after inoculation with TMV. The data suggest that TMV resistance is probably due to a dominant single gene mutation, and this mutation was heterozygously induced in CMT-1 callus line. In a subsequent study, we also examined the multiplication and translocation of TMV inoculated into CMT-1R03 or control tobacco plants (noncultured R2 plants). Inoculated and non-inoculated leaves were harvested separately one month after inoculation, and subjected to the estimation of TMV concentrations by

### Table 4. Selection of TMV-resistant plants from regenerants of a tobacco callus line, CMT-1.

<table>
<thead>
<tr>
<th>Selection steps$^1$</th>
<th>No. of plants</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diseased</td>
<td>Healthy</td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td>967</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td>14</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>3rd</td>
<td>58</td>
<td>3 (30)$^2$</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ Shoots were differentiated from tobacco (*N. tabacum* cv. Bright Yellow) callus line (CMT-1), and symptomless healthy shoots were selected for TMV resistance (first step). Healthy shoots were grown up to intact plats and cultivated for further 1 month (second step). At the third step, selected regenerants were inoculated with TMV and grown for 3 months till seeds were harvested.

$^2$ Regenerants showing delayed mosaic symptoms (at the flowering stage 3 months after inoculation).
Table 5. Segregation of TMV resistance in selfed progeny of a TMV-resistant line (CMT-1R03).

<table>
<thead>
<tr>
<th>Experiments</th>
<th>No. of selfed progeny inoculated with TMV</th>
<th>Ratio</th>
<th>$x^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Susceptible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>29</td>
<td>14</td>
<td>3 : 1</td>
<td>1.30</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>9</td>
<td>3 : 1</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>18</td>
<td>3 : 1</td>
<td>0.58</td>
</tr>
</tbody>
</table>

![Graph showing TMV (mg/g fr. wt. leaves) for control tobacco (A) and selfed progeny of TMV-resistant regenerant (CMT-1R03).](image)

Fig. 2. Estimated quantity of TMV in control tobacco (A) and selfed progeny of TMV-resistant regenerant (CMT-1R03). Each 31 plants were inoculated with TMV, and inoculated (I) and non-inoculated (N) leaves were harvested separately 1 month after inoculation and used to estimate TMV quantity by quantitative immunoelectrophoresis. TMV amounts in CMT-1R03 were shown separately in 24 symptomless (B1) and 7 symptom-appearing plants (B2).

quantitative immunoelectrophoresis method (Toyoda et al. 1983). In control tobacco plants, all of 31 plants inoculated showed mosaic symptoms first in non-inoculated younger leaves 7-10 days and in whole leaves 20-25 days after inoculation. On the
other hand, about 80% of inoculated R2 plants (24 of 31 plants) did not show any symptom in either inoculated or non-inoculated leaves. In these symptomless R2 plants, the levels of TMV quantity in the inoculated leaves were considerably low and those in non-inoculated leaves were below the limit of detection by this method (Fig. 2). These results indicate that resistant plants did not completely suppress the multiplication of virus in inoculated leaves, but inhibited the translocation of TMV from inoculated to non-inoculated leaves.

**Concluding remarks**

The present studies demonstrate that an efficient selection of somaclonal variations would be useful for the production of disease resistant plants, including those in which toxins could hardly be the agents to give selection pressure. In fact, the bacterial wilt-resistant line LNSR-7 was resistant to the disease under field conditions. R$_2$ plants of this line were grown in a field heavily infested with a different strain (KK -101) of *P. solanacearum*. LNSR-7 also showed the strong resistance against a natural infection of this pathogen, while control, susceptible plants were severely wilted under the same conditions. Thus, this line may be commercially utilized as a new tomato cultivar resistant to the disease.

For producing resistant tomato plants to a fungal wilt disease, an *in vitro* selection for resistance to fusaric acid (wilt-inducing toxin produced by *Fusarium* species) has been described by Shahin and Spivey (1986) and our colleagues (Toyoda et al. 1988b). In our investigation, however, tomato regenerants from fusaric acid-resistant callus lines showed strong resistance to the pathogen at an earlier stage, but not at the later stage of infection (unpublished data). Similar results were also obtained when the authors inoculated the wilt-inducing bacterium, *P. solanacearum*, into tomato regenerants, which were obtained from callus lines resistant to the toxic substances produced by this pathogen. These results may imply that resistance selection to toxic substances is not necessarily effective for producing disease resistant plants, especially when the roles of these substances on the pathogenicity or virulence have not been determined. The present strawberry lines were directly selected from regenerated plants using the dual inoculation method (field infection and root inoculation with the pathogen). Although selection pressure was not made with tissue cultures in the present study, utilization of the pathogen-infested soil reflected the natural infection by the pathogen in the farm and moreover promoted the primary selection for resistant lines. Thus, the lines isolated in this system could be available as new resistant cultivars. The present selection system may be applicable to other soil diseases in which the mechanisms for resistance or pathogenicity have not been clarified.
One of the most important devices in the present system was to isolate the callus line which expressed TMV mosaic symptoms in leaflets when redifferentiated into shoots. This enabled us to easily and effectively isolate TMV-resistance mutation occurred during the callus cultures by selection symptomless shoots, without any selection pressure. Thus, the present method may be widely applicable to the selection of resistant clones against other viral diseases as far as mosaic symptoms appear on host plants.

We reported the application of microinjection technique to the introduction of TMV into single cells (Toyoda et al. 1985a) or cell-aggregates (Toyoda et al. 1986) of callus tissues, and showed that callus cell-aggregates obtained from this resistant line permitted TMV to multiply in injected cells, but inhibited translocation of the virus to adjacent cells of the aggregates, whereas TMV in the cells of parental line multiplied and translocated freely. These data strongly support, at the cellular level, the notion that the suppression of cell-to-cell movement is one of the mechanism of plant resistance against viruses.

References

17–24.
体細胞変異選抜による病害抵抗性植物の育成

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本論では、培養細胞における体細胞変異を選抜し、病害抵抗性植物の育成について論述した。選抜対象には、トマトの萎縮病と青枯病、イチゴの萎縮病およびタバコのTMVをとりあげ、それらの選抜結果を説明した。まず、毒素による選抜例として、トマト萎しょう病菌が産生する萎縮型毒素（フサリン酸）をとりあげた。本菌は感染後植物体内でフサリン酸を産生し、その萎しょう毒性によって病状を導入する。それ故、フサリン酸抵抗性を選抜すれば、病状を抑制し被害程度の軽い個体を供用することができる。実際に選抜されたフサリン酸抵抗性個体については、特に感染の初期に強い耐性が認められた。

トマト萎枯病の場合については病原性株の培養ろ液をトマトカルスに毒性作用をもつ点に着目し、ろ液抵抗性変異細胞の選抜を行った。その結果、ろ液抵抗性個体は青枯病菌の接種に対して発病遅延を引き起こす抵抗性であったが、その抵抗性は完全なものではなく、花房形成期に萎しょう状が出现した。このような状況を克服するため、再生個体に直接病原菌を接種し、世代をとおして抵抗性を示す個体の分離（後代選抜法）を試みた。この実験では、抵抗性変異が強力な形質である可能性を考慮して再生体の自殖後代を用いた。その結果、本法によって果実収穫に至る過程で抵抗性を示す系統が分離された。

イチゴの萎縮病についても後代選抜を行った。本実験では、接種操作を簡略化し、効率的な変異体選抜を検討した。すなわち、イチゴ萎縮病菌を導入した汚染土壌に再分化個体を植え付け、再分化後個体ならびにランナーにおける発病を検討した。本法によれば、元株が病原菌の感染を回避した場合でも、ランナーに形成された新株、あるいはその新株から派生したランナーにおいて抵抗性を検討することが可能であった。その結果、ランナーによる継代を少なくとも4世代繰り返せば誤選抜を完全に回避できることが明らかとなった。このようにして、筆者らの研究室では、約1200の再分化個体から2個の抵抗性系統を分離した。

毒素のような選抜薬剤が存在しない病害の選抜例として、筆者らが行ったウイルス抵抗性選抜がある。宿主植物がウイルス抵抗性を獲得するには、ウイルスの侵入を阻断する、侵入細胞での核酸の複製や蛋白質合成を阻害する、あるいは合成されたウイルスの細胞間移行を阻害する必要がある。これらの機能に変異が誘導されれば、いずれの場合にも、結果的に葉にモザイク状が出現しないので、カルスから無病株の病葉を分離すれば抵抗性変異体を育種レベルで選抜できる。このため、実験にはカルス組織を継代しても細胞内でTMVが非常に安定して増殖できる系統を用いた。この方法で、最終的には3個体の抵抗性選抜した。
抗性個体を分離し、そのうち1系統（CMT-1R03）について、自殖R2後代で再度抵抗性発現の有無を検討した。その結果、抵抗性と罹病性の分離比は、CMT-1R03に生じた抵抗性変異が優性に、しかもヘテロに生じていることを示した。次に、機能的にどのような変異が生じたかを検討するためマイクロインジェクション法でカルス細胞にウイルスを注入したところ、注入した細胞ではウイルスの増殖が確認されたのに対し、隣接する細胞へのウイルス移行やそのでの増殖は観察されなかった。これらの結果から、ウイルスの細胞間移行にかかわる何らかの変異が選抜個体に誘導されたことを強く示唆するものと考えた。
CURRENT STATUS OF GENETIC MODIFICATION OF RICE BY GENE TRANSFER

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(3) International Rice Research Institute, P.O. Box 933 Manila, Philippines

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Rice (Oryza sativa L.) is the most important cereal and a source of calories for about 40% of the world population. More than 90% of rice is produced and consumed in Asia. During the last two decades, dramatic increases in rice production have been witnessed in many developing countries. This has been due to the development and adoption of semi dwarf high yielding fertilizer responsive, disease and insect resistant cultivars with improved quality characteristics. Up to now, the genetic improvement in rice has been achieved mainly through the application of classical Mendelian Genetics and conventional plant breeding methods.

Recent advance in biotechnology particularly in cell culture and molecular biology has opened new avenues in the genetic improvement of crop plants. Some of the exciting developments in rice biotechnology include regeneration of plants from protoplasts of both indica and japonica species, production of transgenic plants, development of molecular (RFLP) map consisting of more than 300 DNA markers, availability of species-specific probes and a series of cloned DNA fragments/genes and transposition of maize transposable element (Ac) into rice. These advances have widened the scope and potentials of DNA transformation in rice. However, protocols for stable DNA transformation and the production of transgenic rice plants are still not very efficient. Reproducibility of plant regeneration from protoplasts is poor. Unlike dicots, number of transgenic plants are quite small. Kinds of genes introduced are limited.

This review summarizes the current status of DNA transformation in rice and its potentials to understand the molecular architecture of rice genome and in the transfer of foreign genes for developing transgenic rices with new genetic properties such as

* This review contains research progresses prior to 1991.
resistance to diseases, insects, tolerance to abiotic stress and improved nutritional quality.

**DNA transformation systems and production of transgenic rice**

In dicotyledonous plant species, *Agrobacterium* mediated gene transfer is routine. Numerous examples are available in such species on successful gene transfer using *Agrobacterium* as a vector (1). Also, protoplast culture is not essential for DNA transformation in dicots and simple co-cultivation techniques, leaf disc transformation method (2) and selectable marker genes encoding for kanamycin resistance have been used extensively in DNA transformation. On the other hand, monocots are difficult to transform with *Agrobacterium*. Only, in a few cases, transformants have been produced (3, 4, 5, 6, 7). In view of the absence of natural vectors in rice, transformation

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Source of protoplasts</th>
<th>No. of plants regenerated</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-58/ms (J), Fujiminori (J)</td>
<td>CS</td>
<td>9</td>
<td>(36, 37)</td>
</tr>
<tr>
<td>Nipponbare (J), Sasanishiki (J)</td>
<td>CS</td>
<td>&gt;50</td>
<td>(38)</td>
</tr>
<tr>
<td>Moroberekan (J)</td>
<td>CS</td>
<td>10</td>
<td>(39)</td>
</tr>
<tr>
<td>Taipei 309 (J), Fujisaka 5 (J)</td>
<td>CS</td>
<td>264</td>
<td>(40)</td>
</tr>
<tr>
<td>Yamahoushi (J)</td>
<td>CS</td>
<td>15</td>
<td>(41)</td>
</tr>
<tr>
<td>Norin 8 (J), Nipponbare (J)</td>
<td>CS</td>
<td>300</td>
<td>(42)</td>
</tr>
<tr>
<td>Iwaimochi (J), Koshihikari (J)</td>
<td>Ca, CS</td>
<td>126</td>
<td>(43)</td>
</tr>
<tr>
<td>Norin 8 (J), Nipponbare (J)</td>
<td>Ca</td>
<td>118</td>
<td>(44)</td>
</tr>
<tr>
<td>Fujisaka 5 (J), Iwaimochi (J)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norin 8 (J)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chyokoto (I), Chinsurah Boro II (I)</td>
<td>CS</td>
<td>—</td>
<td>(45)</td>
</tr>
<tr>
<td>Irat 109 (J), 63-83 (J)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-42 (J), 258 (J)</td>
<td>CS</td>
<td>8</td>
<td>(46)</td>
</tr>
<tr>
<td>Akitakomachi (J), Nipponbare (J)</td>
<td>CS</td>
<td>—</td>
<td>(47)</td>
</tr>
<tr>
<td>Blue Bonnet (J), Pantiange-Heigu (J)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1-Breeding line (I), Cl-CMS (J)</td>
<td>CS</td>
<td>—</td>
<td>(48)</td>
</tr>
<tr>
<td>Gulfmot* (I), M201* (J)</td>
<td>Ca, CS</td>
<td>158</td>
<td>(49)</td>
</tr>
<tr>
<td>IR54 (I)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nipponbare (J), Taipei 309 (J)</td>
<td>CS</td>
<td>141</td>
<td>(50)</td>
</tr>
<tr>
<td>Chinsurah Boro II (I)</td>
<td>CS</td>
<td>100</td>
<td>(51)</td>
</tr>
<tr>
<td>Taipei 177 (J)</td>
<td>CS</td>
<td>32</td>
<td>(52)</td>
</tr>
</tbody>
</table>

*J=* japonica, *I=* indica, *CS=* cell suspension, *Ca=* callus
* = green shoots
Table 2. Summary of reported instances of foreign gene expression in rice cells and production of transgenic plants.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Sources of protoplasts/materials</th>
<th>DNA transfer methods</th>
<th>Genes transferred</th>
<th>Gene expression observed in</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tissue</td>
<td>Regenerated plants</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Progeny</td>
</tr>
<tr>
<td>C5924 (J)</td>
<td>CS</td>
<td>PEG</td>
<td>NPT II</td>
<td>+</td>
<td>(8)</td>
</tr>
<tr>
<td>Tai-nung 67 (J)</td>
<td>Leaf</td>
<td>Electroporation</td>
<td>CAT</td>
<td>Protoplast extract (48h)</td>
<td>(53)</td>
</tr>
<tr>
<td>C5924 (J)</td>
<td>CS</td>
<td><em>Agrobacterium</em></td>
<td>spheroplasts+PEG</td>
<td>+</td>
<td>(26)</td>
</tr>
<tr>
<td>Yamahoushi (J)</td>
<td>CS</td>
<td>Electroporation</td>
<td>NPT II</td>
<td>+</td>
<td>(15)</td>
</tr>
<tr>
<td>Taipei 309 (J), P4 (J)</td>
<td>CS</td>
<td>PEG</td>
<td>GUS</td>
<td>+</td>
<td>(9)</td>
</tr>
<tr>
<td>Taipei 309 (J)</td>
<td>CS</td>
<td>Electroporation</td>
<td>NPT II</td>
<td>+</td>
<td>(16)</td>
</tr>
<tr>
<td>Taipei 309 (J)</td>
<td>CS</td>
<td>Microparticle</td>
<td>GUS</td>
<td>48hr after</td>
<td>(23)</td>
</tr>
<tr>
<td>Fujiwara (J)</td>
<td>CS</td>
<td>Pollen tube</td>
<td>NPT II</td>
<td>+</td>
<td>(25)</td>
</tr>
<tr>
<td>Nipponbare (J)</td>
<td>CS</td>
<td>Electroporation</td>
<td>HPH</td>
<td>+</td>
<td>(18)/(31)</td>
</tr>
<tr>
<td>Yamaha (J)</td>
<td>CS</td>
<td>Electroporation</td>
<td>HPH, GUS</td>
<td>+</td>
<td>(17)</td>
</tr>
<tr>
<td>M101 (J)</td>
<td>2-3 day old seedlings</td>
<td>Microparticle</td>
<td>phy</td>
<td>&lt;24hr after bombardment</td>
<td>(24)</td>
</tr>
<tr>
<td>Taipei 309 (J)</td>
<td>CS</td>
<td>PEG</td>
<td>HPH</td>
<td>+</td>
<td>(11)</td>
</tr>
<tr>
<td>Nipponbare (J)</td>
<td>Wounded embryo</td>
<td><em>Agrobacterium</em></td>
<td>(Ti plasmid)</td>
<td>+</td>
<td>(27)</td>
</tr>
<tr>
<td>Fujiwara (J)</td>
<td>6-7 day old seedlings</td>
<td><em>Agrobacterium</em></td>
<td>rhizogenes</td>
<td>+</td>
<td>(28)</td>
</tr>
<tr>
<td>Taipei 309 (J)</td>
<td>CS</td>
<td>PEG</td>
<td>HPH</td>
<td>+</td>
<td>(12)</td>
</tr>
<tr>
<td>IR 54 (J)</td>
<td>CS</td>
<td>PEG</td>
<td>NPT II</td>
<td>+</td>
<td>(13)</td>
</tr>
<tr>
<td>Chinsurah Boro II (J)</td>
<td>CS</td>
<td>PEG</td>
<td>HPH</td>
<td>+</td>
<td>(14)</td>
</tr>
<tr>
<td>Taipei 309 (J)</td>
<td>CS</td>
<td>Electroporation</td>
<td>NPT II, GUS</td>
<td>+</td>
<td>(19)</td>
</tr>
<tr>
<td>Nipponbare (J)</td>
<td>CS</td>
<td>Electroporation</td>
<td>HPH, GUS</td>
<td>+</td>
<td>(34)</td>
</tr>
</tbody>
</table>


has been achieved mainly through protoplasts using direct DNA transfer methods.

During the last 5 years, about 15 reports have become available on regeneration of plants from protoplasts of both japonica and indica rices (Table 1). In addition, over 10 cases, protoplasts have been successfully used to produce transgenic rice (Table 2).
The expression of foreign gene(s) (marker genes) has been observed both at callus and plant levels however, only a limited information is available on the inheritance of foreign gene to the progenies. Some of the methods used for DNA transformation in rice are discussed below:

1. Direct DNA transfer methods

A. Protoplast mediated DNA transfer

Since the first report in 1985 on plant regeneration from protoplasts of japonica rice, several laboratories have successfully regenerated plants from protoplasts of both japonica and indica rice. This has further led to the transformation and production of transgenic rices. Two methods, PEG and electroporation have been commonly used. Parameters employed for such experiments are summarized in Table 3.

(a) PEG methods

Polyethylene glycol (PEG) has been used in delivering DNA into rice protoplasts (8, 9, 10, 11, 12, 13, 14). In general, the method consists of incubating protoplasts, DNA and PEG together, followed by selection of transformants on the selective media. Uchimiya et al. (8) incubated protoplasts (about $5 \times 10^6$) suspended in 0.6 M mannitol with 0.25 ml DNA solution (10μg of the circular form of the bacterial plasmid pCT2T3). The pCT2T3 consisted of a derivative of pGV1122 and a chimeric gene composed of the nopaline synthase promoter, the neomycin phosphotransferase II (NPT II) structural gene from transposon Tn5, and the terminator region from cauliflower mosaic virus (CaMV) DNA. The culture of DNA treated protoplasts was followed by two successive selection by kanamycin-containing medium. The transformation at the rate of 1.5% was recorded.

Zhang and Wu (9) obtained transgenic rice plants after incubation of protoplasts of japonica cultivar with plasmid DNA using PEG method. The plasmid contained the β-glucuronidase (GUS) structural gene and intron 1 of the maize alcohol dehydrogenase (Adh-1) gene and the 3' non-coding region of the nopaline synthase gene. Out of 378 regenerated plants, at least 86 were transgenic as determined by DNA hybridization analysis.

Li et al. (12) used PEG method and produced 628 transgenic rice plants in Nipponbare and Taipei 309. About two thirds were found to have viable seeds. Peng et al. (13) produced transgenic cell lines in indica rice (IR 54) using PEG method. Datta et al. (14) obtained transgenic plants after PEG treatment of protoplasts of indica rice variety Chinsurah Boro II. Plants regenerated from hygromycin-resistant clones grew to maturity and set seeds.

(b) Electroporation

Electroporation involves the application of high-voltage electrical pulses to a
Table 3. Comparisons of parameters employed in DNA transformation in rice.

(a) PEG method

<table>
<thead>
<tr>
<th>Genes transferred</th>
<th>Conditions</th>
<th>Selection</th>
<th>References</th>
</tr>
</thead>
</table>
| NPT II            | 0.9ml protoplasts (5×10⁶)  
                   | + 0.25ml plasmid DNA (10μg)  
                   | + 0.5ml 40% PEG 6000  
                   | Km (100μg/ml) | (8) |
| GUS               | 10⁶ protoplasts, plasmid DNA (10μg), calf thymus DNA (50μg/ml)  
                   | + equal volume of 38% PEG 4000 | Km (25μg/ml) | (9) |
| HPH               | 0.4ml protoplasts (1.5×10⁶)  
                   | + plasmid DNA (6μg), calf thymus DNA (28μg)  
                   | + 0.4ml 40% PEG 6000 | Hm (95μM) | (14) |
| NPT II            | 1ml protoplasts (1~2×10⁷)  
                   | + 100μl plasmid DNA (100μg)  
                   | + 1.1ml 40% PEG (M.W. = 8000) | Km (100μg/ml) | (13) |
| HPH               | 0.25ml protoplasts (4×10⁶)  
                   | + plasmid DNA (10μg)  
                   | + 0.25ml 40% PEG (M.W. = 8000) | Hm (20μg/ml) | (11) |

(b) Electroporation

<table>
<thead>
<tr>
<th>Genes transferred</th>
<th>Conditions</th>
<th>Selection</th>
<th>References</th>
</tr>
</thead>
</table>
| NPT II            | 0.5mM MES  
                   | 7mM KCl  
                   | 4mM CaCl₂  
                   | 6.5% mannitol (pH 5.8)  
                   | 750 V/cm  
                   | 22μFD  
                   | 4 nsec  
                   | (or)  
                   | 500 V/cm  
                   | 125μFD  
                   | 20 nsec | G418 (20μg/ml) | (15) |
| NPT II            | 0.8g/l NaCl  
                   | 0.02g/l KCl  
                   | 0.02g/l KH₂PO₄  
                   | 100g/l glucose (pH 7.1)  
                   | 2000 V/cm  
                   | 40μFD  
                   | 68μsec  
                   | (3 times)  
                   | Km (100μg/ml) | (16) |
| GUS               | 0.1% MES  
                   | 70mM KCl  
                   | 5mM MgCl₂  
                   | 0.4M mannitol (pH 5.8)  
                   | 300 V/cm  
                   | 800μFD  
                   | 10 nsec | Hm (20μg/ml) | (18) |
| HPH               | 0.5M MES  
                   | 70mM KCl  
                   | 5mM MgCl₂  
                   | 0.4M mannitol (pH 5.8)  
                   | 300 V/cm  
                   | 800μFD  
                   | 10 nsec | Hm (20μg/ml) | (18) |

Abbreviations: NPT II : neomycin phosphotransferase II, GUS : β-glucuronidase, HPH : hygromycin phosphotransferase, Km : kanamycin sulfate, Hm : hygromycin B
solution containing a mixture of protoplasts and foreign DNA. Transformation has been achieved through electroporation of protoplasts in several laboratories.

Toriyama et al. (15) used protoplasts of japonica cultivar Yamahoushi and circular form of plasmid DNAs. Electroporated-protoplasts were cultured in B5 medium supplemented with 2 mg/l 2,4-D and 5% mannitol. One month after electroporation, surviving microcalli were transferred to NO3 medium containing 20 mg/l G418 sulfate and 1% agarose. The plating efficiency of electroporated protoplasts was about 0.5% after three weeks. The dividing cells were selected by incubation in G418 containing medium for 2 weeks. Calli larger than 1 mm in diameter were transferred to regeneration medium lacking G418. Several green plants were regenerated from nineteen G418 resistant colonies. These plants were not bleached in the presence of 50 mg/l kanamycin sulfate. The NPT II activity was detected in the leaf extracts of transformed plants. The use of G418 instead of kanamycin is more effective for selecting transformants in rice, because many reverted calli were observed after selection by kanamycin.

Successful transformation studies in rice have been also presented in other laboratories. Using electroporation method, Zhang et al. (16) reported regeneration of transgenic rice plants by somatic embryogenesis from cell suspension derived protoplasts. They used plasmid carrying NPT II gene under control of CaMV 35S promoter. They also emphasized that heat shock treatment of protoplasts prior to electroporation maximized the recovery of kanamycin resistant colonies. Furthermore, essential requirement of omission of kanamycin from the medium for plant regeneration was pointed out.

Matsuki et al. (17) used electroporation and obtained 805 hygromycin-resistant calli from which 26 plants were obtained. Two plants expressed the foreign gene (GUS activity). Shimamoto et al. (18) was successful to regenerate rice plants from protoplasts expressing hygromycin resistance, and GUS activities. They employed electroporation method to introduce foreign genes resided in two different plasmids into rice protoplasts. The non-selectable gene encoding β-glucuronidase was also transferred with the hygromycin phosphotransferase (HPH) gene and its expression was detected in the progeny of stable transformants.

Battraw and Hall (19) produced transgenic plants through electroporation. The protoplasts were electroporated with pNEOGUS 15, a DNA construct carrying two chimeric genes encoding NPT II and GUS.

Transposable elements from maize have been transferred into rice cells. Shimamoto et al. demonstrated the active transformation of maize transposons Activator (Ac)/Dissociation (Ds) (personal communication). Vectors were constructed in which Ac or Ds was inserted in the untranslated leader sequence of the HPH gene.
Upon excision of the elements, the functional HPH gene was restored resulting in the production of hygromycin resistance rice cells. The results indicated that the autonomous Ac element transposes in rice cells and the non-autonomous Ds element is trans-activated by the Ac or CaMV 35S-Ac co-transformed with the Ds. Recently, Murai et al. also observed transposition of the maize Ac elements in transgenic rice (personal communication). Analysis of DNA sequences at 14 empty donor sites indicated that Ac element was excised in rice in a similar manner as maize. The results show that Ac element can be used as an effective heterologous transposon for mutagenesis and gene tagging in rice.

(c) Microprojectile bombardment

Small high density particles are accelerated to high velocity by a particle gun apparatus. These particles penetrate plant cell wall and membrane, and carry foreign DNA into the host cells (20). Transformed cell lines have been obtained through microprojectile bombardment in maize (21) and transgenic plants in soybean (22). Wang et al. (23) bombarded cultured cells of rice with accelerated tungsten particles coated with plasmid containing a β-glucuronidase gene as the reporter. After two days of bombardment, blue transformed cells were detected as an indicative of GUS expression. Bruce et al. (24) also used this method to develop a rapid transient expression assay for the study of DNA sequences involved in the phytochrome-regulated expression of oat phytochrome genes. The 5'-flanking sequence and part of the structural region of an oat phy gene fused to a reporter coding sequence was introduced into intact dark grown seedlings by using high-velocity microprojectiles. The expression was observed after 24th of bombardment.

(d) Pollen tube pathway

A simple method for transformation of rice via pollen tube pathway has been described by Luo and Wu (25). The method involved putting a small amount of a solution of the plasmid DNA, conferring kanamycin resistance, at the cut end of florets using a capillary tube. Usually the florets whose glumes have just opened completely were used for DNA treatment. Seeds from DNA treated florets were collected. Both roots and leaves from the germinated seeds were analyzed for the presence of foreign genes. Of the 259 rice florets, 54 seeds were obtained of which 10 seeds gave DNA hybridization signals to probe DNA. Thus about 20% of the seeds appeared to be transgenic (i.e. 4% of the DNA treated florets). However, inheritance of inserted genes in the progenies has not been shown in this experiment. Although the method is very simple and quite attractive but could not be reproduced in subsequent experiments.

2. Agrobacterium mediated DNA transfer

Agrobacterium has become important DNA vector for transformation in dicot
plant species and is thus used as routine. However, only limited instances of transformation have been reported in some monocotyledonous plant species including the families, Liliaceae (3), Amaryllidaceae (4), Iridaceae (5), Dioscorea (6) and Gramineae (7).

Baba et al. (26) reported the transformation of rice protoplasts by fusing these with Agrobacterium spheroplasts possessing the wild type Ti plasmid by PEG. Protoplasts and A. tumefaciens spheroplasts were mixed in a ratio of 1,000 (spheroplasts/protoplasts) and PEG 4000 was added. The transformed calli capable of growing on a hormone-free medium and of opine synthesis were obtained.

Most recently, Raineri et al. (27) reported Agrobacterium transformation in embryo-derived cultures of two japonica rice cultivars (Nipponbare, Fujisaka 5). Four to five days post germination, the embryos were excised, wounded in the scutellar region and immersed in a culture of preincubated bacteria for 2-3 min with gentle shaking. After a 48 hour co-cultivation on the medium, embryos were transferred onto the same medium supplemented with 200 mg/l cefotaxime and 200 mg/l carbenicillin to kill the bacteria and cultured at 27°C in the dark. Cells transformed were confirmed by drug resistance, tumorigenic growth, opine production, and GUS activity. The transformation is dependent upon Ti-plasmid encoded virulence genes. Hesgans et al. (28) could also obtain transformation through inoculation of young seedlings with Agrobacterium rhizogenes strain LBH 90402.

3. Fate of foreign genes and inheritance

It has been presented the evidence that foreign DNA must be fragmented before the integration into host rice genome (29, 30). Thus, the larger the plasmid size is constructed, the harder the integration of a desired gene would be observed. In order to avoid such problems, co-transformation of two genes located in different vectors demonstrated practical value (13, 18). In view of manner of integration of foreign DNA in rice genome, several information are now available.

Toriyama et al. (15) presented the data of DNA blot analysis of the transgenic plants using the chimeric NPT II gene as a probe. The probe hybridized to a 2.7 kbp fragment of EcoRI/HindIII-digested DNA, which corresponds to the intact chimeric NPT II gene of plasmid pCN. A single or a few copy of DNA were considered to be inserted into host genome of each plant. Zhang and Wu (9) showed that the copy number of GUS gene ranged from one to 10 per cell with majority of plants having two copies per cell. On the basis of GUS activity in the calli, 61 out of 378 plants were transformants. In some transgenic plants, the GUS activity was enhanced 5 to 6 fold under anaerobic conditions in the roots like the maize Adh-1 gene.

In order to examine the possibility of gene amplification and to look at the stability of foreign DNA integrated into rice genome, the following analysis was made
by Morota and Uchimiya (29). The callus was grown in the presence or absence of kanamycin sulfate in the medium for 1 week and then transferred into fresh medium of the same conditions. This subculture was carried out for 3 successive transfers. DNA extracted from one of transformed cell lines indicated that the same copy number of foreign DNA was consistently maintained.

Informations concerning the manner of inheritance of inserted genes in transgenic plants have not been available until recent time. In this respect, Shimamoto et al. (18) presented genetic data with respect to segregation of the hygromycin resistant gene in the progenies of transgenic rice plants. The copy number of integrated hph gene was estimated to be 2-10 per cell. Demonstrated evidence showed that inserted genes segregated as a Mendelian trait, which was proven by the progeny analysis. Co-transformation of a non-selectable gene with a selectable marker was demonstrated. Further instances, also demonstrate the stable inheritance of kanamycin resistant gene into the successive generation (Uchimiya et al. unpublished results). Battraw and Hall (19) found one or two copies of the CaMV 35S promoter/GUS genes in transgenic plants. Hayashimoto et al. (11) analyzed several hygromycin-resistant cell lines and transgenic plants and found that 1 to 10 copies of transferred DNA were integrated at 1 to 4 loci of the rice genome. Further Southern DNA analysis indicates that the introduced plasmid DNA could form concatamers by intermolecular recombination prior to integration. Li et al. (12) analyzed selfed progenies of 29 independent transgenic plants and found that hygromycin resistance gene was integrated at one or two loci.

Peng et al. (13) studied co-transformation of indica rice (IR54) protoplasts with GUS and NPT II genes. GUS assay was made on 104 kanamycin-resistant cell lines. The efficiency of co-transformation was generally 20-30% ; the frequency of kanamycin-resistant calli having both the NPT II and GUS active genes. Southern blot analysis using a probe for GUS indicated integration of several copies of the gene, often as head of tail tandem repeats. There was no clear relationship between foreign gene copy number and the gene (GUS) product.

Datta et al. (14) analyzed foreign gene expression in transgenic plants of indica rice. The presence of transforming DNA and resistance to hygromycin B were complemented by enzyme assay that the gene is functional ; specific phosphorylation of the antibiotic was observed using protein extracts prepared from transgenic plants. Thirty one seeds derived from 5 primary transgenic plants were all hygromycin resistant. The lack of the segregation of the integration pattern of foreign DNA in the progeny indicated that the primary transformant is homozygous.
4. Regulation of foreign gene expression in transformed rice

During the past five years, various genes coding for protein of rice have been cloned in the form of cDNA as well as genomic DNA (Table 4). Extensive studies on the molecular characterization of isolated genes of rice is being carried out. Prior to manipulation of cloned genes, it is needed to investigate the regulatory mechanism influencing foreign gene expression in rice. Thus, experiments on the gene expression by promoters and introns have been presented.

Using transgenic rice plants possessing CaMV 35S–GUS fusion gene, Terada and Shimamoto (31) demonstrated that the level of expression of CaMV 35S promoter in rice was similar to that in tobacco. Matsuki et al. (17) presented the evidence that tissue-specific expression of the rolC promoter of Ri plasmid was the same in both monocots (rice) and dicots (tobacco). Such instances clearly demonstrate that universal existence of basic transcriptional regulatory factors in both monocots and dicots.

Furthermore, McElroy et al. (32) showed that 5'intron of the rice actin 1 gene (Act 1) is essential for efficient foreign gene expression from the Act 1 promoter in transformed cells derived from rice protoplasts.

<table>
<thead>
<tr>
<th>Genes</th>
<th>References</th>
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<tbody>
<tr>
<td>Storage protein</td>
<td></td>
</tr>
<tr>
<td>Glutelin</td>
<td>54</td>
</tr>
<tr>
<td>Prolamin 10 K</td>
<td>55</td>
</tr>
<tr>
<td>Prolamin 16 K</td>
<td>56</td>
</tr>
<tr>
<td>Oryzacystatin</td>
<td>57</td>
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<tr>
<td>Lectin</td>
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<td>Phytohormone inducible protein</td>
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<tr>
<td>α-amylase</td>
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<tr>
<td>Abscisic acid inducible</td>
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<tr>
<td>Photo-regulated protein</td>
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<tr>
<td>Phytochrome</td>
<td>62</td>
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<tr>
<td>Ribulose-1, 5-biphosphate</td>
<td>63, 64</td>
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<tr>
<td>Carboxylase</td>
<td></td>
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<tr>
<td>Others</td>
<td></td>
</tr>
<tr>
<td>Glutamine synthase</td>
<td>65</td>
</tr>
<tr>
<td>ADP glucose pyrophosphorylase</td>
<td>66</td>
</tr>
<tr>
<td>H3 histone</td>
<td>67</td>
</tr>
<tr>
<td>Actin (Act–1)</td>
<td>32</td>
</tr>
<tr>
<td>Gαs 5</td>
<td>68</td>
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<tr>
<td>Sal T</td>
<td>69</td>
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</tbody>
</table>
Dekeyser et al. (33) compared transient expression driven by constitutive and regulated promoters in rice protoplasts and found that 2' promoter of octopine T-DNA is approximately 3 to 4 times more efficient than the CaMV 35S promoter, 10 times more efficient than the nos promoter and the 1'promoter and more than 100 times better than the two other regulated plant promoters. Kyozuka et al. (34) studied the effect of the promoter and intron 1 of the maize Adh-1 gene on the expression of GUS of E. coli in rice. The Adh-1 promoter poorly expressed the GUS gene in protoplasts, however, addition of intron 1 in the untranslated leader sequence of the chimeric constructs increased the activity 11-18 fold. The stimulating effect of intron was also noticed with CaMV 35S promoter. Peterhans et al. (70) reported an accurate processing of the phaseolin intron in transformed rice cells. Thus, the appropriate combination of promoters, enhancers, and intron sequences must be considered in order to achieve highest rate of foreign gene expression in transformed cells.

5. Perspectives

During the last 5 years, dramatic progress has been made in protoplast culture and DNA transformation of rice. Plant regeneration from protoplasts of both japonica and indica rices has been achieved in 15 laboratories, furthermore, in another 10-15 cases transgenic rices have also been produced. Various genes have been cloned in the form of cDNA as well as genomic DNA, comprehensive RFLP genetic map has become available and transposition of maize transposable element (Ac) into rice has also recently been demonstrated. However, protoplast regeneration and DNA transformation systems in rice are still not very efficient. Plant regeneration from protoplasts is often limited to only specific genotypes and the results are often non-reproducible. The cell suspensions often lose regeneration potential. Hence, to incorporate desirable genes into rice plants, it is essential to have efficient plant regeneration systems applicable to a wide range of elite germplasm. Also, there is a need to have cloned genes governing useful agronomic traits such as disease and insect resistance, salinity and draught tolerance, and improved nutritional quality, etc. Some encouraging reports have become available where embryogenic cell lines could be cryopreserved and regenerated into mature plants. Meijer et al. isolated protoplasts from cryopreserved Taipei 309 cell line which upon culturing regenerated into mature plants (personal communication). A transformed cell line could also be cryopreserved, which retained the hygromycin resistance and regenerative capacity of the original cell line.

The availability of useful genes such as Bt gene(s) from Bacillus thuringiensis for insect tolerance, herbicide tolerance, virus coat protein genes could be important to develop transgenic rice germplasm with useful genetic properties. Several storage protein genes have been inserted to different host plants. Such instances could
encourage the attempts to transfer the seed storage protein genes into rice in the aim of improving nutritional quality. Map based gene cloning has become possible after the development of a comprehensive RFLP map. Insertion of transposons into rice would result into the tagging of genes for which mRNA or gene products are not known. Furthermore, the combination of organ specific promoters with target genes would enhance the expression of cloned genes in specific tissues of rice plants.

In spite of the extensive information available in molecular biology on the use of organ specific promoters, yet very little is known about the mechanism of gene expression particularly how to switch the genes on and off. In this respect, the characterization of DNA binding protein from higher plants may facilitate our knowledge concerning regulatory factors in the transcriptional level (35). Isolation of new genes and their introduction into rice plants would accelerate our understanding of regulatory mechanism of gene expression in rice.

Acknowledgments

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References

総合討論

座長 田野茂光（東京大）

座長：昨日から今日にかけて非常に広い範囲に渡ってお話がありましたが、これをひとつつくってディスカッションすることは不可能に近いと思われます。そこで、「イネの突然変異とDNAの解析」、「タンパク質レベルの話」と「培養技術・細胞融合」に分けて、順序をおってディスカッションしてもらいたいと思います。

それでは、DNAレベルの突然変異の解析について何か質問がございましたらどうぞ。

鴨飼（農環研）：矢頭さんにお伺いします。waxyの突然変異で中性子照射由来のものは特にDNAの欠失があるということですが、それはDNAのベースペアの数でいえば、トウモロコシで既に知られている場合の欠失とコンパラティブであるのか。それから、熱中性子の場合は放射線のイオントラックの周辺にどの程度の半径でイオン化が起こるのか。昔Leaがやった計算があると思うのですか。そういう半径とだいたい対応するものかどうかというような検討をされれば幸いでしょうか。

矢頭：お答えにならないと思いますが、それについてはまだデータが十分でないということでお、私の実験を含めていろいろなレポートを見ても、線源とかミュータゲン特異的な傾向を判断する材料がまだ無いということがあります。また、中性子の問題でも初期の物理的な過程に関してはいろいろな仕事がありますが、まだそこまで言うデータはありません。私は今のところ中性子によるミュータントを3つ持っていますが、1つは完全欠失で他の2つはそんなに大きな欠失はありません。初期のLETについてはまだ検討していませんが、いずれにしても数kbpのDNAの範囲をカバーするものではないだろうと思います。

島田（石川農短大）：他の中お話しとも関係あるのですが、放射線による変異と培養による変異でどのような違いがあるのかということを、皆さんはどうお考えでしょうか。例えばイネの場合ですと、培養による変異ではDNAの大きな変異がすごい頻度で起こっているということですが、放射線では同じようなデータがあるのかどうか、お話しの中ではあまり変化がなかったように思ったのですが。全然違う実験でクローンも違うのですが、放射線で起こった突然変異と培養によって起こった変異とでは随分違うような気がします。そののところを、イスの場合やガンマ線照射と培養技術を利用した場合でもどのように考えられているのかお聞きしたいと思います。

矢頭：私がいくつか調べた範囲では、DNAのポリソルフィズムは放射線照射後代ではあまり出てきません。実は昨日お見せしたデータ以外にも多少あるのですが、そんなに多くはありません。助漬さんたちのデータやドイツのグループが研究したデータを読んで少し驚いているのが現状です。むしろ助漬さんに教えて頂きたいのですが、例えばドイツのグループはwaxy遺伝子とかAdh遺伝子でポリソルフィズムを見ている訳ですが、それとその遺伝子座での表現型の関係はどうなっているのでしょうか。
助談：皆さんのに興味のある部分はこの一点に集中するのではないかと思いますが、ガンマ線・中性子線による数々の変異と化学的な薬剤による変異と培養の変異で、出てきたミュータントが同じものかどうかというのはまだ全く検定していないので分かりません。ガンマ線を照射したもののにはDNAの変異が少ないということので、私としてはどちらの方がちょっと驚きます。表現型で見ると、ガンマ線というのは強いダメージを受けて表現タイプが劣悪な形質が構築出てくると思います。それに対して培養変異というのは、極端に劣悪な変異が出るのではなくて結構ゆるやかな彷徨変異というか、段々と変異するような感じで出てきます。また、ドイツMax-PlanckのDr. Brownの実験のwaxy等を使って、それに関する表現型の変異を見てみましたが結果ははいませんでした。DNAレベルでは変異しているのですが、表現型ではその変異はないとしております。

喜多村：私の紹介したミュータントはほとんど欠失型のミュータントですけれども、小松田さんがやられたように、本来ダイズにないような26kDaのタンパクが付加するような変異が培養で出てくるとすれば、優性に遺伝する可能性もあるし非常におもしろいのではないかと思っています。

それに関連して、豊田先生のご発表の中で培養によって優性の病害抵抗性変異が出ていますが、そういうことが本当に培養で起こるのかどうか。中性子でもイネの病害抵抗性等が出ているようですが、その辺のメカニズムをどのように考えたら良いかということが疑問として残ってるのです。

豊田：放射線の照射で誘導される変異と我々が実際に細胞選抜で拾っている変異とはてですが、変異が誘導されるメカニズムについては良く分かりません。我々が拾ってきた、特に病害抵抗性については、タバスコの移行阻害型のような系はむしろクリアーカットな系で解析し易かったのですが、それ以外の病原菌は実際の感染の場面もはっきり分からないケースが多いのが実状です。結果的には抵抗性なんですが、どういった変異が積み重なった状態でそういう結果が得られるのかよく分からない次第です。ですから今この質問に対しましては、これからかなり頑張らないと分からないとしか言えません。むしろ私共の方で示唆を頂きたいというのが正直な印象です。

岸上（植工研）：培養によりますと、再分化する植物では3倍体とか4倍体とか倍数性が増加する再分化個体が出来てくる傾向があると思いますが、放射線の場合にはそういうもののが少ないのではないかという感じを持っております。このことが、培養による変異と放射線をかけた場合の変異でDNAレベルの変化の程度が違うということと関連があるかどうかをお聞きしたいと思います。

助談：培養過程の異数化とDNAの関連ですが、必ずしもDNAレベルの変化が多つかといって異数体・倍数化・トリプロイド・トリトラプロイド・トリソミックが増えるとは考えられなくて、そういう現象というのは細胞同士のカルス継代中の融合とか、そういうところが原因だと思います。そのこととDNAレベルの変異とは関係ないと思いますが、はっきりとは分かりません。

矢田：放射線の方で言えば、放射線や化学物質の初期作用過程というのは、遺伝子に対するものも染色体に対するものも、ヒットの具合が大きいか小さいか、あるいは近傍で2
つのイベントが同時に1回以上起こったかあるいは起こっていないか、ということくらいの非常に物理的な作用だと思います。しかし、それがどのように生物の細胞の中で修復されるかということで多少違ってくると思います。また、遺伝子の中での作用も染色体切片への作用もかなり似た作用ではないかと考えております。ただ現状の頻度は、線量とか線量率によって多少変わってくるのではないかと思っております。実際に線量応応などを取ってみますと、一応結果としてみれば線量に応じた出現頻度の差というのは出ているかもしれません。その程度の事しか言えません。

座長：次にタンパクレベルの一方の話に移りたいと思います。喜多村さんと小松田さんに何か質問はございますでしょうか。

菊池（筑波大）：喜多村先生にお聞きしますが、7Sタンパク質のサブニュートの変異体は、α'は完全欠失型で、α・βは低下型ということですが、全ての完全欠失型というのは作れる可能性があるかどうか。また、α・βというのは同時に作用しているのか、あるいは個別に欠失型を作ることができるのがどうかという可能性について。それから、低下型の場合は他の形質に影響しないということですが、α'・α・β全部の完全欠失型が出来た場合にはどうなるのか。

また、7Sと11Sの関係においてタンパク質の含有量が相対的には変わらないということですが、これはどういうことで起こるか、その関係について非常に重要な問題だろうと思いますのでお聞きしたいと思います。

喜多村：ダイズのタンパク質は大きく分けて7Sと11Sとあると訳ですが、7Sを増やすとか11Sを増やすとか、そういう極限ができるかという質問だと思います。イエスとかノーとかはっきりとは言えませんが、例えばマメ類をダイズの種を越えて見てみると、インゲンマメの仲間は11Sタンパク質に相当するものがほとんどない訳です。またブラジルナッツのように11Sが非常に多いものもありますので、可能性はあるだろうと考えています。

取ってきた変異が大きな変異の場合は致死が多い訳ですが、その致死の理由はジェニックな変異でしょうし、おそらく染色体の断片レベルのものが飛んでいるか、こういう染色体断片レベルの変異であって、その中に重要な他の遺伝子があるために致死に至っているのではないかと考えています。ちなみに、現在アメリカでは11Sを多くすれば経済的にはメリットが大きいということで、育種目標の大きなターゲットの1つに考えられていることは計画書にも書かれているところです。

それから7Sと11Sの相補性の問題で、7Sと11Sの両方ともタンパク体の中に合成されてくる訳ですが、おそらく、アミノ酸ブロックの奪い合いというか、片方がなくなってしまう1つの重要なタンパク質は十分に合成能力があって、合成量が維持されると私自身は考えております。これが本当に7S・11Sがそれぞれ完全に無くなった場合に相補できるかということについては、実験モデルが無いので分かりませんが、そのような想定をしている状況です。

座長：その間に、致死遺伝子とかそういうものは、いわゆる遺伝子としての関与は無いとお考えですか。

喜多村：変異にもいくつかありますが、完全欠失型の場合はほとんどの場合が構造遺伝子
そのものが眩目になっていて、mRNA はほとんど出ていないだろうと考えています。7S の \( \alpha' \cdot \alpha' \cdot \beta \) がほとんど無くなっている変異体については、構造遺伝子というよりも他の何かの別のレギュレーター的な遺伝子が関係しているのではないかと考えています。

萩尾（中国農試）：小松田さんにダイズタンパク質の変異のことでお聞きします。未熟胚から不定胚を誘導される時に NAA を用いられておられますか、2,4-D でも不定胚は出来ますか。

小松田：はい、出来ます。

萩尾：ホルモンの種類を変えられた場合、この変異体のタンパク質を分析して、電気泳動パターンに違いがみられたでしょうか。

小松田：最後のスライドでちょっとお見せしたのですが、26kDa のタンパク質が 2 つの再生個体の後代に出てきました。1 つは 2,4-D で誘導したもの、もう 1 つは NAA で誘導したもので、どちらでも出たということです。

萩尾：培養の期間が長かったり短かったりすると、そのパターンに違いはなかったのでしょうか。

小松田：どちらも長期間培養しておりますので比較はできませんが、24kDa タンパクが出たということでは同じです。

村田（熱研センター）：コメントのようなことで恐縮ですが、先程の DNA の話と今の話とを含めて非常におもしろいと思いましたのは、ガンマ線では欠失などは無くて、あまり大きな変化ではないものが出てくるということかもしれません。そういう話の中で完全欠失型がありますが、東京の場合タンパク質が全く消えているということで完全欠失と言うのですから、DNA は欠失していない。読む取られなければタンパク質は出てこない訳ですから。この辺の所が今回のシンポジウムでいろいろ整理されてきたというか、おもしろいと思います。パクテリアで、ファージの突然変異というのはいろいろ調べられております。パクテリアの場合だいたい紫外線とかケミカルでやっていますが、1 つ例がありまして、ionizing irradiation を当てて変異を作ったところが、ゲノムが小さいせいもあるのですが、出てきたミュータントのほとんどが 1 つのベースのチェンジであったという例があります。ですから今回の話の中で、放射線による変異が、現象としては大きく見えるけれども実際には小さな変異であったということは、ドミナント型の変異が起こり得るということを含めて非常におもしろいと思いました。以上コメントでございます。

座長：どうもありがとうございました。紫外線に関して起こる機構は、たとえばチミングイマーの問題などはかなり分かっていますし、グアニンのメチル化をすることも分かっています。しかし、放射線の場合はそこに機構的な問題が絡んでくるという面があると思います。

では次に、培養技術を利用した育種ということで永富さんのお話と、それからちょっと違うかもしれませんが、非対称融合ということに話を移したいと思います。

久木村（鹿児島県バイテク研）：永富さんに伺いにします。少し前の IAEA の報告書にもありましたが、サトウキビを使ってインドの Jagathesan たち、それからパキスタンのグルー
プがやはり mutation breeding の報告を出しています。それによりますと、急照射が中心だと思いますが、線量が 3krad くらいで実際に非常に有用な変異系を出しています。永富さんの場合はそれよりもオーダー多くて、しかも変異の幅も非常に多いし、実用形質の変異が多いようです。私共もサトウキビを材料にしてこういう仕事をスタートしているところですが、その時にはやはりクロニール照射が良いのか。クロニール照射となると我々のところでは手軽に使えないという問題があるのでその辺を教えて頂きたい。

もう 1 つはキクの話で、花弁からの不定芽がベリクライナル＝キメラということは、キクの不定芽誘導の場合は、シングルセルではなくて多細胞が関与してシュートが出来ると考えて良いのかどうか。この 2 つをお伺いします。

永富：サトウキビの場合適正線量について調べてみましたが、おっしゃるように誘発に有効な線量はだいたい 3 ～ 16kr くらいまで報告があるようです。ただこれにつきましては、それぞれの文献に照射線量率がほとんど明記されていません。そこで照射線量淡水と線量率との関係を調べてみますと、線量率が 1 kR/h 以上の領域では線量線が低くないと芽が死んでしまいます。けれども、100 ～ 500R/h の低線量率の領域になると線量線は 35kR ぐらいまでは照射できるということが分かりまして、おそらくこれは線量率の効果が大きいのではないかと思います。急照射でもぐく低線量になりますと、非常に幅の広い領域で有効な突然変異の誘発ができると思いますので、今後は線量率をもう 1 つの基準にして考えていくべきだと思います。

次に照射と培養の関係ですが、可視的な突然変異の頻度を見ますと、普通の個体照射の場合にはほとんどのものが非常に低頻度で、統計処理もできないくらいのものです。しかし培養系の場合ですと非常に速やかな変異になりまして、従来の交雑育種のような統計処理ができるレベルになります。突然変異の頻度も 2 段のオーダーのパーセントまで上がることが分かりました。ですから、個体のレベルと培養を使ったレベルとは突然変異の頻度に格段の差があると言えると思います。

サトウキビの培養系を用いた照射線照射は、緩照射植物由来の再分化系には正と負の両方向に変異の拡大がみられるので有効な手法です。しかし培養カルスへの急照射の場合には、線量の増加に伴って負の方向へ変異が拡大するので、照射線障害を回避できる範囲で再分化系に照射する必要があります。今後、培養系では細胞周期と照射法との関係を解明して、有効な照射方法を検討していきたいと思っています。

それからキクの話で、花弁培養の場合にはカルスからの再分化になりますので、突然変異体はシングルセル由来だと言うことができると思います。花器培養の場合には、花弁と花托の接着面からカルスが誘導されてくるようです。これも、ほとんどシングルセル由来だと確認されました。緩照射の個体レベルの変異体は従来のように層状キメラになり、培養のレベルですとシングルセル由来の非キメラ性の変異体になるということで、突然変異の質的なものがはっきり分かれて出てきます。

座長：それでは、神代さんのお話に関して何か質問なりディスカッションがございますでしょうか。

蓬原（名大）：葉緑体の DNA について 2 つお伺いします。1 つは、両親の葉緑体 DNA が
ミックスした材料が出たということですが、それは後代まで安定して伝わるかどうかということも。もう1つは、ミトコンドリア DNA は非常に頻繁に組み換えを起こすということです。業績体 DNA の場合にはほとんど組み換えを起こさないと言われています。その理由が分かりましたら教えて頂きたいと思います。

神代：まずミックスした業績体が後代に伝わるかどうかということですが、ミックスしたかどうかというのはマーカーとして3つ用いて、ティントキシナトキシン耐性をマーカーとして用いたときに非常に容易に検出されます。その時に使っている世代は再分化個体にバッククロスでついた種ですから一世代後です。そうすることで、再分化個体がミックスした業績体を持っているということが分かってきます。これが安定して伝わるかということですが、それはソーティングされて sensitive か insensitive かどうかにかかわれてしまいます。しかしそういう個体については、再分化個体で別のマーカーの fraction I protein を使いますとバンドがちゃんと2本見られます。お見せしなかったのですが、ミックスしたものでも sensitive と insensitive の比が極端に違う場合があります。そういうものに関しでは、例えば fraction I protein で当世を検定しても検出されてこないと思います。

それから、なぜミトコンドリアでは高頻度で組み換えが起こり、クロロプラスト DNA は安定であるかということ2番目の点ですが、私としてもずっと疑問に思っております。ミトコンドリアにおける高頻度の組み換えを説明する1つの間接的なデータとして、コスミドライブラリーを作った時に同じ様な配列があって、結局フィジカルマップが出来なかったというようなことがありますが。ミトコンドリアにはよく似た配列があちこちにあり、そういうものの1つがリコンピネーションのホットスポットみたいな役割を果たしていると考えられます。これはあくまでも推察です。それから、クロロプラストの方は非常に安定的でリコンピネーションはほとんどどの形態では起きてません。しかしこのままでに1例だけ、クロロプラストにある薬剤耐性マーカーを selection pressure として使った時に、recombinant が出現したという報告があります。従って、selection pressure をかけるかかえないかによっても変わってくるのではないかというのが私の感じです。

座長：細胞選択に関する問題で、何か意見とかございますでしょうか。

魚住(東京大)：豊田先生にお伺いしたいのですが、細胞選択の判定の利点は、インビトロで検定ができるということと、細胞にした段階で変異が起こるのではないかという2点あると思います。しかし、例えばイチゴの葉黄病の場合だと、インビトロでは選択しないで再生した後で選択しておられます。その時にカルスから再生したことによって変異が増強されたのか、それともランナーだけでも、例えば1000個とってやれば2個ぐらい出るのか。コントロール実験では50株から耐性株が0だったけれども、カルスから再生したものは1200株から2株取れたということですが、カルスから再生することによって変異が上があったかどうかということは検定されませんでしたでしょうか。

豊田：まず、in vitro でセレクションする方が効率的ではないかというご指摘だと思いますが、私自身は必ずしもそう考えておりません。非常にシンプルな系、例えば宿主特異性毒素を作るような系ですと、毒素抵抗性がそのまま病害抵抗性になりますので、ターゲットを毒素抵抗性だけに絞れば in vitro のセレクションで済みますが、実際はそうでない病害
の方がむしろ多い訳です。イチゴの場合でも、感染のプロセスがあまりよく分かっておりませんし、培養過程でいろいろな毒素を作りますが、それが感染のプロセスで働いているのかどうかよく分かりません。今のところ、イチゴの葉変病にしても炭疽病にしても、そういう毒素の関与をきっちりと報告したものはありませんし、実際の感染過程をみておりても、毒素を作るか作らないかということが必ずしも病原性に関わっているのではないと考えています。むしろ、強い病原力を出すか出さないかということになってきますと、in vitro selection の限界がそこに出てくるのではないかと思います。それから炭疽病のセレクションでは、我々が行なった限りでは、カルチャーしないコントロールの方からは 1 例も出ておりません。エスケープしてくる個体はありますが、再生すれば発病します。

選抜した個体の頻度ですが、私共は薬変病の後代選抜で約 1000 個体から 2 個体選抜しましたが、もし有効な selection pressure をかけることができれば、in vitro の段階で 2 個体だけ選抜したこととイコールではないかと考えております。

魚住：選抜の点はそうですが、もう 1 点は、ランナーが出る時のランナーの一番端のどのがすいと鰭を切って考えていたのか、だと思えば、例えばランナーを 1200 個取って選抜した上で抵抗性のものが 2 個体ぐらい出るのはないかという質問なのですが。

農田：お見せしたのはすべて別の個体のランナー由来です。それもう 1 つ、ランナーは最初にセレクションした個体のどのがすいと鰭を切って出るのか、その辺は良く分かりません。私共もこういう病害抵抗性の変異そのものをどう位置付けるかで、特に遺伝的な要因との関係を重視しないといけないのでですが、今までのところは、例えば青枯病のトマトの場合で、交配すると非常に解析の難しい分離をします。そういうことを考えてみると、個体の中に出録のような状態が残っている可能性も含まれておりますし、もう一回いえますと、ああいうふうにランナーが出た時にも、遺伝的なホモジェネスが保持されているかどうかは良く分かりません。ただ、病害に対して抵抗性を示すかどうかというレベルでは十分だと思います。

病害抵抗性になるためには、host と parasite のいろいろな遺伝子の認識の対応があります。その、どの段階で変異を生じても結果的には病害抵抗性になるケースがあります。ですから我々が見ているのは、あくまでも最終的に病気にならなかったというレベルですが、その前段階を見せると抑えているプロセスが違う可能性があります。そこで関与する実際の遺伝子の数とかレベルの問題も含めてこれからの方々を考えております。

座長：誠に残念ですが時間がきてしまいました。この総会会議で全部をまとめて結論を出すというのは無理だと思います。結論はご参加の皆様それぞれにお出し頂くということになってしまいます。

今後、分子レベルでのこういういろいろな解析が進めますし、その間にそういう解析のための手段として変異体を積極的に使っていくと、いろいろな局面での問題が出てくる。しかも、植物種というのは非常に多種多様に分かれてますので、研究もこれからどんどん進むと思います。特にトランジェニックの植物に関してしても、もちろんこのままいろいろな進め方がある訳ですが、今一番問題になっているのはフィールドテストをどうやっているかということです。それに対する規制の問題は今後緩和される方に進む傾向がみら
れますので、そういうことがはっきりしますと研究もどんどんどん進むことと思います。
最後に皆様の今後の研究の発展をお祈りいたしまして、総合討論を終わらせて頂きます。どうもありがとうございました。
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