CONCURRENT SESSION 6

New Techniques and Systems for Mutation Induction

Restriction Endonucleases as a Tool for *In Vivo* Induction of Chromosomal and DNA Damage in Barley Genome

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Abstract

Bacterial restriction endonucleases have been widely utilized to study the significance of DNA double-strand breaks for the formation of chromosomal aberrations based on their ability to produce this particular DNA lesion. Such studies were very scarce in plants until mid-nineties. The stability of maize nuclei towards *in vivo* action of *Eco*RI was investigated, revealing that dry embryo cells were less resistant than meristematic ones actively involved in transcription. Restriction endonucleases were also found to induce structural chromosomal damage in barley genome. They exerted an S-independent mode of action revealing the transition between the G_1 and S phase as the most sensitive stage for aberration induction. Intra-chromosomal localization of chromatid aberrations produced by *Hpa*II, *Msp*I and *Hae*III displayed similar distribution patterns. The most pronounced aberration hot-spots were the Nucleolus Organizing Regions which pointed towards the potential of restriction endonucleases for damage induction in specific genomic locations. Patterns of the localized chromosomal breakage produced by *Hae*III in suitably reconstructed karyotypes showed substantial difference in the aberration hot-spot behavior. Position-specific increase in aberration clustering was found indicating that the incidence of aberration hot-spots generated by restriction endonucleases is dependent on their chromosomal environment. Barley karyotypes with normal and increased expression of rRNA genes were further utilized to evaluate the possible relationship between their transcriptional activity and damage induction. Hybridization profiles obtained after treatment with *Msp*I revealed similar induction kinetics. The potential of barley ribosomal genes to accumulate double-strand breaks with a different structure was also tested by *Alu*I and band intensity reduction followed the pattern found for *Msp*I. Results indicated that the mode of action of restriction endonucleases applied was not substantially influenced by the activity of the nucleolus organizing regions. The data as a whole supports options for the use of restriction endonucleases for directed induction of damage in plant genome.

Introduction

The use of bacterial restriction endonucleases (RE) for induction of chromosomal damage in eukaryotes was the subject of extensive studies during the last two decades of the previous century. This interest was initiated by the need to study the role of DNA double-strand breaks (DSB) in the course of cellular responses to radiation-induced damage and from the necessity to reveal the molecular mechanisms governing the formation of chromosomal damage. The ability of RE to produce one particular DNA lesion, namely DSB, was widely utilized, but data for plants was practically unavailable until the mid-nineties.

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Description of the research

Background studies

Radiation-generated DSBs were found to be the most likely primary lesion leading to the formation of chromosomal aberrations [1, 2]. This suggestion was strongly supported by the successful use of REs as effective inducers of chromosomal damage. The mode of action of REs was found to resemble those of ionizing radiation and radiomimetic chemicals in that it was S-phase independent: chromosome aberrations were induced in G_1 and chromatid in S and G_2 phases of the cell cycle. [3]. It was demonstrated that blunt-ended breaks tended to be more effective, leading to higher frequencies of chromosomal aberrations [4, 3]. Higher efficiency of REs recognizing four bases, and a lower efficiency for those recognizing six bases was also established. Aberration induction was found to also be dependent on a variety of other factors such as cell-cycle progression, cellular repair capacity, DNA methylation and the technique for introduction of REs within the cell nucleus [3, 5, 6] .

It must be pointed out that the majority of the initial studies in the field were performed on mammalian cells, and until recently, the corresponding data from plants was very scarce. The first attempt to analyze the mode of action of RE on plant chromosomes was made by Subrahmanyam and co-authors in 1976 [7]. After treatment of barley root tips with a mixture of the restriction endonucleases *Hind*II and *Hind*III, they found a time-dependent and progressive fragmentation of metaphase chromosomes, as well as a granular appearance of the interphase chromatin accompanied with micronuclei formation. These observations and the ample and decisive evidence of the chromosome-breaking ability of REs in mammalian cells urged us to utilize REs as a tool for induction of chromosomal and DNA damage in the barley genome.

Key findings

Differential response of maize nuclei upon treatment with restriction endonucleases in vivo

It was shown that higher-order chromatin structure in plants resembles that found in other eukaryotes, namely that the nuclear DNA is organized in a series of supercoiled loop domains anchored to a structure termed the nuclear matrix [8]. The germinating maize embryo represents a suitable model system for the study of the transition of an inactive genome of a dry embryo to the higher transcriptional activity of the germinating embryo cells. Electron microscopy studies have shown that the nuclei of dry embryo cells lack internal nuclear matrix, which is however, well-structured in the meristematic cells of the primary roots [9].

Stability towards the action of *Eco*RI of maize nuclei obtained from cells with different transcriptional activity (dry embryo, root tip meristem and epicotyl protoplasts) was investigated. After fluorescent staining of the resulting histone-depleted nuclei, it was found that dry embryo structures are less stable than those actively involved in transcription (**Fig. 1**), most probably due to the absence of a well-defined internal nuclear matrix, which points to the key role of this higher-order chromatin structure in the functioning of the plant genome [10].

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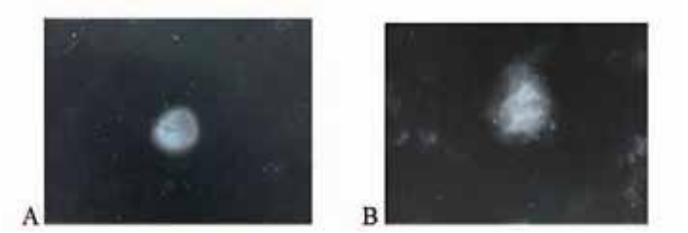

Figure 1 Fluorescent appearance of meristematc **(A)** and dry embryo **(B)** nucleoids treated with *Eco*RI.

Restriction endonucleases induce chromosomal aberrations in barley

The clastogenic ability of *Msp*I, *Hpa*II and *Hae*III in germinating barley seeds was evaluated by Feulgen staining of metaphase spreads. All REs were found to be efficient inducers of structural chromosomal alterations in barley, both of chromosome and chromatid type. The common feature of the induced chromatid aberrations was that isochromatid breaks and chromatid translocations were predominantly observed. Metaphases with multiple aberrations were also found at later recovery periods. The capacity for aberration formation was not significantly influenced by the nature of the DSB generated, but was found to be significantly dependent on the methylation status of the target DNA. Our data indicated that, like in other eukaryotes, REs display an S-independent mode of action. They revealed also that transition between the G_1 and S phases of the cell cycle is the most sensitive stage for induction of chromosomal damage by REs in the barley genome *in vivo* [11]. Examples of different types of chromosomal aberrations observed after RE treatment of barley root tips *in vivo* are presented in **Fig. 2**.

Figure 2 Chromosomal aberrations induced by REs in barley genome *in vivo*. [11]. i, isolocus breaks; s, subchromatid exchange; t, chromatid translocation ; tr, chromatid triradial; d, dicentric chromosome; b, chromatid break.

Intra-chromosomal mapping of chromatid aberrations induced by REs in barley

Chromosomal mutations have been widely used for reconstruction of the barley karyotype to improve its capacity in cytogenetic studies. A rich collection of reconstructed karyotypes in this species was created at our institute [12, 13]. These karyotypes were mainly used for investigation of regional specificity of mutagenic factors and chromosome position effects in the expression of induced aberration hot-spots.

Specific distribution of chromatid aberrations along individual chromosomes produced by *Hpa*II, *Msp*I and *Hae*III in a multireconstructed barley karyotype PK 88, containing three reciprocal translocations, 1H-5H, 2H-7H, 3H-4H, and one pericentric inversion in chromosome 6H, was analyzed further. The REs were found to produce similar intra-chromosomal distribution patterns of the induced aberrations irrespective of their recognition sequence. In all cases, the most pronounced aberration hot-spots proved to be loci representative of both transcriptionally active and condensed (inactive) ribosomal DNA entities, localized within the chromosomal segments comprising the Nucleolus Organizing Regions (NORs). Such a biased aberration clustering (outlined in **Table 1**) demonstrates the ability of REs to induce damage in defined locations of the barley genome [14].

Table 1. Statistical evaluation of hot-spot chromosomal segment sensitivity after treatment with *Hpa*II, *Msp*I and *Hae*III

Segment No	Total number of chromatid aberrations	
	Random distribution–Mean (range)	Observed value
*Hpa*II		
4	5.4 (1.0-11.8)	12
11	5.4(1.0-11.8)	15
38	5.4(1.0-11.8)	20
46	5.4(1.0-11.8)	18
47	4.8 (1.4-11.0)	12
48	4.8(1.4-11.0)	25
*Msp*I		
4	7.6 (0.0-15.2)	17
38	7.6 (0.0-15.2)	36
41	7.6 (0.0-15.2)	18
46	7.6 (0.0-15.2)	27
47	6.9 (0.4-14.2)	29
48	6.9 (0.4-14.2)	17
*Hae*III		
38	8.4 (0.4-16.4)	37
41	8.4 (0.4-16.4)	19
46	8.4 (0.4-16.4)	32
47	7.6 (0.1-15.3)	31
48	7.6 (0.1-15.3)	26

Segment No	Total number of chromatid aberrations			
	Theoretically expected random distribution	Lower confidence limit	Upper confidence limit	Observed value
*Hpa*II				
4	**5.4**	**1.0**	**11.8**	**12**
11	**5.4**	**1.0**	**11.8**	15
38	**5.4**	**1.0**	**11.8**	**20**
46	**5.4**	**1.0**	**11.8**	18
47	4.8	1.4	**11.0**	12
48	4.8	1.4	**11.0**	25
*Msp*I				
4	7.6	0.02	15.24	17
38	7.6	0.02	15.24	36
41	7.6	0.02	15.24	18
46	7.6	0.02	15.24	27
47	6.9	0.37	14.20	29
48	6.9	0.37	14.20	17
*Hae*III				
38	8.4	0.43	16.40	37
41	8.4	0.43	16.40	19
46	8.4	0.43	16.40	32
47	7.6	0.10	15.30	31
48	7.6	0.10	15.30	26

HaeIII induces position-dependent chromosomal breakage in barley

Patterns of the localized chromosomal breakage induced by restriction endonuclease *Hae*III in reconstructed karyotypes T-1586 and T-21, showing respectively, standard and rearranged positions of NOR-bearing segments of chromosomes 6H and 5H, were further investigated. The structural details of these karyotypes are given in **Fig. 3**.

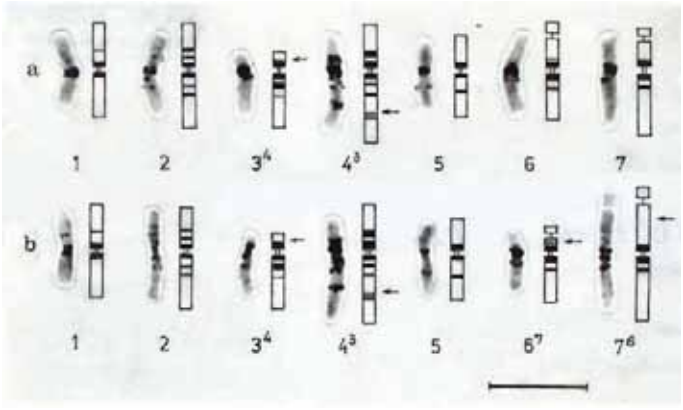

Figure 3 Idiograms of Giemsa-banded chromosomes of barley reconstructed karyotypes T-1586 **(a)** and T-21 **(b)**. Chromosomes are indicated according to the old system, where chromosome 1 corresponds to 7H, 2=2H, 3=3H, 4=4H, 5=1H, 6=6H, and 7=5H [15]. Arrows indicate the putative translocation breakpoints.

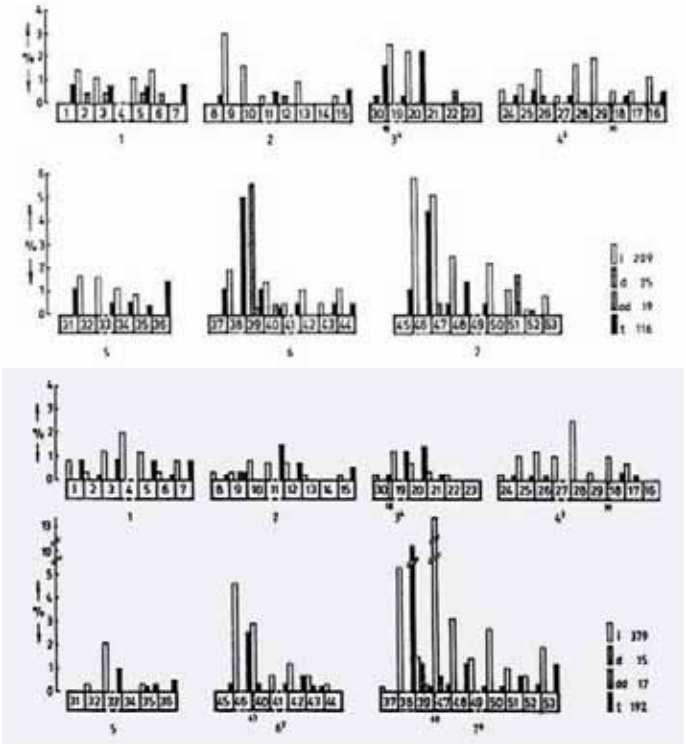

Figure 4 Intrachromosomal distribution of chromatid aberrations induced by HaeIII in karyotypes T-1586 **(A)** and T-21 **(B)**. i- isolocus breaks; d- intercalary deletions; dd- duplication deletions; t- reciprocal chromatid translocations [17].

Due to the reciprocal translocation between the short arms of satellite chromosomes in T-21, the two most pronounced aberration hotspots (segments 39 and 47), after treatment with chemical mutagens [16] become arranged tandemly. In the control line T-1586, it was found that NORs of chromosomes 5H and 6H, respectively segments 46 and 38, containing actively transcribed ribosomal DNA, as well as segments 39 and 47, both representative of condensed rDNA repeats, are the most pronounced aberration hot-spots. Intra-chromosomal distribution of chromatid aberrations in T-21, where the NOR-bearing segments in chromosomes 6H and 5H change their position, revealed substantial difference in the aberration hot-spot behavior. Position-specific increase in aberration clustering was found, most obviously in segments 38 and 47. On the other hand, segment 46 retained its sensitivity, while segment 39 in its new location lost its previous status of aberration hot-spot. The data (**Fig. 3**) are indicative that the expressivity of aberration hot-spots generated by REs might be influenced by their distinct chromosomal location and/or chromatin organization [17].

Induction kinetics of RE-induced double-strand breaks in barley ribosomal DNA

There is a lot of data showing that certain types of DNA damage is more effectively removed from transcriptionally active genes than from silent regions, indicating the existence of intra-genomic repair heterogeneity [18]. It is now widely accepted that transcriptional activity and the higher-order chromatin structure are the main factors influencing the repair efficiency within a particular genetic loci or chromatin domains [19].

Based on our data, which unequivocally displayed that barley NORs behave as a prominent aberration hot-spots after treatment with RE *in vivo*, further studies on the ability of REs to produce DSB in barley ribosomal DNA were performed. Reconstructed barley karyotypes T-1586 and T-35 with normal and increased expression of rRNA genes, respectively, were utilized to evaluate the possible relationship between their transcriptional activity and DSB induction. Due to the enrichment of CCGG sites in barley ribosomal DNA, *Msp*I was first utilized as a tool for induction of DSB. Scanning densitometry of the hybridization profiles obtained revealed similar induction kinetics for both karyotypes. The potential of barley ribosomal genes to accumulate DNA DSB with different structure and eventual dependence of the induction efficiency on DNA methylation was tested by treatment with *Alu*I. Band intensity reduction followed the pattern already observed with *Ms*pI, displaying less amounts of full-length ribosomal repeats three hours after treatment. Histogram presentation of the data is presented in Fig. 5. No substantial difference between the two karyotypes was observed, which indicates that the mode of action of the REs applied was not substantially influenced by the activity of the corresponding NORs [20].

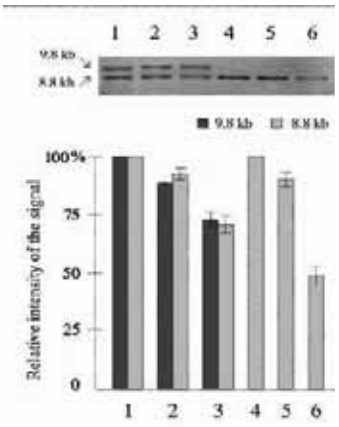

Figure 5 Induction kinetics of double-strand breaks induced by the restriction endonuclease *Msp*I in barley ribosomal DNA. Lines 1, 2 and 3- karyotype T-1586. Lines 4, 5 and 6 – karyotype T-35 [20].

A question arises about the mechanisms maintaining rDNA integrity after DSB induction. Two major pathways are responsible for the recovery of DNA double-strand breaks in eukaryotic cells - homologous recombination (HR) and non-homologous end-joining (NHEJ) [21]. The existence of both repair pathways has been demonstrated in plants. It was shown that tobacco cells are able to repair site-specific DSBs artificially induced by REs via HR [22, 23]. As the search for homology in rDNA should be facilitated due to its repeated nature, it is tempting to speculate that recovery of ribosomal genes might be realized through HR. In plants, however, even in the cases when the finding of homology

is substantially simplified, the repair of DSBs might be also accomplished by NHEJ [24], which leaves the issue open.

Conclusions and likely outputs

The ability of REs to induce chromosomal and DNA damage in the barley genome *in vivo* is unequivocally established. The results concerning the mode of action on a chromosomal and gene-size scale point towards the potential of REs for inducing of DSB in defined genomic entities. Such data, along with the capacity of REs to facilitate genetic transformation, also points towards the options for site-directed induction of DNA breaks in a specific locations of the plant genome.

ACKNOWLEDGEMENTS

This work was supported by the National Science Fund of Bulgaria, Grant K-422 and the IAEA CRP Project Bul 5010.

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Achievements and Perspectives of Crop Space Breeding in China

L X Liu*, H J Guo, L S Zhao, J Wang, J Y Gu & S R Zhao

Abstract

Aerospace provides a special environment with strong cosmic radiation, microgravity, weak geomagnetic fields, and a super-clean super-vacuum. A large amount of experimental data showed that environmental conditions in space affect plant growth and development, as well as inducing genetic changes in crop seeds. The frequency of chromosomal aberrations is greatly increased in seeds carried into space and subsequently germinated on the ground. The combined effects of both cosmic radiation and microgravity, together with other spaceflight factors, appear to be the main causes of genetic changes in seeds from space flights. Since 1987, China has been conducting experiments of space-induced mutagenesis for crop improvement using recoverable satellites, Shenzhou spacecrafts and high-altitude balloons. *Shijian-8*, the first world satellite specially designed for the space-breeding programme, was launched on September 9, 2006. It carried over 2,000 plant accessions from 133 species. So far, 66 new mutant varieties of crops, including rice, wheat, cotton, rapeseed, sesame, pepper, tomato and alfalfa have been developed by the space-breeding programme and officially released in China. A catalog of useful rare mutant germplasm was also obtained. A new technique and method of mutation induction has been set up by simulating the space environment. It is concluded that space-induced mutation can be a novel and effective way to enhance genetic diversity from which to breed new crop varieties.

Introduction

Induced mutation in plants has been an effective breeding strategy in China for more than 50 years. According to incomplete statistics, by 2007 the total number of mutant varieties and mutant-derived varieties officially registered in China was 741, including 45 crops and ornamental species [1, 2]. The popularization and utilization of these mutant varieties has made an important contribution to China's food production and social and economic development. Mutation induction has become one of the most fruitful and widely used methods for crop improvement in China [3]. In the last 20 years, new mutagens, such as mutagenesis in space, have been explored and developed to actively upgrade mutagenic efficiency in crop breeding. This paper briefly reviews the recent advances and future perspectives of space-induced mutagenesis for crop improvement.

Space-induced mutation technique

The main characteristics of the aerospace environment are strong cosmic radiation, microgravity, weak geomagnetic fields, and a hyper-clean super-vacuum. A large amount of experimental research has shown that space environment affects seed germination and growth of plants [4, 5]. The mitotic index declines remarkably, and the type and frequency of chromosomal aberrations in space-flown seeds are greatly increased compared to earth-bound controls. These mutagenic effects are universal among plants. Results from space flight experiments show that space conditions are mutagenic [6, 7].

The spaceflight-induced mutation technique, or "space breeding," is a technique that uses the genetic variations produced in plant seeds by the space environment that can be reached by recoverable spacecraft (such as recoverable satellites and space shuttles) and high altitude balloons to identify novel germplasm on the ground from which to develop new crop varieties [8].

Biological effects of space environment factors on plant seeds

The sensitivity to space flight differs between plant species and between varieties of the same species. After space flight and subsequent germination on the ground, seed vigor enhanced and germination rate increased markedly in seeds of wheat, triticale, barley, maize, cotton, sunflower, soybean, cucumber and tomato. There was no significant difference in germination rate compared to the ground control in seeds of rice, millet, pea, sweet pepper, lettuce and tobacco, while seed germination rate decreased in seeds of sorghum, watermelon, eggplant, radish and towel gourd [4, 5]. After space flight, seed germination potential, germination index, seedling height and seedling vigor index of wheat, barley and triticale were all significantly higher than those of ground controls and Gamma-ray irradiated seed. Activities of peroxidase isozymes and esterase isozymes also increased in these species by space flight. In contrast, germination and seedling growth of sorghum seeds was greatly reduced, and flowering was also delayed. The growth habit and maturation period of soybean were also affected by space flight [9].

Compared with traditional Gamma-ray irradiation and other mutagenic treatments, the greatest difference in agronomic performance of the first generation (SP_1) produced from seeds following space-flight was a lack of damage effects, such that space-flight could even produce a beneficial effect on the growth of SP_1 seedlings. In the SP_2 generation from dry seeds of *japonica* rice variety Zhongzuo 59 carried by a high altitude balloon, all 11 characters investigated, which included plant height, growth period duration, spike length, grain husk color and light sensitivity, differed widely. Some high-quality rice types were selected, which could be stabilized easily in later generations [10]. Although the variation in space-induced mutations in the SP_1 generation of wheat was lower than that of a Gamma-ray irradiation treatment, wide variation was observed in the SP_1 generation following space flight, with variation ranging from 2.2% to 11.1% [11].

Mutant varieties developed by space breeding

Since 1987, China has conducted experiments of space-induced mutagenesis for plant improvement 21 times using recoverable satellites, Shenzhou spacecrafts and high-altitude balloons to carry plant seeds into space. Seeds were planted after returning from space flight for the selection of useful mutations. Shijian-8, the breeding satellite specially designed for the space-breeding programme, was launched

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Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009,**213-215**

on September 9, 2006. It carried over 2,000 accessions of plant seeds belonging to 133 species [2].

So far, China has officially approved 66 new varieties of crops including rice, wheat, cotton, rapeseed, sesame, pepper, tomato and alfalfa developed by the space-breeding programme. These new varieties have characters of high yield, good quality and multiple resistances. Some useful rare mutations that might make a great breakthrough in crop yield were also obtained [12, 13, 14]. The space breeding programme has begun to reap excellent social and economic benefits.

It has been shown that space-induced mutation breeding of crops can be a novel and effective way to create distinctive genetic resources with which to breed new varieties, due to its wide mutation spectrum, high frequency of useful genetic variation and short breeding period.

Mechanism of space mutagenesis

Some recent research on the effects of the space environment suggests that space flight is effective in inducing changes in crop genomes. The genomic polymorphisms in 201 rice plants developed from space flown seeds were investigated with RAPD analysis and 30.2% more polymorphisms were found compared with plants from ground control seeds [16].

The reasons why space environment causes chromosomal aberrations that result in alterations of genetic characters are not very clear at present. Space radiation is one possibility. More multiple chromosomal aberrations were observed in seeds hit by HZE (high-charge and high-energy) particles of cosmic radiation in space, and the frequency of abnormal plant development increased. The aberrations were different if HZE particles hit different parts of seeds. The frequency of aberration was the highest when the root meristem or hypocotyl was hit. However, many experimental results show that an increased frequency of aberrations can also be observed in seeds not hit by cosmic particles during space flight. The longer the seeds were kept in space, the higher the frequency of aberration, suggesting that microgravity might also have a mutagenic effect on seeds [6, 15].

It is evident that the combined effects of both cosmic radiation and microgravity are the main causes of the genetic changes in plants induced by space conditions. Once the seeds of plants that have evolved under the effects of earth gravity for billions of years were put in the microgravity environment of space and were affected by various physical radiation factors at the same time, their genomes were inevitably be strongly affected. In addition, the strong vibration and blast force associated with spacecraft launch and landing cannot be neglected as causal agents contributing to the increased frequency of chromosomal aberrations during space flight [5].

Study on simulating space environment factors

Because of the need for major investment and technological support, the chance of space flight experimentation is very limited. It is therefore, important to make ground simulations of space factors to investigate the mechanism of space-induced mutation and apply these to plant breeding.

Experiments were conducted to simulate factors of the space environment using accelerator or geomagnetic-field free space [9]. The mutagenic effects of various space-flight factors on wheat and other crops were studied from various angles of particle biology and physical field biology. It has been proven that the space environment has significantly different biological effects and mutagenic effects from traditional Gamma-rays. A new technique and method of mutation breeding by simulating the space environment has been set up.

Magnetic field-free space

Equipment of the magnetic field-free space (MF) is a large magnetic screening installation which uses a two-layer magnetic screening structure combined with coil compensation. Its magnetic strength is 20 nT, which is 4×10^{-4} of the magnetic strength of the earth's magnetic field.

Air-dried seeds of wheat, and of other plant species, were treated by MF at room temperature for various periods of time [17]. The results showed that the seed germination and seedling growth were obviously inhibited by over 180 days of MF treatment. The striking morphological features of the wheat seedlings treated by MF were that the first leaf of the seedling was shorter, wider and thicker, the leaf apex became round, and leaf color turned dark green. MF treatment during the process of anther callus induction of wheat, stimulated the development of male gametes and the final formation of calli, resulting in the production of high-quality anther calli and a high percentage of green plants. These significant differences from the traditional Gamma-ray treatment showed that MF had an obvious mutagenic effect on wheat seeds and could be used as a new physical mutagen for wheat improvement. Some new mutant varieties have been developed by MF treatment in rice and alfalfa [18, 19].

Single high-energy ion beam implantation

Air-dried seeds of wheat cultivars were irradiated using a single heavy 7Li ion beam generated by a tandem accelerator at an energy level of 42.3 Mev. Studies of biological effects showed that ^{7}Li ion beam implantation inhibited germination rate, seedling height and root length in the M_1 generation. There was no linear dose-effect relationship, but the "Braag peak" effect in ^{7}Li ion beam implantation. It was observed that 50Gy could be the appropriate dose of ^{7}Li ion beam to irradiate wheat seeds at an energy level of 42.3 Mev. Compared with Gamma-ray radiation, ^{7}Li ion beam implantation produced less biological damage. Various morphological and cytological aberrations of the seedlings occurred. The most significant variations were chlorophyll deficiency of the main vein, leaf split, leaf curl and tufted seedlings [20, 21, 22].It was observed that ^{7}Li ion beam implantation into crop seeds can produce the effects not only of the energy transfer, mass deposition and charge exchange, as observed with other ion beams, but also the reaction of ^{1}H(^{7}Li, ^{7}Be) n in the irradiated seeds .

Analysis of mutation types and frequencies of M_2 populations from seeds of wheat treated with ^{7}Li ion beams, demonstrated that this treatment could induce significant mutations. Mutation phenotypes of ^{7}Li heavy ion beams were mainly spike types and plant height. Due to the very limited M_2 population sizes from ^{7}Li heavy ion treatments, the statistics of frequency of mutations and mutants was incomplete. However, the data indicated that 50Gy of ^{7}Li heavy ion treatment could produce the highest mutation rate in both frequency and types, further suggesting that 50Gy could be the optimal irradiation dose for ^{7}Li heavy ion beam treatment in wheat [23, 24].

Mixed high-energy particles

Dry seeds of various genotypes of winter wheat and alfalfa were irradiated by a mixed high-energy particle field generated from E2 beam lines of LINAC of Beijing Electron Positron Collider [9, 25]. The cytological effects on the root tip cells of wheat seeds were studied, with the same dosage of Gamma-ray irradiation as a control. The results showed that irradiation with mixed high-energy particle field inhibited mitosis and produced various chromosomal aberrations such as micronucleus, chromosome bridges, circular chromosomes and dissociative chromosomes in root tip cells of wheat, with significant dose-effects. Higher rates of cytological damage and percentage of chromosome circles and fragments were found in the M_1 generation of wheat following mixed particle field irradiation than following Gamma-ray irradiation, indicating in the greater biological effects of irradiation with mixed high-energy particle fields than with Gamma-ray irradiation [26, 27]. A wider mutation spectrum and higher mutation frequencies, as well as a greater number of useful mutations for earlier maturity, shortness and spike type, were

observed in the M_2 generation following mixed particle field irradiation than following Gamma-ray treatment. The mixed particle field could be used as a new mutagen for mutation induction and crop improvement [16].

Conclusions and Prospects

Space-induced mutation is an effective new way not only to develop new crop varieties, but also to obtain rare mutants that may make great breakthroughs in important economic characters of crops, such as yield and quality, which are difficult to obtain using conventional breeding methods on the ground.

Research on the application of space-induced mutation techniques needs to be strengthened. It is necessary to undertake further work on several problems associated with space-induced mutation techniques, such as the selection of material, methods of treatment, molecular screening of mutants and the identification of quality characters in early mutant generations.

Because of the need for major investment and technological support, the chance of space flight experimentation is very limited. It is therefore, important to make ground simulations of space factors to conduct research for revealing the mechanism of space-induced mutation and apply this to crop breeding.

Recently developed biotechnological tools facilitating selection, characterization and genetic analysis of desired traits have significantly stimulated the use of space-induced mutation breeding and basic research. It is clear that the development of the space breeding programme will lean heavily on and be associated with, not only effective use of the approach, but also advances in plant biotechnology, particular plant molecular biology. Therefore, international cooperation will be very important for the sustainable progress of this research.

ACKNOWLEDGEMENTS

Acknowledgements: Research was successively supported by NSFC 39870192, 30270345 and 30670488, National Space Breeding Project (2003-138), National 863 Project (2002AA241011, 2007AA100102), National S & T Support Plan (2008BAD97B01), MOA Project (200803034), Core Research Budget of the Non-profit Governmental Research Institutions (ICS, CAAS) and the International Atomic Energy Agency, Vienna through Projects IAEA/RAS5045, RC12610, RC14195 and CPR5017.

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Establishment of Ion Beam Technology for Breeding

A Tanaka

Abstract

We have begun to investigate the characteristics of ion beams for inducing mutation from a molecular to a phenotypic level. Mutation induction rates were investigated using known visible *Arabidopsis* mutant phenotypes, such as *glabra* (*gl*) and *transparent testa* (*tt*). These observations indicated that mutation frequencies induced by carbon ions were twenty-fold higher than those induced by electrons. Molecular analyses showed that half of the mutants induced by ion beams possessed large DNA alterations, while the rest had point-like mutations. The common feature of mutations induced by ion beams is the deletion of several bases. It is possible that ion beams induce a limited number of large and irreparable DNA lesions, resulting in the production of null mutations that show a new mutant phenotype. Novel mutants, such as those that are UV-B resistant, have serrated petals and sepals, or lack anthocyanins, have been induced by 220 MeV carbon ions in *Arabidopsis*. The mutated genes were found to encode novel and key proteins for each process. In chrysanthemum and carnation, several kinds of flower-color and flower-form mutants that have never been produced by Gamma-rays or X-rays were induced by carbon ions. These observations indicate that the characteristics of mutations induced by ion beams are high frequency and broad spectrum, and produce novel mutants. Many breeding programmes are using mutants induced by ion beams and successfully producing useful new crop varieties.

Introduction

A number of studies on plant mutagenesis by ionizing radiation have been carried out since mutations were induced using X-rays in maize and barley by Stadler in 1928 [1]. The biological effects of ion beams have also been investigated and it has been found that ion beams show a high relative biological effectiveness (RBE) in lethality, mutation, and so on, compared to low linear energy transfer (LET) radiation such as Gamma-rays, X-rays and electrons [2]. As ion beams deposit high energy on a local target, it has been suggested that ion beams induce predominantly single- or double-strand DNA breaks with damaged end groups that are unable to be repaired easily [3]. Therefore, it seems plausible that ion beams frequently produce large DNA alterations, such as inversions, translocations and large deletions, rather than point mutations. However, the characteristics of mutations induced by ion beams have rarely been studied. On the base of the Consultative Committee for Advanced Radiation Technology in Japan, the Takasaki Ion Accelerator Advanced Radiation Application (TIARA) was established and basic research on plant mutation by ion beams was began in 1991. We first investigated the characteristics of ion beams on mutation induction. For over 16 years, the biological effects of ion beams have been studied and novel mutants and varieties of crops have been consistently and efficiently produced using mutations induced by ion beams. At present, more than 100 research projects utilize ion beam irradiation produced by several irradiation facilities in Japan.

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Irradiation methods

Ion beam irradiation of plant materials has been carried out in the following facilities: TIARA of the Japan Atomic Energy Agency (JAEA), the RIKEN Accelerator Research Facility (RARF), the Wakasa Wan Energy Research Center Multi-purpose Accelerator with Synchroton and Tandem (W-MAST), and the Heavy Ion Medical Accelerator in Chiba (HIMAC) of National Institute of Radiological Sciences (NIRS). As a representative ion source, TIARA was established as the ion beam facility for exclusive use on biological and material science [4]. The effects of ion beams on plant materials have been investigated using several plant species including *Arabidopsis*, rice, barley, tobacco and chrysanthemum. Several kinds of energies and ions, such as helium (He), carbon (C), neon (Ne) and argon (Ar), were used in these studies, with 220MeV C ions being the most common. All ions were generated from the AVF-cyclotron in TIARA (**Fig. 1**) [5]. The physical properties of the 220 MeV carbon ions are as follows: Incident energy at the target surface was 17.4 MeV/u, mean linear energy transfer (LET) in a target (0.5 mm thick) was estimated to be 121.5 keV/µm as water equivalent, and the range of ions was ca. 1.1 mm **(Table 1)**. These physical properties were calculated using an ELOSS code programme, a type of modified OSCAR code programme. Particle fluences of the ions were determined using a diethyleneglycol-bis-allylcarbonate (CR-39) film track detector. In general, ion beams are scanned at around 70 x 70mm, and exit the vacuum chamber through a beam window of 30µm titanium foil. The sample is placed under the beam window and irradiated in the atmosphere. In the case of *Arabidopsis* or tobacco seeds, 100-3,000 seeds are sandwiched between kapton films (8µm thick) to make a seed monolayer for homogeneous irradiation. In the case of rice or barley seeds, the embryo side faces the ion beams. Tissue cultures, such as those of ornamental explants, calluses and shoot primordia are contained in an aseptic petri dish that is irradiated directly, except that the lid of dish is replaced by a thin film in order to decrease the loss of ion beam energy. A sample is irradiated within 2 minutes.

Table 1. Physical parameters of ions produced in TIARA. Modified from [5]

Ions	Total energy (MeV)	Specific energy (MeV/u)	Projectile range (in water, mm)	LET (keV/µm)
$^{4}He^{2+}$	100	25.0	6.2	9.1
$^{4}He^{2+}$	50	12.5	1.7	19
$^{12}C^{6+}$	320	26.7	2.3	86
$^{12}C^{5+}$	220	18.3	1.1	122
$^{20}Ne^{7+}$	260	13.0	0.3	504

Biological effects of ion beams

Biological effects, such as seed germination, plant survival and chromosome aberrations were first investigated because they provide important

Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009, **216-219**

indices to determine the properties of the ion beams used for mutagenesis in plants [5]. Most efforts were focused on the LET-dependence of survival of plants from treated seeds. In general, all the experiments showed a peak of RBE for the survival of treated seeds above ca. 230 keV/μm (**Fig. 2**). Using carbon ions with different LET, Hase, *et al.* [6] found that the RBE for plant survival and chromosome aberrations in tobacco increased with increasing LET and showed the highest value at 230 keV/μm. Shikazono, *et al.* [7] found that the LET of the peak RBE for lethality of *Arabidopsis* was over 221 keV/μm for carbon ions and over 350 keV/μm for neon and argon ions. This data suggests that the LET for maximum of RBE for plant lethality is higher in seeds than in mammalian cells.

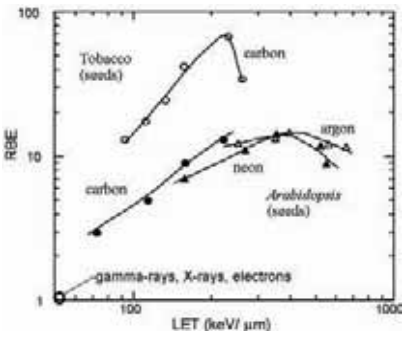

Figure 1 AVF-cyclotron (Left) and Irradiation Apparatus for Seed (IAS) (Right) in TIARA.

Figure 2 The relative biological effectiveness (RBE) of lethality as a function of linear energy transfer (LET) (from [6] and [7]).

The effects of LET on chromosomal aberrations was also investigated. The frequencies of mitotic cells with chromosome aberrations, such as chromosome bridges, acentric fragments and lagging chromosomes, were much higher for ion beams than for Gamma-rays [8]. The highest RBE was 52.5 at 230 keV/μm of LET [6]. The frequency of cells with chromosome aberrations did not decrease after fractionated irradiation with carbon ions, although a clear decrease was observed after exposure to electrons [9]. Recently, DNA double-strand breaks (DSBs) were quantified in tobacco protoplasts using pulsed-field gel electrophoresis [10]. Initial DSB frequency depended on LET and the highest RBE was obtained at 124 and 241 keV/μm carbon ions. These results indicate that the biological effects induced by ion beams were greater than those induced by low LET radiation, and that DNA damage induced by ion beams is likely to be irreparable.

Recently, Kazama, *et al.* [11] showed that a LET of 30 keV/μm (N ion) was most effective for inducing albino plants of *Arabidopsis*, indicating that the relationships with LET differ between mutation induction, plant lethality, chromosome aberrations and DSBs.

Characteristics of mutations induced by ion beams

Mutation frequency

Mutation frequency was investigated on a gene locus basis using visible known Arabidopsis mutant phenotypes, such as *transparent testa* (*tt*), in which the seed coat is transparent because of the lack of pigments, and *glaborous* (*gl*), in which no trichomes are produced on leaves and stems [12]. The average mutation frequencies of *tt* and *gl* loci induced by C ions was twenty-fold higher than those induced by electrons (**Table 2**, [13]). Mutation frequency is generally calculated as a unit per dose for radiation induced mutations. However, it is important to compare the mutation frequency as number of mutants per irradiated population when considering the use of mutants for practical purposes, such as agriculture for example. Carbon ions can produce *Arabidopsis* mutants at a rate four times higher than electrons, because carbon ions need one fifth of the dose (i.e. RBE=5) to induce the same biological effects as electrons [5].

Table 2. Mutation frequency induced by carbon ions and electrons [13]

Mutagen (dose)	No. of M2 plants	Locus	Mutation frequency / locus / diploid cell / dose (Gy) (X 10^{-6})
Carbon ions (150 Gy)	104,088	tt (tt3~tt7, tt18, tt19) gl (gl1~gl3, ttg1, ttg2)	1.9 (20 times)
Electrons (750 Gy)	80,827	tt (tt3~tt7, tt18, tt19) gl (gl1~gl3, ttg1, ttg2)	0.097

Mutation spectrum

In order to elucidate the features of ion beams as a new mutagen, the mutation spectrum induced by ion beams was compared with that induced by low LET radiation. To do this, Nagatomi, *et al.* [14] investigated the spectrum of mutations in flower color using chrysanthemum cv. Taihei with pink-color petals. We studied mutation induction in plants regenerated from irradiated explants of floral petals. Most flower color mutants induced by Gamma-rays were light pink, while a few were dark pink in color. By contrast, the color spectrum of the ion beam-induced mutants shifted from pink to white, yellow and orange. Furthermore, flower mutants induced by C ions showed complex patterns of coloration, and striped color types, that have never been obtained by Gamma-ray irradiation of this cultivar. It was suggested, therefore, that the mutation spectrum of flower color induced by ion beams is broad and that novel mutation phenotypes can be obtained.

Table 3. Mutation spectrum of flower color in carnation. Modified from [15].

Mutagen	Mutation frequency (x 10-1 %)									
	Light pink	Pink	Dark pink	Red	Salmon	Yelow	Cream	Stripe	Minute striped	Com-plex
EMS	0	5.2	0	1.0	0	0	0	3.1	0	0
Soft X-rays	1.7	8.4	0	3.4	0	0	0	0	0	0
Gamma-rays	1.7	2.6	0	1.7	0	0	0	0	11.3	0
Carbon ions	2.4	4.7	2.4	3.5	2.4	2.4	1.2	3.5	0	2.4

The mutation spectrum of flower color and flower shape of carnation was investigated by Okamura, *et al.* [15]. When the carnation variety Vital, whose phenotype is spray type with cherry pink flowers and frilly petals, was investigated, flower color mutants such as pink, white and red were obtained by X-ray irradiation, whereas the color spectrum of the mutants obtained by carbon ion irradiation was far wider and included pink, light pink, salmon, red, yellow, complex and striped types (**Table 3**). In addition, many kinds of round shaped petals were induced. These data indicated that ion beams can induce novel flower color and shape with high frequency.

Molecular mechanisms of mutation

C ion and electron-induced Arabidopsis mutants were compared at molecular (DNA) level [12, 13]. In the case of C ions, 14 loci out of 29 possessed intragenic point-like mutations, such as base substitutions, or deletions of several to 100 of bases (**Table 4**). Fifteen out of 29 loci however, possessed intergenic DNA rearrangement ('large mutations') such as chromosomal inversions, translocations, and deletions. In the case of electrons, nine alleles out of 12 loci had point-like mutations and three out of 12 loci had DNA rearrangements. Sequence analysis revealed that C ion-induced small mutations were mostly short deletions. Furthermore, analysis of chromosome breakpoints in large mutations revealed that C ions frequently deleted small regions around the breakpoints, whereas electron-irradiation often duplicated these regions. These results could imply that different types of non-homologous end joining pathways operate in response to the mutations induced by the two radiation types and that C ion-induced mutations are mostly likely to result in nulls.

Table 4. Characteristics of mutation induced carbon ions and electrons. Modified from [13]

	Carbon ions		Electrons	
	Point-like mutation	Large DNA arrangement	Point-like mutation	Large DNA arrangement
Mutation (TT, GL loci)	48%	52%	75%	25%
	(as 100%)		(as 100%)	
Deletion	79%		44%	
Base substitution	14%		44%	
Insertion	7%		11%	
		(as 100%)		(as 100%)
Deletion		65%		13%
Duplication		24%		75%

New varieties and mutants

Model plants

Several new *Arabidopsis* mutants, and the gene responsible for these mutations, were identified following ion beam mutagenesis [16]. Ultraviolet light-B (UV-B) resistant or sensitive mutants were obtained, and the genes responsible have been identified [17, 18, 19]. New anthocyanin-accumulating or anthocyanin-defective mutants were also obtained and some of the genes responsible have been identified [20, 12, 21]. A novel flower mutant, *frill1*, which has serrated petals and sepals, and the gene responsible for this phenotype have been found [22, 23]. A novel auxin mutant, the *aar1-1*, was also obtained [24]. In *Lotus japonicus*, which is used as a model leguminous plant, a novel hypernodulation mutant, named *klavier* (*klv*), was isolated following irradiation with helium ions [25]. Thus, not only new mutants but also new genes can be discovered using ion beam-induced mutagenesis.

Crops

In crops, ion beams have been used for inducing mutants resistant to major diseases. Mutants resistant to bacterial leaf blight and blast disease were induced in rice [26]. Higher mutation frequency was found in the ion beam treatment compared to Gamma-rays or thermal neutrons. Two mutant lines of yellow mosaic virus-resistant barley were found in a screen of ca. 50,000 M_2 families [27]. By exposure of tobacco anthers to ion beams, mutants resistant to potato virus Y have been obtained [28]. A high frequency (2.9~3.9%) of resistant mutants was obtained by irradiation with carbon and helium ions. Recently, banana mutants tolerant to black Sigatoka *in vitro* were induced by carbon ions [29]. Eight candidates from two varieties were selected for resistance in the field.

In addition to disease resistance, chlorophyll mutants were frequently observed in these crops. About 2.1% of the M_2 generation derived from barley seed exposed to carbon ions were chlorophyll-deficient mutants [30]. An albino mutant of tobacco was obtained by irradiation with nitrogen ions at an early stage of embryonic development [31]. A high frequency (11.6 %) of chlorophyll-deficient mutants, including an albino mutant, were obtained in rice by irradiation with neon ions [32]. A variegated yellow leaf mutant of rice, which could be induced by activation of endogenous transposable element, was induced by carbon ions [33].

Ornamental flowers

As described above, complex and stripe types of flower color have been obtained in chrysanthemum [14]. Morphological mutant phenotypes have also been observed in chrysanthemum [34]. One of these mutations, a reduced axillary flower bud mutant, was induced by carbon ions for the first time [35]. Recently, by re-irradiation of this mutant using carbon ions, the ideal characters of not only a few axillary flower buds but also low temperature flowering were obtained [36]. In addition to the carnation varieties described above [15], mutants of petunia with altered flower color and form have been induced by ion beams [37]. In rose, mutants with more intense flower colors or mutants in the number of petals, flower size and shape have been obtained [38]. Mutants induced by nitrogen or neon ions in *Torenia* include two groups of flower color mutants, ones that lack genes required for color or pigment production and others in which their expression is altered [39]. In cyclamen, ion beam irradiation of the tuber was found to be much more useful for changing flower characteristics than irradiation of other materials such as callus, somatic embryo, and so on [40].

Trees

Ion beams have also been used for generating mutants for tree breeding. Wax mutants and chlorophyll mutants such as Xanta and Albino were obtained in the forest tree, Hinoki cypress [41]. Shoot explants of *Ficus thunbergii* were irradiated with several kinds of ion beams to increase the capability of plants to assimilate atmospheric nitrogen dioxide [42]. A mutant variety with 40-80% greater capability to assimilate atmospheric nitrogen dioxide has been induced.

Figure 3 Novel varieties and mutants induced by ion beams. From top left to right: New chrysanthemum complex-color variety, "Ion-no-Seiko"; New rose-flower type carnation variety; New Osteosperumum variety "Vient Flamingo"; New chrysanthemum variety "Aladdin," which has reduced axillary flower buds. From bottom left to right: one-month old plants of *Arabidopsis* wild-type (upper rank) and UV-B resistant mutants (lower rank) under high UV-B condition; Flavonoid accumulating seed of the *Arabidopsis tt19* mutant; New barley mutant resistant to barley yellow mosaic virus; New variety "KNOX" of *Ficus thunbergii*, which has a high capability for the uptake and assimilation of atmospheric nitrogen dioxide.

Future prospects

Ion beams can be utilized as a novel mutagen to generate new mutants for basic research and to create new varieties as novel genetic resources. New genes, found using ion beams for mutagenesis, deliver new knowledge that can be exploited in biotechnology and molecular biology. In

the applied sciences, several flower varieties have already been commercialized following ion beam mutagenesis. For example, new varieties of carnation with different flower colors and/or shapes have been produced both in Japan and Europe, and new chrysanthemum varieties, with few axillary flower buds, named Aladdin and Aladdin 2, are being produced by more than 30 companies and associations. At present, not only Japanese but also other Asian scientists have started to use ion beams to induce mutations suitable for crop breeding. In the future, induced mutagenesis using ion beams will undoubtedly be used not only for model or decorative plants but also to develop plants that resist biotic and abiotic challenges or have potential for phytoremediation. These will have the potential to increase food security and resolve global environment problems.

ACKNOWLEDGEMENTS

This work has been achieved with the persistent help of the people involved in the research and development of ion-beam plant breeding. Therefore, I respectfully thank all the technical staff who established the ion beam irradiation systems and the researchers who undertook plant mutagenesis and breeding by the use of ion beams.

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Mutagenic Mechanisms of Ion Implantation in Plants

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Abstract

Ion beam implantation, as a new mutation technique, has been widely used in mutation breeding, and great achievements have been made for both the agriculture and fermentation industry. The mechanism underlying ion beam-induced mutagenesis has been a topic of research in recent years. In this paper, we focus on the initial physical process of ion implantation into organisms, noting that energy deposit, mass deposit and charge transfer of the implanted ions into target organisms are the main contributors to the biological effects. Recent studies of remote damage following ion beam implantation in plant samples are also included. It was observed that targeted ion implantation of the shoot apical meristem (SAM) of *Arabidopsis* embryos induces damage to the root apical meristem (RAM), indicating long distance systemic effects in intact organisms. Further studies showed that the generation of reactive oxygen species upon ion implantation could play important roles in the observed systemic effects.

Introduction

In the mid-1980 's, some pioneering work was carried out by the authors' laboratory, where 30 *keV* nitrogen ions were implanted into dry rice seeds. When the implantation dose was sufficiently high, yellow stripes were seen on the leaves of rice plants grown from the seeds, and these characteristics could be stably inherited by later generations. This phenomenon indicated that ion implantation could become a new mutation technique. In China, six key laboratories of ion beam bioengineering have been established in six provinces, and ion beam mutation is one of the most important projects undertaken by three key ministries' laboratories and three national biochemical engineering centers. These laboratories and centers have been equipped with 19 ion beam bioengineering facilities to serve mutation breeding for 42 plant breeding units in 22 provinces. From the authors' laboratory alone, 23 new varieties and 35 new strains of industrial microbes have been bred by ion beam mutation since 1994 [1]. These varieties cover almost all main crops, including rice, wheat, maize, cotton, soybean, tomato and sweet potato. The varieties are highly welcomed by peasants, due to their high yield and quality, and widely planted in certain areas along the Yangzi River in southern-eastern China. Of the microbial strains, two particularly excellent new stains, for producing vitamin C and arachidonic acid, respectively, increased sugar-to-acid transformation rate greatly, thereby increasing fermentation efficiency. Both strains have been adopted by manufacturers. Presently China is the main producer of vitamin C in the world, and the second largest supplier of arachidonic acid.

While ion beam mutation breeding has achieved great profit in its application, the interactions between the implanted ions and complicated organisms have also been studied intensively. In a previous publication [2], the authors proposed that a combination of energy absorption, mass deposition, and charge transfer of energetic ions in the seeds resulted in the biological effects. Since then, scientists have been trying to demonstrate the interactions between the energy, mass and charge of the implanted ions and organic molecules, and obtained some significant results. A key question is the traveling range of the implanted ions in the seeds. According to stopping power theory for ions penetrating into dense matter, e.g. metal and semiconductor, the range of incident ions with energy 30-200 *keV* cannot exceed 1μm, but a typical thickness of the rice seed coat is 40-200μm. It seems impossible that ions with such a low energy level can penetrate through the coat. To address this question, the National Natural Science foundation of China supported a series of studies in this field. It has been discovered, through various experiments, that the penetrating depth of 30-200 *keV* ions in plant seeds can be in the range of 60-135μm, and the damage distance can be as far as 800μm [1].

Recently, studies on the mechanisms of mutagenesis by ion beam mutation have seen substantial progress. It has been observed that targeted ion implantation of shoot apical meristems (SAM) of *Arabidopsis* embryos induces long-distance systemic effects on root apical meristems (RAM). This provided important new information for understanding the effects of ion beam mutagenesis.

Here, we focus on the interactions between the implanted ions, biomolecules and plant seeds, discussing the ion beam-induced mutation from two main aspects, the original physico-chemical process and the systemic effects.

Initial process of ion implantation into organisms

To reveal the interaction process of the implanted ions with complicated biological objects, some simplified models, which use simplified organic molecules or botanical slices consisting of complicated biological objects, were used as targets. Using these models, an initial process of ion implantation into organisms could be studied independent of subsequent or final biological effects.

Energy loss features [2]

According to stopping power theory, the total energy loss per unit path of a single incident ion in the target material is calculated by:

$$-\frac{dE_1}{dx} = N\left[S_n\left(E_1\right) + S_e\left(E_1\right)\right] \quad (1)$$

where E_1 is the incident ion energy, N is the number density of the target material, and $S_n(E_1)$ and $S_e(E_1)$ are the nuclear stopping and electronic stopping powers, respectively.

The basis of (1) is the assumption of a continuous, homogeneous distribution of the atomic volume density, which is much greater than the

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Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009, **220-222**

ion range. For organisms, there are a lot of the free voids, e.g. biological holes or channels in organisms, which can be considered to be transparent to the implanted ions in vacuum. Furthermore, the dimensions would be larger and larger with increasing doses of the implanted ions, or new holes or channels could be produced. So, the target atom volume density along the ion incident direction after implantation to a certain dose is a periodic function. In a period interval (-l, l), atom volume density can be regarded as a Fourier series, and after operation the total energy loss of a single incident ion in the target material is calculated by:

$$-\frac{dE_1}{dx} = \left\{\frac{(1-\alpha)}{2} + \sum_{n=1}^{\infty}\frac{N}{n\pi}\left[\left[\cos n\pi - (-1)^n\right]\sin n\pi x/l - \sin n\pi \cdot \cos n\pi x/l\right]\right\} \times [S_n(E_1)+S_e(E_1)] \quad (2)$$

where E_1 is the incident ion energy, N is the number density of the target material, and $S_n(E_1)$ and $S_e(E_1)$ are the nuclear stopping and electronic stopping powers, respectively. α is related to the ion implantation dose and the voids fraction in organism, calculated by:

$$\alpha = \frac{2rD}{N_A} + 2\eta - 1 \quad (3)$$

where D is dose of the implanted ions, N_A is the target area density. η is the vacancy volume intrinsically in the organism (e.g. for crop seeds, $\eta > 10\%$), r is a coefficient related to the particle emission induced by the ion, electron sputtering, and chemical sputtering. The coefficient r is considerable. For example, the emission coefficient r is 290 lactamine molecules/ion or 3.8 × 103 atoms/ion with nitrogen ion sputtering on a lactamine film, and is 3 to 4 orders of magnitude greater than that of for the elements of C, H, N and O calculated using classical collision cascade theory.

It can be seen from eq. (2) that fluctuation of the incident ion energy loss along the direction of the ion trajectory is a feature of beaded energy deposition, because of the voids in the target organism. Ion beams can etch away the coat from the surface of the biological sample and dig paths that could connect the voids (that in a natural situation can be isolated from each other) in the direction of ion incidence. Thus, later incident ions can penetrate longer and longer distances until finally they enter the embryonic cells, as in the case of ion implantation into crop seeds.

Mass deposit effects

For ion implantation of organisms, when the ion energy is decreased down to chemical reaction range, the implanted ions are deposited in the target, and may react with nearby atoms and molecules. The process can be demonstrated by nitrogen ion implantation into NAA (Naphthyl Acetic Acid, with MW of 186) [3]. There is no nitrogen atom in the molecular structure of NAA. After nitrogen cation implantation into NAA, there are new molecular structures in the implanted NAA sample analyzed by GC-MS. Its mass spectrum suggests a molecular weight of 267g/mol. This new molecule could contain a nitrogen atom according to chemical valence theory (**Fig. 1**). This means that ion implantation not only induces damage of the target molecules, i.e. producing molecular fragments, but also results in the formation of new molecules because of reaction between displaced atoms, interstitials or fragments with the implanted ions if they are active elements.

At the same energy, Fe^+, P^+ and N^+ with doses of 1×10^{13}, 1×10^{14}, 1×10^{15} and 1×10^{16} ions/cm^2 were separately implanted into *Arabidopsis thaliana* seeds. cDNA-AFLP analysis was performed to show variations at the mRNA level and genetic stability for three generations (**Fig. 2**). The gene variation rate of the M_0 generation implanted by nitrogen ions was the highest among the three types of ions, reaching 8.15%. Some gene variation was repaired in the successive generations for all three types of ions, but the gene mutation rate of nitrogen ion implantation was also the highest in M_2. These results illustrate that the implantation of ions with different mass could lead to biological effects at different levels.

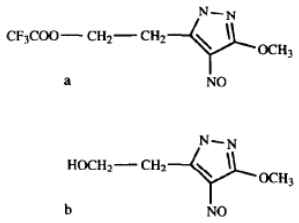

Figure 1 The structure of main product in ion implantation of α-NAA. (a) derivatives of product; (b) product.

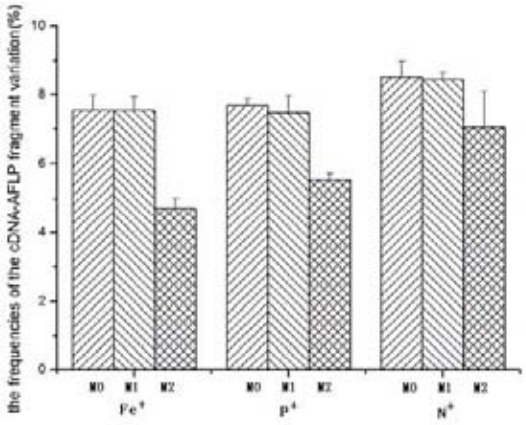

Figure 2 The frequencies of the cDNA-AFLP fragment variation in the M_0, M_1, M_2 generation Arabidopsis after Fe^+, P^+, N^+ ion implantation (provided by Prof. G. F. Zhang).

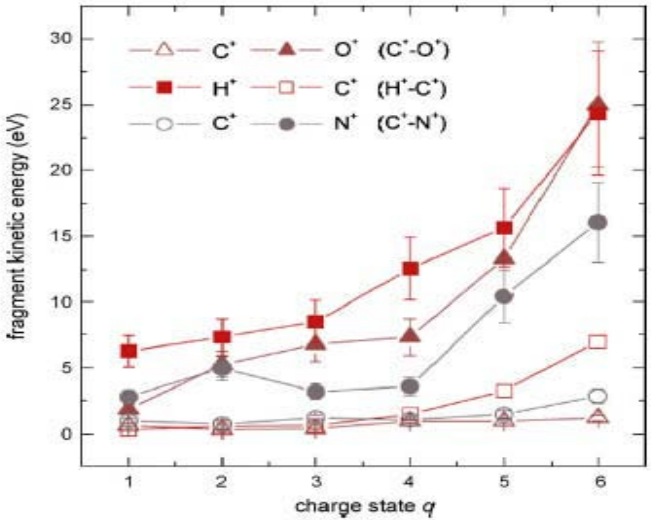

Figure 3 Kinetic energies of atomic fragment ions from 4 keV/amu (C+: 2 keV/amu) Cq+-induced fragmentation of thymine [4].

Charge transfer effects

In terms of ion-sputtering bio-molecules, the energies of the ion-induced fragment ions and emitted electrons are particularly important in explaining mechanism of long-distance damage in ion implantation of an organism (see Section 4). If the energy of emitted fragments is sufficiently high, these fragments will induce further damage to surround-

ing molecules. **Fig. 3** shows that the energies of fragments are depend on the charge number of incident ions [4].

Since biological organisms are not good electrical conductors, the accumulated surface charge is not immediately released. Instead, the charge is maintained for a time long enough for changes in the electrical characteristics of the sample surface to be examined by a capillary-electrophoresis method. The accumulation of surface charge not only affects the electrical characteristics of biological organisms, but also may lead to changing an electrostatic field across the cellular membrane, influencing various complicated biochemical processes, further resulting in cellular damage.

The charge transfer effects of ion implantation into *Arabidopsis thaliana* seeds were studied using H^+, O^+ and O^- with doses of 1×10^{13}, 1×10^{14} and 1×10^{15} ions/cm^2 at a same energy level. Peroxidase activity, isozyme, semi-quantitative PCR and Western blotting analyses indicated that peroxidase level, protein contents, and enzyme activity caused by ions with higher charges were two to four times greater than that of the control group. However, the difference of these effects caused by ions with different charge numbers became less significant when the implantation dose increased. **Fig. 4** shows that at the mRNA level, the expression of POD isoenzyme is clearly dependent on the ion charge state.

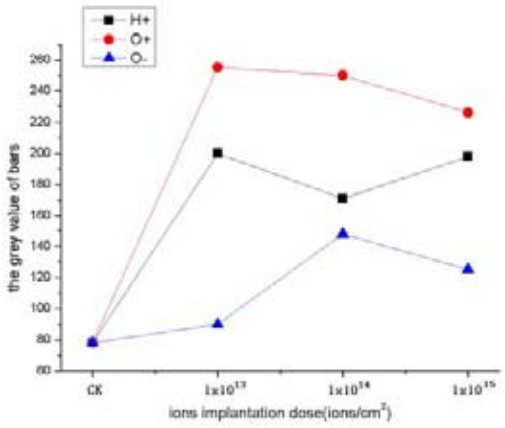

Figure 4 mRNA expression of POD isoenzyme after H+, O+, and O- ion implantation. (Provided by Prof. G. F. Zhang)

Long- distance damage induced by ion implantation

The integrity of the embryo in the *Arabidopsis thaliana* seed is shown in **Fig. 5** (middle). The average embryo was measured to be 300-500μm long, 200-270μm wide, and 50-70μm thick. The SAM (shoot apical meristem) and RAM (root apical meristem) cell groups of an *Arabidopsis* seed (**Fig. 5**, left) are almost entirely responsible for postembryonic development to elaborate plant architecture. The SAM and RAM are pivotal in generating a series of highly reproducible stages that imply tight control of the orientation and frequency of cell division as well as cell morphology and differentiation in development. The SAM is responsible for development of the aerial parts of the plant, while the RAM is responsible for development of the subterranean root system.

The right panel in **Fig. 5** shows the 5×5 etching spots on CR-39 after being hit by 1,000 protons. The diameter of each etching spot, showing the damaged area by proton irradiation, is about 9μm. Such a target resolution allows precisely targeted irradiation of cells in the defined SAM area of the intact embryo and ensures that no cells located more than 10 micrometers away receive any radiation exposure. It is impossible that protons could hit at cells in the RAM which is located ~150μm away from the SAM.

To determine the radiation-induced long-distance damage effects, embryos irradiated at the SAM and un-irradiated controls were examined for their root development. The density and length of the root hairs of seedlings grown from SAM-irradiated and control embryos were measured at day five post-irradiation. The root hair number of SAM-irradiated seedlings was 11 ± 3, significantly lower than un-irradiated controls (16 ± 2, $P < 0.01$), and the root hair length of irradiated seedlings (0.26 ± 0.14mm) was only half of that of un-irradiated controls (0.58 ± 0.06 mm, $P < 0.01$). Similarly, the primary root elongation and lateral root initiation of SAM-irradiated seedlings were significantly inhibited. The mean length of the primary roots and the number of lateral roots per centimeter of primary root of irradiated seedlings were about 2.95 ± 0.46 mm and 0.94 ±0.31 mm, respectively, significantly shorter or less than that of the controls (4.35 ± 0.53 mm for the length of primary root, $P < 0.01$, and 1.35±0.32 for the number of lateral root per centimeter of primary root, $P< 0.05$) [5]. As mentioned above, 1,000 protons aimed at the defined SAM target could not irradiate the RAM. These results indicate that the non-irradiated RAM undergoes damage, leading to the inhibited root pattern development.

How the SAM irradiation induces damage in the RAM of the *Arabidopsis* embryo is not clear. In the present study, a treatment with DMSO recovered the primary root length compared with the un-irradiated controls, indicating that ROS (reactive oxygen species) induced by SAM-irradiation, or probably ROS-related auxin and auxin-dependent transcription processes, might be involved in radiation-induced SAM long-distance systemic responses.

Discussion

In Section 2 we reviewed the primary effects of implanted ions on organisms. Similar to other radiation, there are many coexisting factors, including energy, mass and charge of the implanted ions, affecting the biological effects, making it difficult to distinguish between direct and indirect effects of these factors. In Section 3 we reported recent studies that there are remote systemic responses in ion implantation into plant seeds. These studies also bring forward new questions. What is the connection between initial process of ion implantation and the remote effects? It was suggested in the experiments that ROS could be one of the factors, and there were fragment molecules and radicals produced in ion implantation into organisms. What other factors or signaling molecules would be induced and emitted/transferred in ion implantation into organisms, and how could the signals be transferred to longer distance from the irradiated site?

ACKNOWLEDGMENTS

The authors would like to thank Prof. G. F. Zhang and Dr. G. Yang for providing their experimental results.

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Zinc Finger Nuclease-Mediated Gene Targeting in Plants

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Abstract

Zinc finger nucleases were used to facilitate homology driven repair and site-specific transgene integration in transgenic tobacco cell cultures. A target DNA sequence containing a non-functional, partial 3' PAT gene sequence flanked by zinc finger binding sites was stably integrated into BY2 suspension cultures using *Agrobacterium*-mediated transformation. A transgenic event containing a single integrated copy of the target sequence was used for gene targeting through co-transformation with two different *Agrobacterium* strains containing: i) donor DNA sequences comprising the 5' partial DNA fragment necessary to correct the non-functional PAT gene flanked by sequences homologous to the pre-integrated target DNA and ii) DNA that encoded a zinc finger nuclease that specifically recognized binding sites within the pre-integrated target. Two gene targeting strategies differing with respect to the distance between the zinc finger binding site and the homologous sequences were used. Gene targeting was demonstrated for both strategies as evidenced by the re-constitution of a functional PAT gene and was confirmed via molecular and biochemical analyses. Sequencing of recombined DNA confirmed that PAT gene reconstitution resulted from homology-driven repair at the zinc finger nuclease cleavage site. However, imperfect recombination resulting from non-homologous processes was also observed.

Introduction

Gene targeting can be used for making site-directed mutations, gene editing, deleting or inserting DNA sequences to pre-determined loci in the genome. Unfortunately, gene targeting in plants is not yet routine [1]. Attempts to enhance gene targeting efficiency in plants have included the use of negative selectable markers to enrich for cells that have undergone rare targeted integration [2] and over-expression of genes encoding proteins believed to be involved in natural recombination processes [3]. Nonetheless, the overwhelming occurrence of random DNA integration via non-homologous processes compared to homology-directed repair appears to be a major limitation to the routine application of gene targeting in plants [4].

Most recently, substantial increases in the frequency of gene targeting have been observed following the induction of DNA double stranded breaks in host cells and the apparent stimulation of cellular repair mechanisms [5]. Restriction enzymes whose recognition sites are rare in the plant genome have been shown to stimulate gene targeting following the formation and repair of DNA double stranded breaks in the host DNA [6]. Strategies to achieve targeted DNA double stranded breaks have been developed by fusing zinc finger DNA binding proteins with sequence-independent nuclease domains derived from Type IIS restriction endonucleases [7-8]. Site-specific mutagenesis in Arabidopsis [9] and targeted transgene integration in tobacco [10] using zinc finger nuclease-mediated double stranded break formation have been demonstrated. In addition, endogenous gene correction frequencies up to 18% have been observed in human cell cultures following zinc finger nuclease-mediated gene targeting [11].

In the present study, a target construct, comprising zinc finger nuclease binding sites and a non-functional partial selectable marker gene, was stably integrated into tobacco cell cultures. Efficient gene targeting was demonstrated through the correction of the selectable marker gene following the co-transformation with a zinc finger nuclease gene and donor DNA comprising sequences capable of complementing the non-functional marker gene and homologous to the integrated target construct.

Materials and Methods

Vectors

The following four vectors were designed and constructed in this study:

Target vector

The target construct contains the following five components as shown in **Figure 1A**: i) a hygromycin phosphotransferase (HPT) expression cassette comprising an *A. thaliana* ubiquitin-3 (ubi-3) promoter [12] driving the *E. coli* HPT gene [13] terminated by an *A. tumefaciens* open reading frame-24 (orf-24) 3' untranslated region (UTR) [14]; ii) homologous sequence-1 (Homo 1), consisting of the *N. tabacum* RB7 matrix attachment region (MAR) [15]; iii) a β-glucuronidase (GUS) expression cassette containing a Cassava Vein Mosaic Virus (CsVMV) promoter [16] driving a GUS gene [17] terminated by the *A. tumefaciens* nopaline synthase (nos) 3'UTR [18]; iv) homologous sequence-2 (Homo 2), consisting of *A. thaliana* 4-coumaroyl-oA synthase (4-CoAS) intron-1 (Locus At3g21320, GenBank NC 003074) and; v) a *S. viridochromogenes* phosphinothricin phosphotransferase (PAT) [19] 3' 256 bp partial gene fragment terminated by *A. tumefaciens* ORF-25/26 3' UTR [14]. Two types of zinc finger binding sites, BS-1 and BS-2, were integrated into the target sequence. One binding site (BS-1) was inserted downstream of the CsVMV promoter and fused with the GUS coding sequence at the N-terminal. Two copies of a second binding site (BS-2) were placed next to Homo-1 and Homo-2, respectively. The transformation vector (pTARGET) comprising the target sequence is shown in **Figure 1A**.

Donor vector

The donor DNA construct consisted of Homo 1 from the *N. tabacum* RB7 MAR [15], a full-length *A. thaliana* ubi10 promoter [12], 299bp of 5' partial PAT gene coding sequence [19] and Homo 2 from *A. thaliana* 4-CoAS intron-1 (Locus At3g21320, GenBank NC 003074). Both Homo-1 and Homo-2 in the donor vector were identical to the corresponding Homo 1 and Homo 2 in the target vector (pTARGET). The transformation vector comprising the donor sequence (pDONOR) is shown in **Figure 1B**.

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Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009,**223-226**

Zinc Finger Nuclease

The zinc finger-Fok1 fusion protein genes were driven by a CsVMV promoter and 5' UTR [16]. Also included in the cassettes were *N. tabacum* osmotin 5' and 3' UTRs [20]. A schematic representation of the ZFN-1 and ZFN-2 transformation vectors (pZFN-1 and pZFN-2) is shown in **Figure 1C**.

Positive Control

A vector containing an intact PAT gene expression cassette comparable with the expected homologous recombinants included the *A. thaliana* 4-CoAS intron-1 (Locus At3g21320, GenBank NC 003074) inserted at the 299/300bp of the 'complete' PAT coding sequence [19]. The resulting positive control vector (pCONTROL+) which comprised the intron-containing PAT gene driven by the *A. thaliana* ubi10 promoter and terminated by the *A. tumefaciens* orf25/26 3' UTR is shown in **Figure 1D**.

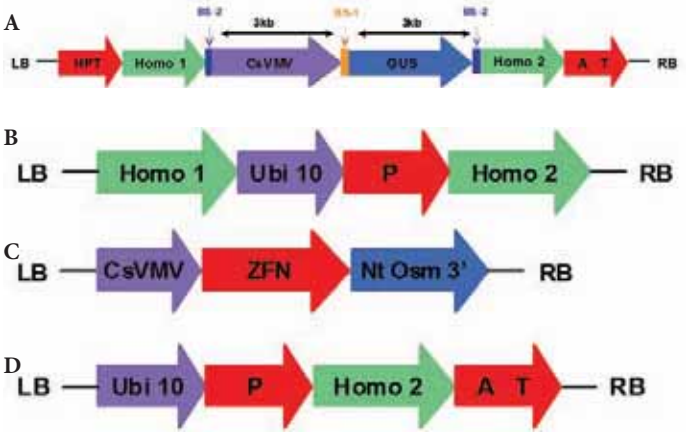

Figure 1 Schematic representation of vectors. **(A)** the integrated target vector pTARGET; **(B)** donor DNA, pDONOR; **(C)** zinc finger nuclease 1 (ZFN-1, pZFN-1) and 2 (ZFN-2, pZFN-2); **(D)** positive control pCONTROL+.

Transformation

Target Sequence Integration

BY2 tobacco cell suspension cultures were used for *Agrobacterium*-mediated transformation to stably integrate the target sequence. The BY2 cell suspension cultures were maintained in media containing LS basal salts (PhytoTechnology Labs M524), 137.4 mg/L K_2HPO_4, 30 g/L sucrose, 2.22 mg/L 2,4-D, 1 mg/L thiamine-HCL, 100 mg/L myo-inositol and 0.5 g/L MES at a pH of 5.7. The BY2 cells were sub-cultured every seven days by adding 40 mL of fresh LS-based medium to 1 mL packed cell volume (PCV). The BY2 cell suspension culture was maintained in 250-mL flasks on a rotary shaker at 25°C and 125 RPM.

To generate transgenic BY2 cell cultures with the integrated target sequence, a four-day post sub-culture tobacco suspension was divided into 10-12 four mL aliquots which were co-cultivated in 100 x 25 mm Petri dishes with 100 µL *Agrobacterium* strain LBA4404 harboring pTARGET grown overnight to an OD600 ~1.5. Dishes were wrapped with parafilm and incubated at 25°C without shaking for three days after which 11 mL of LS medium containing 500 mg/L carbenicillin. Following re-suspension of the tobacco cells, 1 mL suspension was dispensed onto 100 x 25 mm plates of appropriate base medium containing 500 mg/L carbenicillin and 200 mg/L hygromycin solidified with 8 g/L TC agar, and incubated unwrapped at 28°C in the dark. This resulted in 120-144 selection plates. Individual hygromycin-resistant isolates appeared 10-14 days after plating and were transferred to individual 60 x 20 mm plates (one isolate per plate) where they were maintained as callus on a 14-day subculture schedule until needed for analysis and subsequent re-transformation experiments.

Gene Targeting

A hygromycin-resistant, transgenic cell culture, BY2-380, containing a single, full-length integrated copy of the target sequence, was selected and used to re-initiate suspension cultures. *Agrobacterium*-mediated transformation of BY2-380 was performed as described above. For each experiment, 10 co-cultivation plates were generated as follows: one plate comprised cells co-cultivated with 100 µL of an Agrobacterium strain harboring pDONOR (donor DNA); one plate was co-cultivated with 100 µL of an *Agrobacterium* strain harboring pCONTROL+ (intron containing PAT selectable marker, the positive control); four plates were co-cultivated with 50 µL of an *Agrobacterium* strain harboring pDONOR and 250 µL of an Agrobacterium strain harboring pZFN-1; and four plates were co-cultivated with 50 µL of an *Agrobacterium* strain harboring pDONOR and 250 µL of an *Agrobacterium* strain harboring pZFN-2. Following co-cultivation, the cells were plated out on LS medium containing 500 mg/L carbenicillin and 15 mg/L Bialaphos®. Individual Bialaphos®-resistant isolates appeared 2-4 weeks after plating and were transferred to individual 60 x 20 mm plates (one isolate per plate) where they were maintained as callus on a 14-day subculture schedule until needed for analysis.

Analysis

Genomic DNA Extraction and Quantification

Genomic DNA was extracted from tobacco calli and cell suspension cultures using DNeasy 96 Plant kit (Qiagen, Valencia, CA USA) and quantified using PicoGreen ds DNA Quantitation kit (Molecular Probes, Eugene, Oregon USA). An aliquot of 2 µL extracted genomic DNA was checked through agarose gel electrophoresis to ensure the DNA quality.

PCR and Sequence Analysis of Target and Recombinants

To confirm the full length of target sequence in the selected target lines, nested PCR was performed using Takara LA Taq polymerase (Takara, Japan). To further confirm the sequence in the selected target lines, The PCR products were cloned into pCR-Blunt II TOPO vector (Invitrogen, Carlsbad, California) and sequenced by Cogenics (Houston, Texas).

To confirm the herbicide resistance events from site-directed recombination experiments, all putative recombination events were analyzed by PCR assay initially with primer pair of TAAGGATCCAACCATGGCTTCTCC and AGATCTGGGTAACTGGCCTAACTG. To further enhance the sensitivity of assay, a nested PCR was then performed using primer pair of TACCCTTGGTTGGTTGCTGAGGTT and GAAGGCCTATAACAGCAACCACAG. To confirm if the recombination was occurred on site of target sequence rather than off site due to ectopic recombination, all events that were positive on initial PCR reaction were further analyzed through nested PCR.

The 5' end of the recombined sequence was analyzed through another set of nested PCR reactions using primer of GTGTAGAAGTACTCGCCGATAGTG) and GACGACTAGGTCACGAGAAAGCTA in the first PCR, and CCATGTT GGCAAAGGCAACCAAACandTGATAAACACGACTCGTGTGTGCC in the nested PCR reaction. A subset of recombinant events were further analyzed by PCR that amplifies a DNA fragment across the entire recombined region, from the 3' end of the HPT gene to the 3' end of the PAT gene in the target sequence. This PCR analysis can eliminate the possibility that the positive PCR results in the above described reactions resulted from contamination of positive control, i.e., the intron-containing PAT expressing vector. To further confirm the sequence of the PCR products from recombinant events, the PCR fragment was purified from the gel using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and then either sequenced directly using the Dye terminator Cycle Sequencing Kit (Beckman Coulter) or sub-cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, California) then sequenced using the Dye terminator Cycle Sequencing Kit.

Results and Discussion

Production, Screening and Characterization of Target Events

A total of 224 hygromycin-resistant transgenic events were generated from BY2 suspension culture cells co-cultivated with *Agrobacterium* harboring the target sequence. Of the 123 events derived from BY2, 224 displayed GUS expression. Southern blot analysis was performed on all 123 GUS-positive isolates to determine the copy number of the integrated target sequence. Two probes representing the PAT and HPTII coding sequences were used to ensure there was no additional partial target sequence integrated into the selected event. One of the events (BY2-380) which showed GUS expression and contained a single full-length copy of the target sequence was selected for suspension culture re-establishment and subsequent re-transformation.

Gene Targeting

The two strategies deployed for zinc finger nuclease-mediated gene targeting are outlined in Figure 2. In one strategy the zinc finger binding site (BS-1) was centrally located in the target construct with ~ 3 kb of non-homologous sequence between BS-1 and the nearest homologous sequence. In the presence of ZFN-1 and donor DNA, which contained homologous sequences identical to that in the target, the 5' partial PAT gene along with its promoter, replace the entire ~ 6 kb fragment between the homologous sequences in the target through simultaneous gene replacement and addition whereby the two partial PAT gene sequences reconstitute a functional PAT gene, resulting in PAT expression and an herbicide resistance phenotype. A second strategy involved two zinc finger binding sites (BS-2) directly downstream and upstream of the left and right side homologous sequences, respectively. Between the two BS-2 sites was ~ 6 kb of DNA sequence. Expression of the ZFN-2 gene results in excision of the ~ 6 kb fragment and, in the presence of donor DNA, PAT gene correction via gene addition.

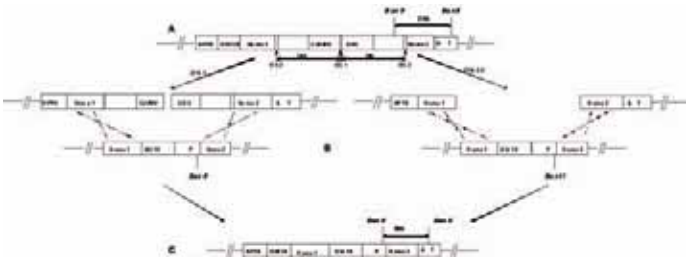

Figure 2 Predicted site directed gene replacement and addition mediated by zinc finger nucleases, ZFN-1 and ZFN-2.

Table 1. Summary of Re-transformation of Target Cell Cultures with Zinc Finger-Fok1 Fusion Protein Genes and Donor DNA.

Treatment	# of Selection Plates	# of Herbicide Resistant Events	Ave. # of Events per Selection Plate
Positive control (pCONTROL+, PAT gene only)	46	536	11.7
Donor DNA only (pDONOR)	46	0	0
Donor DNA + ZFP-1 (pDONOR + pZFN-1)	214	43	0.2
Donor DNA + ZFP-2 (pDONOR + pZFN-2)	214	47	0.2

Table 1 summarizes the results of retransforming target culture BY2-380 with zinc finger nuclease-containing constructs pZFN-1 and pZFN-2. Out of 214 selection plates, 43 and 47 herbicide resistant isolates were obtained from the re-transformation with the donor DNA together with ZFN-1 and ZFN-2, respectively. When compared to the re-reformation with the positive control, the intron-contained PAT gene, the frequency of recombinants was 1.7% for both ZFN-1 and ZFN-2. No herbicide resistant isolates were obtained from the re-transformation with the donor DNA only.

All putative recombination events obtained following herbicide (Bialaphos®) selection were first analyzed by PCR using primers specifically for amplifying a DNA fragment spanning the re-constituted PAT gene, more specifically, the forward primer anchored to the 5' end of the PAT coding sequence in the donor DNA and the reverse primer anchored to the 3' end of the PAT coding sequence in the target DNA. A 2.3kb PCR fragment would be obtained only if the two partial PAT coding sequences were joined together through recombination. Figure 3 shows the PCR results from a set of 20 isolates selected from the co-transformation of BY2-380 target line with ZFN-1 or ZFN-2 gene along with donor DNA. As predicted, a 2.3kb PCR product was obtained from all of these isolates selected (**Figure 3A-I**). The 2.3kb PCR product from multiple independent isolates representing those derived from both ZFN-1 and ZFN-2 transformations were purified from agarose gels, cloned into the pCR2.1 TOPO vector, and then sequenced. The sequencing results confirmed that all of the PCR products cloned in the TOPO vector contained the recombined sequence as predicted, including the 5' and 3' partial PAT gene sequences with the intervening 4-CoAS intron-1. This result confirmed the predicted recombination for both strategies tested and exemplified gene targeting via zinc finger nuclease gene expression.

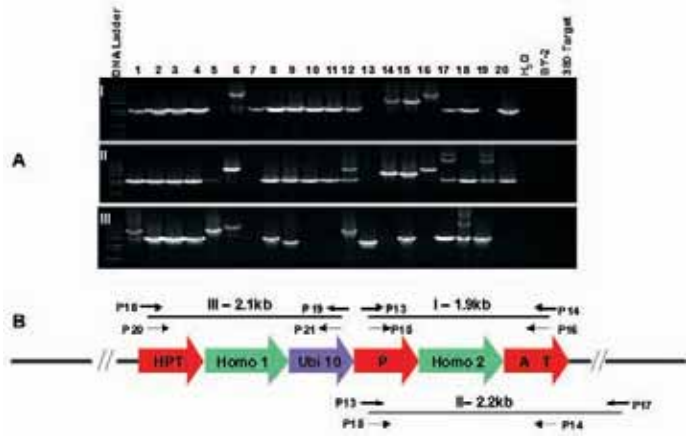

Figure 3 PCR confirmation of inter-chromosomal recombination events from BY2-380 target line. 1-16: recombinants derived from ZFN-2 treatment, 17-20: recombinants derived from ZFN-1 treatment. **(A)** PCR amplification of recombinants: I. 3' of predicted recombinants from 5' partial PAT to 3' partial PAT gene. II. 3' of predicted recombinants from 5' partial PAT to the flanking genomic sequence at 3' end. III. 5' of predicted recombinants from HPT to 5' partial PAT gene. **(B)** Predicted recombinants.

To further characterize the putative recombination events, two additional nested PCR reactions that allowed for a detailed analysis of the recombinant sequences were carried out (**Figure 3B**). In the four samples generated from co-transformation with ZFN-1 and donor DNA, two of the samples displayed amplification of the expected size fragment in all three reactions (lanes 17 and 18). The other samples displayed no amplification in one of the three PCR reactions. Of the 16 samples generated from co-transformation with ZFN-2 and pDONOR, five amplified the expected size fragment in all three reactions (lanes 2, 3, 4, 9 and 15). Six of the samples displayed amplification of the expected size fragment in the PCR reactions associated with the corrected PAT gene sequence. In one of the samples only the 5' reaction resulted in the expected size

fragment (lane 13). The remaining samples had either no amplification or altered size in at least one of the reactions. Thus, among this set of 20 Bialaphos®-resistant isolates 7 of the 20 samples gave results consistent with high fidelity recombination across the entire integrated sequence.

Conclusion

Zinc finger-mediated gene targeting was achieved in tobacco cells. This conclusion is based on the fact that functional herbicide resistance was observed following re-transformation of target events (containing a partial 3' PAT gene) with donor DNA (containing the corresponding 5' PAT gene sequence) and PCR amplification of expected recombinant fragments was demonstrated. In addition, sequence analysis of PCR products confirmed recombination.

Zinc finger nuclease expression stimulates homology-driven repair in a large region surrounding a double strand break in tobacco suspension cells (~3kb on each side). This conclusion is based on the simultaneous gene replacement and addition strategy employed with ZFN-1.

Imperfect recombination apparently resulting from non-homologous DNA repair was also observed.

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Genetically Unstable Mutants as Novel Sources of Genetic Variability: The Chloroplast Mutator Genotype in Barley as a Tool for Exploring the Plastid Genome

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Abstract

The presence of clonally variegated seedlings was used as a criterion to isolate putative genetically unstable mutants (GUMs) from the M_2 or further generations arising from X-rays and/or chemical treatments applied to barley seeds. Analysis of seedlings in the glasshouse revealed that in some of the families isolated, a particular spectrum of mutant phenotypes was repeatedly observed over several generations of auto pollination. By reciprocal crosses it was noticed that some of these GUMs produced maternally-inherited changes and they were classified in two groups manifesting either a narrow or a wide spectrum of mutant phenotypes. One case of the latter, designated as a "chloroplast mutator" genotype, has been studied in our Institute since 1985. In several mutants obtained from this GUM, evidence of major plastid-DNA changes were not detected, but interestingly, sequencing of some plastid genes showed that single nucleotide mutations were present. Mutational changes, consisting in transitions T/A – C/G which were located at three different positions in the plastid gene *infA*, were detected in three independently-originated *albo-viridis* mutants. Additionally, one transition and one base insertion on the *ycf3 locus* were observed in a temperature-sensitive *viridis* type and one transition on the plastid gene *psbA* was observed in families selected for atrazine tolerance. Both the wide spectrum of mutants and the subtle DNA changes induced in this barley chloroplast mutator genotype, suggest that it can be an exceptionally valuable tool to explore the potential functionality of the otherwise highly conserved plastid genome.

Introduction

Induced mutation techniques have contributed impressive amounts of genetic variability to plant research and plant breeding. For the most-part, variability has been observed in nuclear genes, while little progress has been made in the induction of variability in genes of the plastid and mitochondria. Looking for novel sources of genetic variability that could fill this gap, we isolated several putative genetically unstable mutant (GUM) families originating from diverse X-ray and/or chemical treatments applied to barley seeds. Selection was based on the presence of clonally variegated seedlings (*striata* type) in M_2-spike progenies or in plant progenies of further generations. Seedling analysis carried out in the glasshouse over several generations of autopollination revealed that in some of the families isolated, a particular spectrum of mutant phenotypes was repeatedly observed, suggesting that some parts of the genome were differentially affected depending on the different GUMs. Reciprocal crosses and glasshouse screening allowed the detection of cytoplasmically inherited mutations in some of these GUMs, which could be clearly classified into two groups according to whether they induced either a narrow or a wide spectrum of mutant phenotypes, as previously proposed [1]. GUMs belonging to the last group seem to be more interesting to use as new sources of genetic variability. To our knowledge, this kind of experimental material has been reported several times in dicot species, but there is only one example in monocots [2]. So far, we have isolated only two GUMs of this type. In this paper we summarize results from molecular analyses of mutants isolated from one of them, previously reported as the barley "chloroplast mutator" genotype [2].

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Results

The barley chloroplast mutator genotype and the isolation of genetically stable chlorophyll deficient types

The barley chloroplast mutator genotype was previously described as a nuclear gene that when homozygous, induces several types of cytoplasmically inherited chlorophyll deficiencies [2]. It was postulated that the nuclear genotype responsible for the recurrent occurrence of plastome mutants was probably related to failures in a DNA-repair mechanism [2]. It was observed that homozygous mutator plants had a normal vigor phenotype, that the expression of the mutator activity was limited to a low percentage of F_2-seedlings from crosses using mutator plants as male parent and the phenotype was only manifested as narrow isolated streaks [2]. Chlorophyll-deficient types were mostly observed as clonally variegated plants, but in the F_3 and the F_4 some solid phenotypes were also observed at second-leaf seedling stage. In order to obtain genetically stable families, some of the chlorophyll mutants that were viable at the field nursery were backcrossed as female to wild type plants. Later on, selection of stable families was carried out in the F_3 and further generations. The first four genetically stable families showed particular chlorophyll deficient types and also differed in their genetic instability. They were designated as cytoplasmic lines (CLs) and a basic description was published [3].

No structural changes in plastid DNA were detected in four chloroplast mutator induced CLs

Extensive RFLP analysis showed no differences in the restriction patterns of CL1, CL2, CL3 and CL4 compared to the control genotype, suggesting that the barley chloroplast mutator did not induce major changes in plastid DNA [4].

Three different transitions on the *infA* plastid gene

CL2 is a chlorophyll deficient *albo-viridis* type that has a time-dependent phenotype mainly restricted to the upper part of the first leaf blade [3] and shows a pattern of greening and plastid development opposite to that usually observed in monocots [5]. Results from experiments in which embryogenesis occurred at different temperatures and with seeds with or without antibiotic treatments, indicated that CL2 syndrome produces a delay in plastid protein synthesis during embryogenesis and on this basis, the *infA* gene was proposed to be responsible for that syndrome [5]. This gene encodes a protein that is homologous to the bacterial translation initiation factor 1 (IF1) [6]. This is a highly conserved protein that belongs to the family of oligonucleotide binding fold

Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009,**227-228**

proteins, to which ribosomal protein S1 and the cold shock proteins CspA and CspB also belong [7]. The comparison of *infA* gene sequences found in wild type or CL2 seedlings showed a mutational change, it was a **T→C** transition at nucleotide 157 (GenBank AY488513), corresponding to a semi-conserved amino acid change of serine to proline at residue 52, which is a highly conserved residue of the IF1 protein in Poales [8]. Fifteen other CL2-like isolates from the same chloroplast mutator pool presented an identical mutation. However, on new pools made by crossing the mutator genotype as a male parent, two new CL2-like mutants showing maternal inheritance were isolated. One of these new mutants showed a **T→C** substitution at nucleotide 97 (GenBank AY743911), giving a conserved amino acid change from phenylalanine to leucine at the universally conserved residue 32 of the IF1 protein [8]. The third point mutation in the *infA* gene was recently identified (A.M. Landau and A.R. Prina, unpublished); it is an **A→G** transition at position 185 and it also corresponds to a highly conserved residue of the IF1 protein. To our knowledge, these three mutants carrying the CL2 syndrome are the first detected in the *infA* gene of higher plants and consequently, they can greatly contribute to the understanding of the functions of this plastid gene that are at present inferred from information in bacteria.

A transition and one-base insertion in *ycf3* locus

For CL3, a homogeneous light green (*viridis*) type [3] that is very sensitive to high temperature [9], *ycf3* and *ycf4* plastid *loci*, each encoding a different PSI-assembly chaperone, were postulated as candidate genes. In CL3 and in the wild type the sequences of *ycf4* were the same, while, in the *ycf3 locus* two differences were found, a transition (**T→C**) at position 528 and a base insertion (**T**) at position 150. Both changes were localized in one of the two introns of the *ycf3 locus*, intron 1, which interestingly resulted in a temperature-sensitive defective splicing (A.M. Landau, unpublished).

A transition on *psbA* gene

In order to test the generation of inheritable changes in traits other than chlorophyll deficiencies, we used selection experiments with atrazine as a model, due to the well-understood molecular basis of this character. Two atrazine-tolerant families were obtained after two generations of selection applied on a pool of mutator plants [10]. Molecular characterization was performed by PCR amplification of an internal fragment of the plastid gene *psbA*. The *BstXI* restriction pattern of the amplified fragments showed one band in the wild-type, but two in atrazine-tolerant plants. Sequence analysis of a 277 bp fragment showed a transition (**A→G**) at position 790 of the *psbA* gene, which created a *BstXI* restriction site, and corresponds to an amino acidic change of serine-to-glycine in the D1 protein of PS II. This result was in agreement with the molecular basis previously described for other atrazine-tolerant plants, such as rapeseed and several weed species [10]. As the two identical tolerant families were isolated from the same pool of plants carrying the mutator genotype it is not possible to determine the independence of the mutational events originating them.

Discussion and Conclusions

Based on more than 20 years of glasshouse and field observations in progenies from autopollination, reciprocal crosses and backcrosses, we can conclude that the barley chloroplast mutator genotype induces a wide spectrum of mutants with cytoplasmic inheritance, including several viable and normal vigor types. Homozygous mutator plants show a normal vigor phenotype, suggesting that no massive disturbances are induced by the mutator action [2]. The lack of major plastid DNA changes in CLs and the point mutations observed in plastid genes, suggest that the barley mutator genotype presented here is an important source of genetic variability allowing the induction of mutant alleles with remaining functionality. This enables the induction of allelic series, instead of gene knockouts, for the highly conserved plastid genes. This is exemplified by the induction of the three different viable *infA* gene mutants mentioned above, the first ones reported in higher plants [8].

It is important to state here that most of the mutants we isolated from the barley chloroplast mutator genotype were selected from a single pool of plants maintained for several generations by autopollination. The isolation of 15 CL2-like mutants carrying identical *infA* gene sequences from that pool of plants, alerted us to the necessity of handling the plant material in a more controlled manner, in order to avoid duplications of mutants arisen from the same mutational event [8].This is especially important for plastome mutants in higher plants for which the allelic tests used for nuclear genes are not available.

It must be remarked that the exploration of the plastid genome by postulating and sequencing candidate genes, as we have been doing so far, is limited to those genes that can be inferred from a particular mutant phenotype, or by using certain artificial selective pressures. During the last few years, the TILLING strategy (Targeting Induced Local Lesions in Genomes), which combines chemical mutagenesis with a powerful screening methodology [11, 12], has made allelic series of nuclear genes available and has become a powerful method for elucidating the function of nuclear genes in higher plants. In this context, the impact of the barley mutator genotype as a tool for exploring the plastid genome can be dramatically improved by developing a TILLING strategy targeted to the plastome.

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Mutagenesis of Genes for Starch Debranching Enzyme Isoforms in Pea by Zinc-Finger Endonucleases

H Hussain

Abstract

Starch debranching enzymes in plants are divided into two groups based on their ability to hydrolyze different substrates. The first group, pullulanases, hydrolyze α-1,6-glucosidic linkages in substrates such as pullulan, amylopectin and glycogen. The second group of debranching enzymes, isoamylases, hydrolyze glycogen and amylopectin and are not active on pullulan. Three isoforms of isoamylase and a pullulanase have been isolated from a cDNA library of *Pisum sativum*. These isoamylases have been characterized following their heterologous expression in *E. coli*. Based on the DNA sequence that encodes these debranching enzymes, a specific mutagenesis targeting these enzymes will be attempted. The technique involves the homologous recombination of DNA mediated by zinc-finger endonucleases. Vectors will be constructed to include a fragment that will modify these genes. Using this technique, it is hoped that null mutants for each enzyme will be created and the exact role of these enzymes for the synthesis and degradation of starch in plants will be elucidated.

Introduction

Synthesis of starch involves several major enzymes such as ADP-glucose phyrophosphorylase (AGPase), starch synthases (soluble and granule bound), starch branching enzymes (SBE) and starch debranching enzymes (DBE). Of the four main enzymes, the isoforms of starch DBE are the least described in terms of their exact role in the process of starch biosynthesis. Starch DBE can be divided into two major groups, namely isoamylases and pullulanases. The differences between these two groups are in their ability to hydrolyze different type of starch substrates. The isoamylases hydrolyze glycogen and amylopectin and are not active on pullulan. Genes encoding three different isoforms of isoamylases have been characterized in potato [1]. In addition, to the isoamylase isoforms from potato, similar isoforms from wheat have also been described [2]. These isoforms showed different catalytic properties towards starch substrates. The pullulanases hydrolyze α-1,6-glucosidic linkages in substrates such as pullulan, amylopectin and glycogen. Genes encoding pullulanases have also been described in several plants [2, 3]. This paper describes the identification of starch DBE from pea, including isoamylase isoforms and pullulanase. The data can be used for further characterization of starch biosynthesis in plants, such as using the gene sequence to produce null mutants. Mutational studies of pullulanase in *Zea mays* have been described [3]. However, no studies report the effects of mutagenesis of isoamylase isoforms and pullulanase on starch formation.

There are several techniques for mutagenesis of plants, such as TILLING methods, ion-beam implantation, and *in planta* transformation methods using *Agrobacterium*. Another method of plant mutagenesis is by homologous recombination of the targeted gene using zinc-finger endonucleases or zinc finger nucleases (ZFN). In this method, a specific gene can be targeted for mutation *in situ* leaving the rest of the genome unperturbed. This strategy has several advantages over gene addition procedures, which include the risk of mutations arising from random insertion, because the approach aims to incorporate exogenous DNA at a predetermined site in the chromosome. In addition, the exogenous DNA does not have to include a complete protein coding sequence or separate signals to ensure its expression because it is incorporated at an endogenous locus. Inappropriate tissue specificity, timing, level and duration of expression are not issues, because the targeted gene remains under normal, endogenous controls [4]. Thus, if targeted mutation can be accomplished with high efficiency, other genetic factors affecting the mutant can be ruled out. Therefore, based on the identification of starch DBE from pea as described here, we propose to develop a series of mutant plants harbouring frame-shift mutations (knockout mutants) for the starch DBE in pea.

Methodology

Methodology for identification of isoforms of isoamylase and pullulanase followed the methods that have been described previously [1]. They can be outlined as follows:

- Design of degenerate primers for isoamylase isoforms and pullulanase based on the sequences of these enzymes from other plant species that can be obtained from available databases.
- PCR of coding regions of isoamylase isoforms and pullulanase with degenerate primers.
- Screening of a pea embryo cDNA library using fragments obtained using the PCR described above.
- *In silico* characterization of nucleotide sequences including sequence alignments and structural determination.
- Heterologous expression of DBE genes in *E. coli* for enzyme analysis.
- Dinitro-salicylic acid (DNS) assay to determine debranching activities of isoamylase.

For the mutagenesis work, the methodology that is going to be employed will be based on the work described by Wright and co-workers [5]. The work is summarized as follows:

- Identify ZFNs target sites near the targeted locus within the gene of interest.
- Design and select zinc finger proteins (ZFPs) that recognize the chosen ZFN target sites.
- Convert the designed and/or selected ZFPs into ZFNs.
- Deliver ZFNs alone to induce a targeted double strand break (DSB) in starch DBE genes of normal cells and stimulate non-homologous end-joining to generate a pool of mutants, some of which will be frame-shift mutations resulting in functional deletion of DBE gene, i.e. knock-outs of DBE genes.
- Monitor for gene correction (or reverse mutagenesis) at the targeted gene loci using appropriate PCR techniques.

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Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009,**229-230**

- Monitor the resulting mutants so that the mutated fragment does not integrate elsewhere within the genome of the cell by appropriate PCR and hybridization techniques.

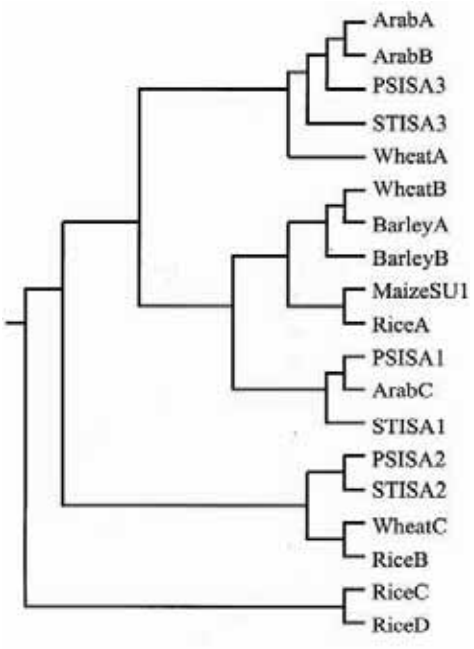

Figure 1 Phylogenetic tree of predicted amino acid sequences of isoamylase proteins from pea (PSISA1, PSISA2 and PSISA3) and other plants.

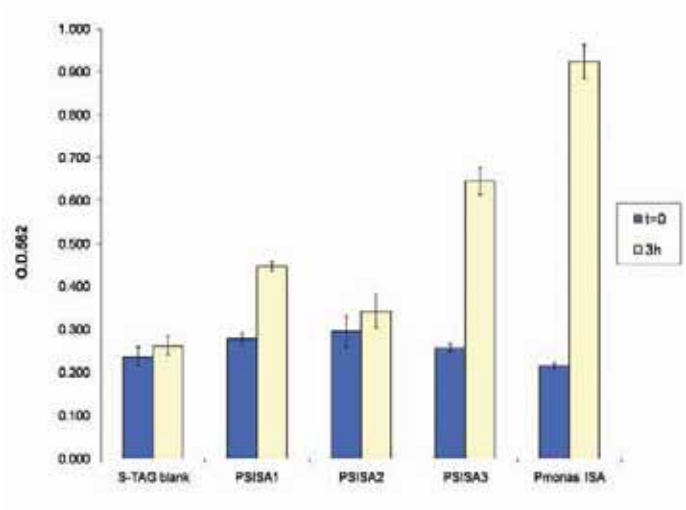

Figure 2 Debranching activities of pea isoforms of isoamylase expressed in *E. coli* tested using the DNS assay. PSISA1 and PSISA3 show the presence of activity, while PSISA2 did not show any significant difference to the control.

Results and Discussion

Comprehensive screening of a pea embryo cDNA library has shown that there are three different genes that encode isoamylases in pea (**Fig. 1**). Analyses of the primary structure of these isoamylase isoforms showed that two of them are most likely to have catalytic activity. However, one of the isoform, PSISA2 is unlikely to have the catalytic activity. This was further confirmed by the DNS assay using amylopectin as a substrate (**Fig. 2**). *In silico* analysis of the PSISA2 isoform also showed that it retained the characteristics to bind to glucans. Therefore, it is likely that non-active isoamylase isoforms might play a non-catalytic role during the formation or degradation of starch. *In silico* characterization of the pea pullanase showed that the translated amino acid sequence of this gene was similar to pullulanases isolated from other plants and bacteria. The amino acids sequence also suggested that the gene encoded a plastidial form of pullulanase similar to that found in spinach and rice. These results can be used to design oligonucleotides that can be used in gene targeting studies.

The ZFC-mediated gene mutagenesis is rapidly becoming a powerful and versatile tool for targeted genome engineering of many different organisms and cells, including plant and human cells. Previous studies of gene targeting using ZFN has shown successful application. Since data from the *in silico* characterization of isoforms of isoamylase and pullulanase showed the complete sequence of the pea genes, the process of producing ZFNs to induce double strand break is likely to be successful.

Conclusion

The screening of a pea cDNA library for gene sequences of starch debranching enzymes indicated the existence three different isoforms of isoamylase and one isoform of pullulanase. This is similar to results obtained for potato [1]. Further investigation of the exact roles of these enzymes in starch biosynthesis can be undertaken by null mutation, employing ZFN-technology. Gene targeting offers great promise for studying the functions of various genes through reverse genetics, which is sometimes difficult to achieve using other methods. It is hoped that the mutagenesis study can reveal more details of exact role of starch DBE in the synthesis and degradation of starch in plants.

ACKNOWLEDGEMENTS

The author wishes to thank IAEA and University Malaysia Sarawak for awarding grants to attend the symposium. Also, special thanks to Cathie Martin (John Innes Centre, UK) for her help with the work.

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Generation of New Rice Cultivars from Mature Pollen Treated with Gamma-Radiation

H Wang, S Qiu, J Zheng , L Jiang, H Huang & Y Huang*

Abstract

Two new high-quality and early-yielding *indica* rice cultivars, Jiahezaozhan and Jiafuzhan, have been developed, certified, and cultivated by farmers in the provinces of Southern China. These new rice varieties were created by a new mutation breeding technique in which mature rice pollen irradiated with Gamma (γ)-ray was used to produce parents for crossing. The optimal dose for the irradiation was approximately 46Gy. The cumulative effects of the mutations increased in advanced generations and most of the mutant traits became stable in the fifth generation. These results showed that the mutations generated by Gamma- radiation on mature rice pollen were largely of quantitative trait loci.

Key Words:

Rice (*O. sativa* L. indica);Mature pollen;γ-ray irradiation;Mutation breeding

Introduction

The creation of new germplasm resources, such as dwarf, cytoplasm male sterile (CMS) and photo-thermo-period sensitive genic male sterile (PTGMS) rice derived from natural mutations, has brought significant changes to rice breeding and production in China [1]. In contrast, few human-assisted (induced) mutations have resulted in the development of new plant varieties, despite ample demonstration that the various mutation techniques can induce mutation and create new genotypes for the generation of novel germplasm by mutation breeding [2].

We have used Gamma-radiation of mature rice pollen to generate mutants with novel traits. When the haploid rice pollen is irradiated by Gamma-rays, there are different degrees of damage to the male gametes in the pollen and the induced mutation has no chimerism. However, the traits exhibited by progenies generated from the fusion of male gametes and eggs are variable. Our data demonstrates that the mutant traits of progenies generated using this technique are inheritable. This technique has been exploited to generate new plant varieties. The high-quality early *indica* rice cultivars, Jiahezaozhan and Jiafuzhan, were developed by the combination of this mutation technology and sexual hybridization. Our data is consistent with previous findings that irradiation of rice mature pollen with Gamma-rays can produce a wide mutation spectrum of progenies [3], but contradicts the argument that the rice pollen was more resistant to irradiation and that the variation of traits in the second-generation was similar to that produced by conventional hybridization [4, 5].

Materials and Methods

Rice varieties: Rice varieties, Ma 85 and Bai 85 (*Oryza sativa* L. *indica*), were obtained from the Rice Research Institute of Fujian Academy of Agricultural Sciences, China (FJRRI). They are genetically stable, resistant to blast, and have a seed set rate of more than 90%. The grain was short and elliptical in shape and basically impossible to thresh.

Radiation source: The ^{60}Co radiation facility at FJRRI was used for the experiments.

Irradiation method: Spikes were collected in the proper time, placed in a glass Petri dish, and irradiated with Gamma-rays in doses of 0, 23, 46, 93, and 186Gy. The Petri dish maintains a moist environment, such that the floral glume of the rice spike can blossom and expose itself to others without dispersing the pollen. This method is practical and repeatable, and the pollen is still viable one to two hours after the radiation treatment.

Pollination and observation: Seeds were cut in half after a hot-water-emasculation treatment, pollinated with mature rice pollen irradiated by Gamma-rays, and placed in dark envelopes until maturity. First-generation hybrid seeds (H_1) were cultivated from a single plant. Each individual plant was harvested. Second-generation hybrid seeds (H_2) were grown from a single plant from each line of 60 plants. From the third-generation (H_3) on, every generation was also cultivated from a single plant of each line. Plants in each generation and group were observed closely for their height, heading stage, seed setting rate and grain shape.

Results and Discussion

Establishment of sexual hybridization technology for rice mature pollen irradiated by Gamma-rays

The life span of rice pollen is only a few minutes. In order to treat the mature rice pollen with Gamma-radiation, whole plants were used. Irradiation of cultured spikes stripped from tillers and kept with wet tissue was not effective, as it was difficult to locate the pollen and keep it viable. However, over the past years, we have successfully performed hundreds of radiation treatments by preserving the spikes in Petri dishes. This overcomes the technological bottleneck of Gamma-radiation treatment of the mature rice pollen and is routinely applied in our research.

Table 1. Mutant traits in H1 plants derived from mature pollen irradiated by different Gamma-ray doses.

Dose (Gy)	No. of individuals	Plant height (cm)	Heading stage (day)		Seed setting rate (%)
			Ahead of time	Postponed	
0	50	95-110	0	0	90
23	150	80-110	0	0	20-90
46	200	80-110	0	1-3	10-90
93	200	70-110	0	1-5	50-90
186	100	70-105	0	1-10	0.5-10

Mutant trait performance of H_1 plants derived from mature pollen treated with Gamma-radiation

Original plants of Ma 85 and Bai 85, with their glumes cut in half after a hot-water-emasculation treatment, were pollinated with mature rice

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pollen irradiated by Gamma-rays, and placed in dark envelopes until maturity. The H_1 was cultivated from a single plant and the plant height, heading stage and seed setting rate were recorded (**Table 1**).

The results showed that increasing radiation doses increased detrimental phenotypes in H_1 plants. The H_1 population traits showed no obvious difference to the control group, except a slight decrease of seed setting rate, when the radiation dose was 23Gy. However, with more than 46Gy dose, there were distinct inhibitory effects on the plant height, heading stage and seed setting rate of the H_1 population. However, for each treatment, the degree of inhibition differed between individual plants. It was obvious that the induced mutation of rice mature pollen irradiated by Gamma-rays was random.

Mutant trait variation of H_2 plants derived from mature pollen treated with Gamma-radiation

Because of different seed setting rates, each H_1 plant was harvested separately. Each H_2 plant was cultivated from single lines of 60 entries. Then, the variation of plant height, heading stage and seed setting rate of the H_2 population was recorded (**Table 2**). The results indicated that the H_2 generated from pollen exposed to radiation over 46Gy had two-way variation in plant height and heading stage. The variation was much greater than in mutagenesis experiments performed with dry seeds.

Table 2. Variation frequency of mutant traits in H_2 plants derived from mature pollen irradiated by different Gamma-ray doses.

Dose (Gy)	No. of individuals	Variation frequency of plant height (%)		Variation frequency of heading stage(%)		Variation frequency of seed setting rate(%)	
		Dwarf	Tall	Early	Later	<50%	50-85%
0	500	0	0	0	0	0	0
23	500	3.1	0	3.5	0	0	3.4
46	1000	19.5	5.7	6.4	3.6	13.8	26.9
93	200	20.3	4.9	4.7	5.3	15.2	30.1
186	500	21.5	6.8	4.2	7.2	19.7	27.3

We selected 121 individual plants of high quality, treated with different doses of Gamma-rays to investigate their panicle traits (**Table 3**).

Table 3. Variation of some panicle traits of 121 selected H_2 individuals.

	Panicle length		Spikelets per panicle		Grain shape				Length-width ratio		1000-grain weight (g)		waxy	drop
	-	+	-	+	long	short	wide	slim	-	+	-	+		
No. of individuals	52	15	811	15	16	1	6	8	2	12	1	6	8	2
(%)	43.0	12.4	67	12.4	13.2	0.8	5.0	6.6	1.7	9.9	0.8	5.0	6.6	1.7

* + = increase, - = decrease

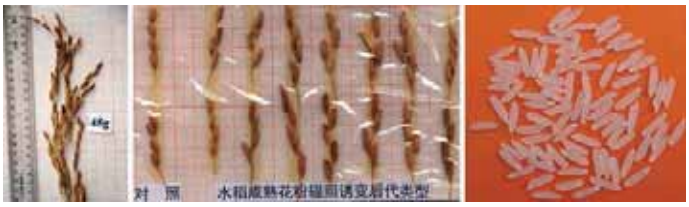

Figure 1 Panicle mutants derived from mature pollen irradiated by Gamma-rays.

Panicle traits of H_2 plants showed a distinct two-way variation. Individuals showed 1000-grain weight lower (3g) or greater (8g) than the control group. The change in grain shape of individuals was mainly due to an increase of grain length to 8.4-10.6 mm (**Fig. 1**), with an increase in length-width ratio from 2.5 to 3.2.

There were new characteristics in the H_2 population, including individuals with seed of a waxy appearance, low seed setting rate, similarity to multiploids (long awn and big grain), and altered color of the glume hull. Among the 121 plants chosen, 112 (92.6%) were from the group treated with 46Gy. Moreover, 27 of the 30 best plants (90%) were treated with 46Gy of Gamma-radiation, suggesting that this group had a higher frequency of useful variation.

Genetic variation of mutant traits of H_3, H_4 derived from mature pollen irradiated by Gamma-rays

Plant height, heading stage, seed setting rate and grain shape of each individual of the H_3 and H_4 generations were recorded (**Table 4**). The results showed that separation of traits of each individual of the H_3 and H_4 generations was still very complex. There was a wider mutation spectrum in H_3 and H_4 plants than that of H_2 plants. Three mutant quantitative traits of plant height, heading stage and grain length all had cumulative phenomena. Seed setting rate gradually became normal with further generations. For grain shape mutation, the large grain mutants of H_2 and H_3 showed high variability and grain weight exhibited a cumulative phenomenon.

Table 4. Comparisons of the range of separation of some traits in the H_2, H_3 and H_4 generations.

	Plant height (cm)	Heading stage (d)	Seed setting rate (%)	Grain length (mm)	1000-grain weight (g)
CK	15-105	0	Above 90	8.0-8.8	29-31
H2	40-115	3-3	Above 0.2-90	8.0-10.6	24-39
H3	57-140	15-15	Above 10-90	8.0-13.4	21-54
H4	70-155	15-not heading	Above 20-90	8.0-13.8	21-51

In all generations, the length, width and 1000-grain weight of rice grain and brown rice were measured at random (**Table 5**). The results indicated that the grain shapes generated by Gamma-irradiation of mature pollen were heterogeneous. The change in the grain shape for each generation was mainly in the grain length with an absolute increase of 5 mm (4.4 mm), or an increase of 56.8% (68.8%), but the grain width could increase and decrease for each individual grain. In all, the length-width ratio rose from 2.5 (2.1) in the original seed to 5.3 (4.5), with absolute increase of 2.8 (2.4). The appearance of rice had been greatly improved because of reduced chalkiness or no chalkiness.

Table 5. Ranges of variation of grain shapes of progenies derived from mature pollen irradiated by Gamma-rays.

	No. of individuals	Rice grain			Brown rice			1000-grain weight (g)
		Length (mm)	Width (mm)	Length-width ratio	Length (mm)	Width (mm)	Length-width ratio	
Ma85	5	8.4-8.8	3.4-3.8	2.2-2.5	6.0-6.3	3.0-3.3	1.9-2.0	29-31
Bai85	5	8.4-8.7	3.6-3.7	2.3-2.4	6.2-6.4	3.0-3.1	2.0-2.1	29-31
H_2	18	8.3-10.6	3.0-3.5	2.4-3.2	6.0-8.4	2.4-3.1	2.0-3.3	24-37
H_3	356	8.0-13.4	2.6-4.0	2.2-4.2	6.0-10.8	2.4-3.6	1.6-3.6	21-54
H_4	110	8.4-13.8	2.6-4.2	2.2-4.3	6.2-10.2	2.0-3.4	1.6-4.0	21-54
H_5	55	8.5-13.8	2.0-3.4	2.3-5.3	6.2-9.0	1.8-3.4	1.8-4.5	21-54
H_6	42	8.4-11.6	3.0-3.8	2.2-4.6	6.2-9.0	2.4-3.6	1.9-4.5	21-54
H_7	44	8.4-11.7	2.6-3.7	2.5-4.3	6.3-9.3	2.3-3.1	2.1-3.6	21-54

The mutant individuals with altered 1000-grain weight also showed positive and negative two-way variation (**Fig. 1**), with variation in H_2

being smaller and variation in H_3 being larger. From the H_4 generation on, its change was within the range of the H_3 generation. The absolute decline of 1000-grain weight was 8g (27.6%) and the absolute increase was 23 g (72.4%). Rice had a reduced rate of chalkiness and good quality within 10 g of a decrease or increase of the 1000-grain weight. When the 1000-grain weight was over 40g, rice had a big white belly, loose structure and poor quality. Grain shape was the major characteristic affected by of mutating mature pollen. There have been no previous examples of changes of such magnitude in rice mutation breeding programmes.

Genetic stability of mutant progenies derived from mature pollen irradiated by Gamma-rays

Genetic stability of various lines of mutant progenies derived from rice mature pollen irradiated by Gamma-ray was studied further. The results showed that mutations in H_2 and H_3 lines derived from mature pollen irradiated by Gamma-rays all appeared to separate, and that the H_3 generation had more mutant types and a broader spectrum of mutations than the H_2 generation. These mutant types included: extremely incompact, double gulme hulled, extremely dwarf and sterile plants. The generations above H_4 gradually became stable and the H_5 generation had more than 70% stable lines. Some individuals with low-seed setting rate and large grain were very difficult to stabilize and these traits still exhibited great variability in the H_{15} generation, indicating that mutation of some male gametes is very complicated.

Developing good quality early *indica* cultivars using mature pollen irradiated by Gamma-rays

Jiahezaozhan, a high-quality and yield early *indica* rice cultivar, was developed in 1996, using the hybridization of a mutant generated from mature pollen irradiated by Gamma-rays as its original female parent plant. Jiahezaozhan was the first early *indica* rice cultivar in Fujian province with its quality meeting the standards of Second Class Good Edible Rice issued by the Agriculture Ministry.

Jiafuzhan, a new early *indica* rice cultivar with a 1000-grain high quality weight of 30 g, high yield, resistance to blast, resistance to planthopper, and wide adaptability, was bred by crossing "Jiahezaozhan" as the a female parent with a mutant having a 1000-grain weight of 36.4 g derived from mature pollen irradiated by Gamma-rays. This cultivar received the first prize in Science and Technology in Fujian province, having an annual planting area of 66,700 hm^2 in Fujian province, and additional hectares in neighboring provinces [6].

Conclusion

A mutation technology using mature rice pollen irradiated with Gamma-rays was established, and has produced more than 200 stable mutant rice lines with large grain, high quality and good plant types, to date. It is a potent technology by which new mutant types can still be produced in the third generation, probably as a consequence of multiple recessive mutations. The inheritance of traits for very large grain and very low seed setting rate tend to be unstable, and progenies with these traits continue to produce new types of mutant.

The mutation technology using mature pollen irradiated by Gamma-rays, mainly induces point mutations. It is especially effective in producing mutations relating to yield components controlled by minor genes. These include mutants in growth stage, grain shape and grain weight. There is no other report describing such a rich repertoire of changes in the grain shape and rice quality generated by irradiating mutation of mature rice pollen.

Bautista [7] and Wang [8] have observed chromosome aberrations in progenies derived from irradiating rice pollen. Tang [3] found that phenotypic variation in the second generations derived from rice pollen irradiated with different doses of Gamma-rays was larger than the control group. Our work has demonstrated unambiguously that Gamma-ray irradiation can elicit mutational effects on mature rice pollen, in contrast to the previous reports by Chin, *et al.* [4, 5].

ACKNOWLEDGEMENTS

This work was supported by China National 863 Project (2007AA10Z179), Fujian platform of Science and Technology Innovation Project (2007N2005), Fujian Science and Technology Key Project (2008N0122) and by Xiamen Science and Technology Project (3502Z20083003).

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