เซนติเมตร รองลงมาได้แก่ ชุดการทดลองที่ 3 และ 2 มีความกว้างทรงพุ่มเท่ากับ 48.90 และ 48.85 เซนติเมตร ส่วนชุดการทดลองที่ 1 มีความกว้างทรงพุ่มน้อยที่สุด เท่ากับ 48.10 เซนติเมตร

ระดับความรุนแรงในการเกิดโรค พบว่า ระดับความรุนแรงในการเกิดโรคในระดับ 1 มีพริกจากชุดการทดลองที่ 3 เพียงชุดการทดลองเดียวที่เกิดโรคในระดับ 1 เท่ากับ 4.27 เปอร์เซ็นต์

ความรุนแรงในการเกิดโรคระดับที่ 2 พบว่าชุดการทดลองที่ 1, 2, 3 และ 4 มีเปอร์เซ็นต์ต้นพริกเกิดโรคเท่ากับ 26.31, 21.05, 27.80 และ 27.88 เปอร์เซ็นต์ตามลำดับ

ความรุนแรงในการเกิดโรคระดับ 3 พบว่า ชุดการทดลองที่ 1 มีเปอร์เซ็นต์การเกิดโรคระดับ 3 สูงที่สุด เท่ากับ 55.26 เปอร์เซ็นต์ รองลงมาได้แก่ ชุดการทดลองที่ 3 และ 2 มีเปอร์เซ็นต์ ต้นพริกที่เกิดโรคระดับความรุนแรงระดับ 3 เท่ากับ 54.01 และ 50.00 เปอร์เซ็นต์ และชุดการทดลองที่ 4 มีเปอร์เซ็นต์ต้นพริกเกิดโรคในความรุนแรงระดับ 3 น้อยที่สุด เท่ากับ 48.80 เปอร์เซ็นต์

ความรุนแรงในการเกิดโรคระดับ 4 พบว่า ชุดการทดลองที่ 2 มีเปอร์เซ็นต์การเกิดโรคสูงที่สุดเท่ากับ 21.05 เปอร์เซ็นต์ รองลงมาได้แก่ ชุดการทดลองที่ 4, 1 และ 3 มีเปอร์เซ็นต์การเกิดโรคเท่ากับ 11.53, 10.52 และ 6.41 เปอร์เซ็นต์ ตามลำดับ

ความรุนแรงในการเกิดโรคระดับ 5 พบว่าชุดการทดทดลองที่ 3 มีเปอร์เซ็นต์การเกิดโรคสูงที่สุดเท่ากับ 4.27 เปอร์เซ็นต์ รองลงมาได้แก่ ชุดการทดลองที่ 4 เท่ากับ 2.88 เปอร์เซ็นต์ ส่วนการทดลองที่ 1 และ 2 มีเปอร์เซ็นต์การเกิดโรคเท่ากันคือ 2.63 เปอร์เซ็นต์

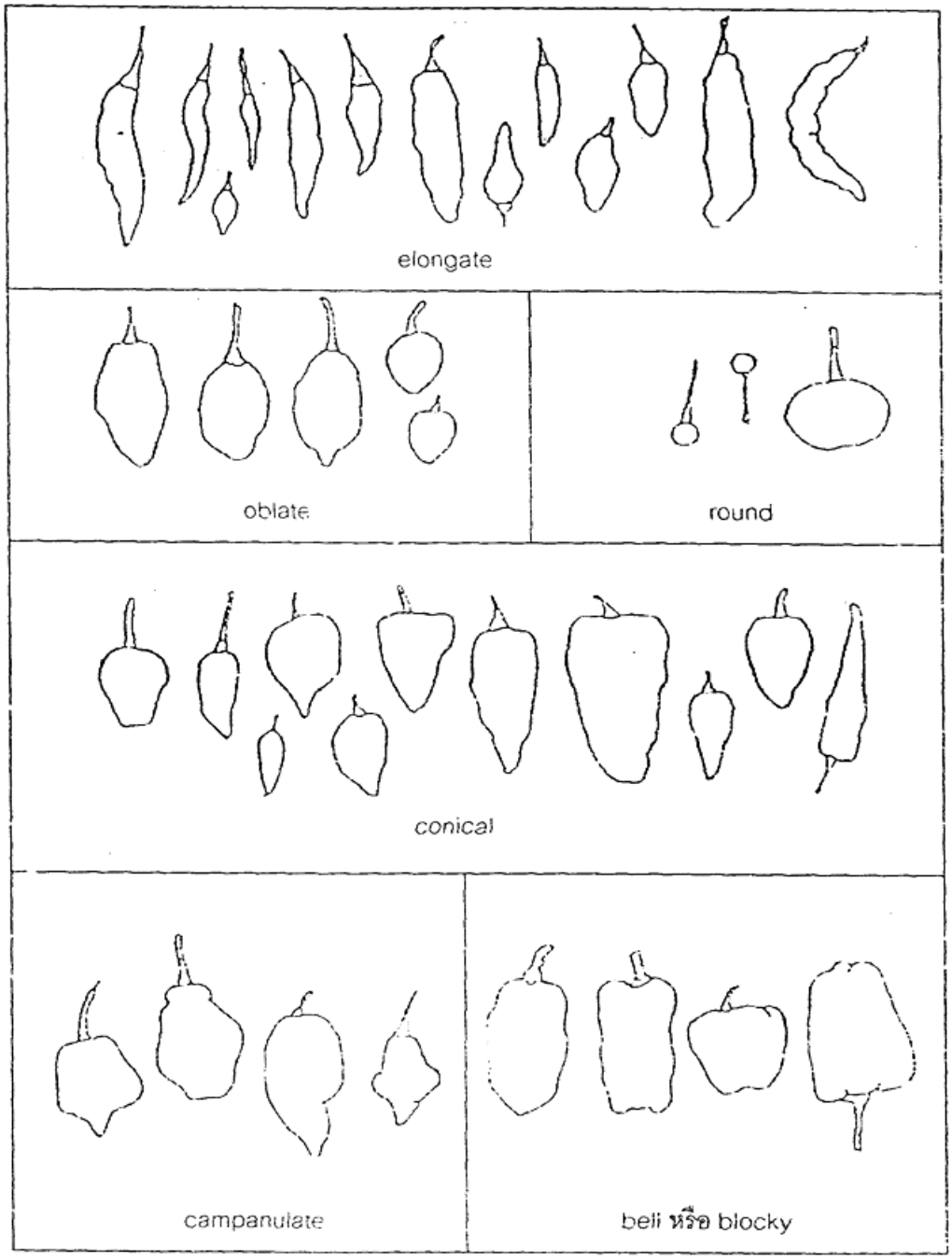
ลักษณะของผลพริก พบว่า ผลพริกจากทุกชุดการทดลองมีสีผลเป็นสีเขียวอ่อนและมีรูปทรงผลแบบ elongate

จากการทดลองนี้ เมื่อพิจารณาเรื่องของการเกิดโรคใบด่างจากเชื้อโรคไวรัสในพริก แสดงให้เห็นถึงแนวโน้มของผลการทดลองที่ลำไนโตรเจนไอออนทำให้พริกที่ถูกระดมยิงด้วยลำไนโตรเจนไอออนปริมาณ 6×10^{16} ions/cm^2 มีระดับความรุนแรงของการเกิดโรคต่ำกว่าชุดการทดลองอื่นๆ (ระดับ 1) จึงเป็นไปได้ว่า การใช้ลำไนโตรเจนไอออนสามารถทำให้พริกมีความต้านทานโรคเพิ่มขึ้น แสดงอาการของโรคเพียงเล็กน้อยและยังสามารถให้ผลผลิตได้ ดังนั้นจึงควรนำเมล็ดพันธุ์พริกที่ได้จากการผสมตัวเองของต้นพริกที่มีความต้านทานโรคนี้มาพัฒนาต่อไป

เอกสารอ้างอิง

IBPGR. 1983. **Genetic Resources of *Capsicum***. International Board for Plant Genetic Resources, Rome. 49 p.

ภาคผนวก



ภาพที่ 4 รูปร่างผล (IBPGR, 1983) แบบยาว (elongate), oblate, rond, conical, campanulate และ bell หรือ blocky

บทที่ 5: ผลสัมฤทธิ์ (Output) จากโครงการวิจัยที่ได้รับทุนจาก สกว.

5.1) รายชื่อผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)

1) B. Phanchaisri , R. Chandet , L.D. Yu , T. Vilaithong , S. Jamjod , S. Anuntalabhochai**.** (2207). Low-energy ion beam-induced mutation in Thai jasmine rice (*Oryza sativa L*. cv. KDML 105). *Surface & Coatings Technology* 201:8024–8028.

2) S. Mahadtanapuk , L.D. Yu , R. Cutler , T. Vilaithong , S. Anuntalabhochai. (2007). Mutation of Bacillus licheniformis using low-energy ion beam bombardment. *Surface & Coatings Technology*. 201 : 8029–8033.

3) L.D. Yu,., S. Sangyuenyongpipat , S. Anuntalabhochai, B. Phanchaisri , T. Vilaithong , and I.G. Brown. (2007). Effects of low-energy ion beam bombardment on biological cell envelopes. *Surface & Coatings Technology.* 201: 8055–8061

4) R. Norarat, N. Semsang, S. Anuntalabhochai, and L.D. Yu. (2008). Low-energy Low-fluence Ion Beam Bombardment of Naked Plasmid DNA, to be presented and submitted to The 16th International Conference on Ion Beam Modification of Materials, Aug. 31 – Sep. 5, 2008, Dresden, Germany; the Proceedings will be published in *Nucl. Instr. and Meth. B.*

5.2). การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)

-

- เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)

- เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

- เกิดความร่วมมือในการทำวิจัยร่วมกับ Dr. Liang Den Yu ภาควิชาฟิสิกส์ คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ และ ผศ. ฉันทนา วิชรัตน์ สาขาพืชผัก และภาควิชาพืชผัก สาขาพืชผัก ภาควิชาพืชสวน คณะผลิตกรรมการเกษตร มหาวิทยาลัยแม่โจ้

นอกจากนั้นยังมีผู้เชี่ยวชาญร่วมกับได้แก่ Dr. Robert cutler จาก Bard colledgeม New York ได้ลา sabbatical leave มาร่วมทำวิจัยภายใต้โครงการนี้เป็นระยะเวลา 2 ปี

- เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

1) ผลงานวิจัยบางส่วนได้นำไปใช้ในการสอน ในกระบวนวิชาสำหรับทั้งบัณฑิตศึกษา ได้แก่ กระบวนวิชา 202736 (Molecular biology) และระดับปริญญาตรีได้แก่ 202432 (Molecular Biology of the genes)

2) ได้รับเชิญเป็นวิทยากรพูดเรื่อง ลำไอออนเทคโนโลยี ภาควิชาพืชสวน คณะทคโนโลยีการ เกษตร สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง ฉลองกรุง ลาดกระบัง กรุงเทพ 10520

3) ได้รับเชิญเป็นวิทยากรพูดเรื่อง การประยุกต์เทคนิคลำไอออนในการปรับปรุงพันธุ์พืช มหาวิทยาลัยนเรศวรพะเยา ตำบลแม่กา อำเภอเมือง พะเยา

4) นักศึกษาปริญญาเอก

น.ส นวลอนงค์ เสมสังข์

น.ส กันตา แสงวิจิตร

น.ส สุกัญญา พิทักษ์รัตนานุกูล

5) นักวิจัยหน้าใหม่ที่ได้ร่วมทำวิจัยในโครงการนี้ได้แก่

ดร. รัฐพร จันทร์เดช จากคณะวิทยาศาสตร์ มหาวิทยาลัย แม่โจ้

ดร.สุภัค มหัธนพรรค จากมหาวิทยาลัยนเรศวรวิทยาเขตพะเยา

5.3) อื่น ๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

5.3.1) การเสนอผลงานในที่ประชุมวิชาการทั้งในและต่างประเทศ

1) เสนอผลงานแบบปากเปล่า (oral presentation) ในงานการประชุมระดับนานาชาติ the 2nd Thailand Nanotechnology Conference : Nonmaterials for Health, Energy and Environment. Phuket Thailand 2008 , 13-15 August 2008, Phuket Granceland Resort and Spa

2) เสนอผลงานโปสเตอร์ในการประชุมสัมมนาวิชาการระดับนานาชาติ ในงาน BioAsia 2007 : การวิจัยและพัฒนาพืช (The 6th Asian Crop Science Association Conference) และการประชุมนานาชาติข้าวเพื่ออนาคต (The 2nd International Conference on Rice for the Future) วันที่ 5-9 พฤศจิกายน 2550 ณ ศูนย์การประชุมแห่งชาติสิริกิติ์

3) เสนอผลงานโปสเตอร์ในงานการประชุมวิชาการวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ครั้งที่ 33 “วิทยาศาสตร์และเทคโนโลยีเพื่อประเทศไทยที่ยั่งยืน” (Science and Technology for Sustainable Thailand) วันที่ 18 – 20 ตุลาคม 2550 ณ มหาวิทยาลัยวลัยลักษณ์ จังหวัดนครศรีธรรมราช

4) เสนอผลงานแบบปากเปล่า (oral presentation) ในงานการประชุมระดับนานาชาติ “The 15th International Conference on Surface Modification of Materials by Ion Beams (SMMIB-15)” Mumbai, INDIA 30th September to 5th October 2007

5) เสนอผลงานแบบปากเปล่า (oral presentation) ในงานการประชุมระดับนานาชาติ “14th International Conference on Surface Modification of Materials by Ion Beams”, September 4-9, 2005, Pine Bay Resort Hotel, Kusadasi, Turkey.

6) รางวัล 1 ใน 10 ของ poster ดีเด่นจำนวน 160 เรื่อง จากผู้เข้าร่วมประชุม 33 ประเทศ ในการประชุม14th International Conference on Surface Modification of Materials by ion beam ระหว่างวันที่ 4-9 SEPT 2005 , Kusadasi, Turkey.

5.3.2) รางวัล

รางวัลงานวิจัยดีเด่นสกว. ประจำปี 2549 ชื่อผลงาน เทคนิคปรับปรุงพันธุ์ข้าวหอมมะลิด้วยลำไอออนพลังงานต่ำ โครงการเรื่อง ลำไอออนพลังงานต่ำกับงานด้านเทคโนโลยีชีวภาพ จัดขึ้นวันที่ 20 ธันวาคม 2549 ณ. ที่โรงแรม สยามซิตี้ กทม

5.3.3) สิทธิบัตร

ได้ยื่นขอรับสิทธิบัตรเรื่อง วิธีการชักนำให้เกิดการกลายพันธุ์ในสิ่งมีชีวิตโดยเทคนิคลำไอออนพลังงานต่ำ (อยู่ระหว่างการขอสิทธิบัตร)

5.3.4) Abstracts

บทคัดย่อข้างล่างเป็นบทคัดย่อที่ได้นำเสนอในการประชุมดังกล่าวข้างต้น

1) Induction of anthocyanin accumulation in Thai jasmine rice mutant by low-energy ion beam

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Abstract.

Using a low-energy N+ / N2+ ion beam, a mutant of Thai jasmine rice (*Oryza sativa* L. cv. KDML105), BKOS line, was detected. One of its phenotypes was black seeds. Anthocyanin accumulation and their gene expressions were investigated. Anthocyanin production which is absent in the KDML105, was present in all tested tissues of the mutant excluding young roots. Anthocyanin synthase (ANS) was found to be expressed in all mutant tissues but not in wild type and two enzymes, F3'H and F3'5'H , which were also expressed. The expression of *Ra*, a known anthocyanin upregulator, in the mutant is proposed to be caused by the inactivation of a repressor gene present in the wild type which was inactivated in the mutant by the ion beam bombardment. The increased production of anthocyanin and the additional photoperiod insensitivity mark this mutant as a possible new improved crop variety for Thai rice cultivation.

Keywords : intestinal bacteria**,** carboxymethyl cellulose, termite

2) Analysis of Purple Rice Mutant Induced by Low Energy Ion Beam

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[(a)]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

[(b)]Fast Neutron Research Facility, Department of Physics, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

[(c)]Department of Agronomy, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand

Abstract

The application of low ion beam bombardment for mutation induction in purple glutinous rice was investigated. About 4800 seeds were bombarded using a low energy nitrogen ion beam at energies of 60, 80,125 keV and fluences of 1,4, 8 x 10^{16} ions / cm^2. With increasing energies and fluences, both germination and survival of the rice seedling was reduced. Eight mutants with green leaf and stem sheath in the M1 generation were detected at 60, 80 and 100 keV but not at 125 keV. Seeds from the M1 generations were cultivated for M2 phenotypic analysis. The mutants exhibited a broad spectrum of phenotypes including: stable green leaf blade and stem sheath, green leaf and purple stem sheaths, purple leaf and stem sheaths, white seed coats and pericarp as well as a changed starch property from amylopectin to amylose content.

The high annealing temperature random amplified polymorphic DNA method (HAT-RAPD) was chosen to determine genetic variation among the mutants. Of 7 tested primers, the three primers OPH15, OPX13 and OPW09 revealed genetic variation between the mutants and wild type

Keywords: purple rice, low energy ion-beam, mutation, HAT-RAPD

3) การโคลนและการศึกษาคุณสมบัติของซีดีเอ็นเอที่เข้ารหัสการสังเคราะห์ GTP - BINDING PROTEIN ขนาดเล็ก (RAB7) จากข้าวขาวดอกมะลิ 105 (*Oryza sativa* L. cv. KDML105) (MOLECULAR CLONING AND CHARACTERIZATION OF cDNA ENCODING THE SMALL GTP-BINDING PROTEIN (RAB7) FROM *Oryza sativa* L. cv. KDML105)

สุกัญญา พิทักษ์รัตนานุกูล,[1] รัฐพร จันทร์เดช,[2] และ สมบูรณ์ อนันตลาโภชัย[1]

Sugunya Pitakrattananukool,[1] Ruttaporn Chundet,[2] and Somboon Anuntalabhochai[1]

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บทคัดย่อ : Rab7 เป็น GTP-Binding protein ขนาดเล็กที่เกี่ยวข้องกับกระบวนการ Endocytosis และมีบทบาทสำคัญตั้งแต่ระยะเริ่มแรกจนถึงระยะสุดท้ายของกระบวนการ Endosome/lysosome vesicular transport ของสิ่งมีชีวิตทุกชนิด จากการศึกษาสามารถโคลน Rab7 cDNA ได้จาก cDNA library ของใบข้าวขาวดอกมะลิ KDML105 (*Oryza sativa* L. cv. KDML105) cDNA ที่ได้มีความยาว 958 คู่เบส สามารถแบ่งได้เป็น บริเวณด้านปลาย 5’ ที่ไม่เข้ารหัสการสังเคราะห์โปรตีนมีความยาว 65 คู่เบส บริเวณปลาย 3’ ที่ไม่เข้ารหัสการสังเคราะห์โปรตีนมีความยาว 325 คู่เบสและบริเวณที่เข้ารหัสสังเคราะห์โปรตีนมีความยาว 628 คู่เบส ซึ่งสามารถแปลรหัสได้เป็นกรดอะมิโนที่มีความยาว 209 กรดอะมิโน คำนวณน้ำหนักโมเลกุลได้เป็น 23 กิโลดาลตัน และลำดับกรดอะมิโนที่ได้มีความคล้ายคลึงกับ Rab7 จากพืชชนิดอื่น ได้แก่ *Oryza sativa* L. indica, *Oryza sativa* L. japonica, *Cucumis sativus* และ *Lotus japonicus* คิดเป็น 98 , 97 , 90 และ 88% ตามลำดับ

Abstract: Rab7 is a small GTP-binding protein which is believed to be involved in endocytosis that is important in early to late endosome/lysosome vesicular transport in all organisms. We have isolated a *Rab7* cDNA clone from a rice leaf (*Oryza sativa* L. cv. KDML105) cDNA library. The full length cDNA is 958 bp and the sequence contains a 65 bp 5’ noncoding region, 3’ noncoding region of 325 bp and a 628 bp open reading frame corresponding to a translation product of 209 amino acid with a calculated molecular mass of 23 kDa. The predicted amino acid sequence displays significant resemblance to known Rab7 from *Oryza sativa* L. indica, *Oryza sativa* L. japonica, *Cucumis sativus* and *Lotus japonicus* which show 98 , 97 , 90 and 88% identity, respectively.

Keywords : small GTP-binding protein , *Oryza sativa indica* cv. KDML105, Endocytosis

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Low-energy ion beam-induced mutation in Thai jasmine rice (*Oryza sativa* L. cv. KDML 105)

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Abstract

Low-energy ion beam bombardment at energy levels in the range of 60–125 keV and ion fluences (dose) of 1×10^{16}–5×10^{17} ions/cm^2 was chosen for mutation induction in Thai jasmine rice (*Oryza sativa* L. cv. KDML 105) at Chiang Mai University. One of the rice mutants designated BKOS6 was characterized. The rice mutant was obtained from KDML 105 rice embryos bombarded with $N^+ + N_2^+$ ions at an energy level of 60 keV and ion fluence of 2×10^{16} ions/cm^2. Phenotypic variations of BKOS6 were short in stature, red/purple color in leaf sheath, collar, auricles, ligule, and dark brown stripes on leaf blade, dark brown seed coat and pericarp. The mutant's reproductive stage was found in off-season cultivation (March–July). HAT-RAPD (High Annealing Temperature-Random Amplified Polymorphic DNA) was applied for analysis of genomic variation in the mutant. Of 10 primers, two primers detected two additional DNA bands at 450 bp and 400 bp. DNA sequencing revealed that the 450 bp and the 400 bp fragments were 60% and 61% identity to amino acid sequence of flavanoid 3′hydroxylase and cytochrome P450 of *O. sativa japonica*, respectively.

Keywords: Ion beam bombardment; Mutation; Thai jasmine rice; HAT-RAPD

1. Introduction

Since the discovery of X-ray-induced genomic mutation in 1927 by Muller et al. [1], many types of mutagenic sources, for example, gamma-ray, UV irradiation, chemical agents, neutron, and ion beam bombardment, have been developed and applied in crop mutation. High-energy ion beam irradiation at MeV energy levels was applied as a mutagenic source in Japan and succeeded in induction of mutation in many plant species such as *Arabidopsis* [2], rice [3], carnation [4], and rose [5]. Moreover, applications of low-energy ion beam bombardment at energy levels lower than 100 keV in biotechnological research work in gene transfer and as a new mutagenic source have been intensively carried out in China since 1986. In 1993, the first evidence of gene transfer (GUS) into intact rice cells by low-energy ion beams was reported by the Chinese research group [6]. In Thailand, at Chiang Mai University, application of low-energy ion beams to biological research work has been addressed since 1998. In 2001, we reported the first evidence of the application of low-energy ion beam bombardment to gene transfer into bacterial cells [7]. Many studies concerned with the mechanisms of ion–biomaterial interactions causing pathways or nano-holes for gene transfer into ion-bombarded cells have been carried out [8–12]. By using N^+ ion implantation on rice seeds, Yu et al. [13] discovered genetic mutation in rice in M_2 generation and opened a new field: low-energy ion beam mutation. The method has provided many advantages, such as low damage rate, higher mutation rate, and wider mutation spectrum. During 1986–1987, 11 new lines of rice mutants with higher yield rate, broader disease resistance, and shorter growing period but higher quality were bred in China [13].

Thai jasmine rice (*Oryza sativa* L. cv. KDML 105) is very famous in the world rice market for its exotic characteristics.

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When cooked, it is shinny white like jasmine flower color, soft and tender with aromatic fragrance. The demand of KDML 105 in world markets is increasing year by year. But to increase the grain yield of the rice by industrial cultivation is limited by its two disadvantageous characteristics: tall and photoperiod sensitivity. The specifics of the tall variety, that is 140–150 cm in height with delicate and slender internodes, cause Thai jasmine rice to easily tangle and fall over in the maturity stage. The tall variety makes it impossible to be harvested by mechanized methods. It also has a limitation of direct-seeding cultivation which has a lower cost than transplant cultivation. And its photoperiod sensitivity allows it to be cultivated only once a year in the in-season period (July–December).

The aim of the study was to induce mutation in Thai jasmine rice to screen for short-in-stature and photoperiod-insensitive varieties that would fulfill multiple cropping and direct-seeding cultivation needs.

2. Experiment

2.1. Ion bombardment

The ion beam facility used in the experiment was a 150-kV non-mass-analyzed heavy ion implanter self-developed at Chiang Mai University [14]. The ion beam system is comprised of a high-current duoplasmatron ion source, a broad beam transport system and versatile target chambers. Under a low pressure, 10^{-3}–10^{-4} Pa, nitrogen ions ($N^+ + N_2^+$) were produced with a beam current of about 2 mA, accelerated by an electrostatic field, defocused by magnetic lens and finally introduced into a target chamber to bombard rice seeds in a special target holder. The adjusted beam with a spot size of 10 cm in diameter was able to bombard 800 rice seeds altogether.

In preparation of Thai jasmine rice seeds, fresh harvested seeds were heated at 49 °C in an oven system for 5 days in order to break the rice dormancy. The rough seeds were then carefully husked to avoid any damage to the embryo tissues. The seeds were put in the sample holders, a copper plate with a diameter of 10 cm and 1.2 cm thick. The sample holder has 800 tiny holes that are fitted to the seed size and let the embryo part be exposed to the ion beam. Four sample holders holding the rice seeds were then installed onto a rotatable sample holder stage in the target chamber. Several thousands of the rice seeds were ion bombarded at varying ion energy levels of 60–125 keV and ion fluences of 1×10^{16}–5×10^{17} ions/cm^2. To prevent overheating of the rice seeds during ion bombardment, the rice seeds were bombarded using a pulsed mode with a 10-to-10-second interval and also water-cooled. The seeds kept in the vacuum condition and in air, without the ion bombardment, were positive control (vac^+) and negative control (vac^-), respectively.

2.2. Rice mutant screening

After ion bombardment, the ion-bombarded seeds and controls were kept in moist conditions and then cultured in soil for 3–4 weeks. The seedlings were then transferred to grow as transplanted rice in soil in plastic pots at 1 seedling/pot. The cultivations were carried out in in-season cultivation (July–December) to screen for short-in-stature characters, and cultured in off-season cultivation (March–July) to screen for photoperiod-insensitive characters. Phenotypic and genomic variations in all growth stages, dates of flowering, dates of harvesting, plant height as culm length at harvesting day, etc., were recorded and analyzed.

2.3. DNA analysis

In DNA analysis, samples of leaf tissue were ground in liquid nitrogen to a fine powder and DNA extraction was performed following Doyle and Doyle method [15] but with minor modifications. Amplification of the DNA was carried out by the HAT-RAPD [16] technique. The amplification products were separated by electrophoresis. Additional DNA bands found in mutants were subcloned into plasmid pGEM-T easy vector (Promeca) and transformed into *E. coli* by electroporation and sequenced by the method of Sanger et al. [17]. The base sequences of PCR products were then compared with amino acid sequences in the GenBank database.

Mutants obtained from the ion-bombarded rice seeds with both phenotypic and genomic variation were further cultured from M_2 to M_3 to investigate stability of the mutants.

3. Results and discussion

3.1. Rice mutants

$N^+ + N_2^+$-ion beam bombardment in the KDML 105 seeds resulted in induction mutation in their genomes (Fig. 1). Three interesting new lines were derived and selected from the ion-bombarded rice seeds, named as PKOS1, TKOS4, and BKOS6. PKOS1 was selected from the seeds bombarded with nitrogen ions accelerated at 60 kV with fluences of 2×10^{17} ions/cm^2. This line was photoperiod insensitive and short in stature. TKOS4 was selected from the seeds bombarded with nitrogen ions accelerated at 80 kV with fluences of 8×10^{16} ions/cm^2, and it was photoperiod insensitive, tall, and early-flowering. BKOS6 was selected from the seeds bombarded with nitrogen ions accelerated by 60 kV with fluences of 2×10^{16} ions/cm^2, and showed photoperiod-insensitive, short-in-stature, early-

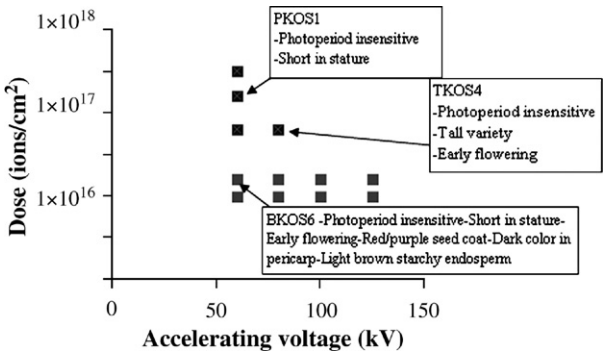

Fig. 1. Induction mutations found in KDML 105 rice seeds bombarded with $N^+ + N_2^+$ ions at various ion energy levels and ion fluences (doses).

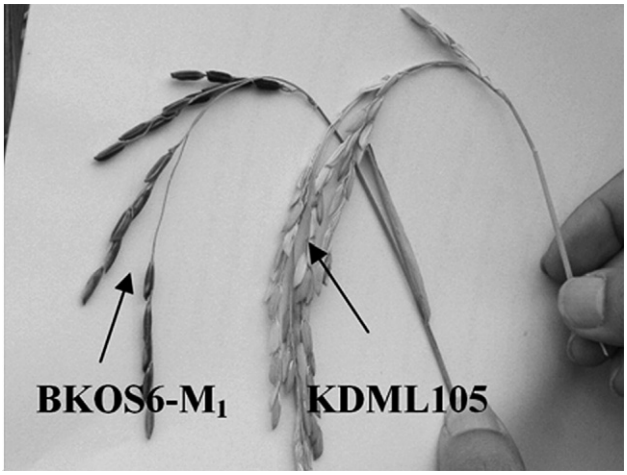

Fig. 2. BKOS6-M_1 rice with dark brown to black seed coat (left) compared with straw color of the KDML 105 control (right).

maturing properties, and obvious variations in phenotypic appearances. Hereby, BKOS6 was chosen for investigation and analysis in this work.

3.2. Characterization of BKOS6

Phenotypic variation found in BKOS6 in M_1 generation was a single culm rice plant, very short in stature at 49 cm in culm length on the harvesting day, compared with an average of 94 cm in height of the control group ($N=20$). The plant had a dark brown to black seed coat (Fig. 2), reddish purple collar, auricles, ligule and leaf sheath, compared with those of control that were white, and dark purple stripes on the leaf blade. Fig. 3 shows difference in morphology and color variation in paddy and in brown rice seeds of the KDML 105 control, purple rice, and BKOS6. The light brown color in the seed coat was found in BKOS6 rice seeds in the maturity stage, compared with a straw color of the KDML 105 control and dark brown of purple rice. Pericarp of BKOS6 brown rice was dark purple to black as well as purple rice's whereas the KDML 105 control's pericarp

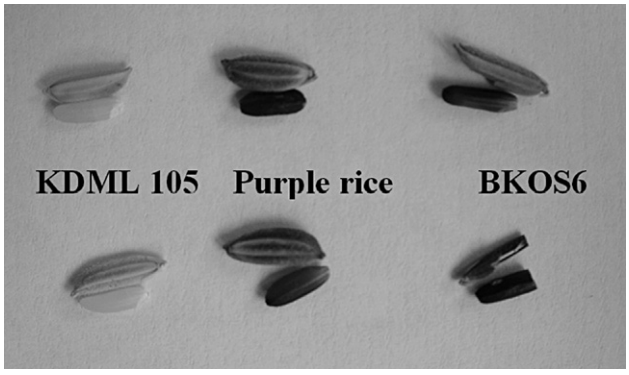

Fig. 3. Comparison of seed shape and color among KDML 105, purple rice, and BKOS6. In the first row, seed coat of BKOS6 in maturity stage was light brown compared to straw color in the KDML 105 control and to dark brown in purple rice. In the second row, pericarp of BKOS6 brown rice was dark purple to black as purple rice's but the KDML 105 control's was light straw or light amber.

Fig. 4. Appearance of BKOS6 in M_2, and M_3, generations cultured in pot (13 in. in diameter) was short in stature of approximately 67 cm in culm length at harvesting day compared to 94 cm of the control.

was light straw or light amber. Moreover, color pericarp and tegmen color of BKOS6 were dark purple to black while in starchy endosperm was light brown. Seed shape of BKOS6 was long and slender grain as *indica* rice type. Phenotypic variation of red/purple color was found in apiculus of the BKOS6 seed coat in the flowering stage and the red/purple color was found in all areas of the seed coat in the early ripening stage.

The gross appearance and some phenotypic variations of BKOS6 were shown in Fig. 4 and Table 1. For M_2 and M_3 generations cultured in pot in in-season and off-season of cultivations, the short in stature of around 67 cm in culm length compared to 94 cm (ANOVA, analysis of variance, $p<0.05$) of the control was recorded. Shorter panicle length of 20 cm compared to 24 cm (ANOVA, $p<0.05$) of the control, lower number of 65 spikelets per panicle compared with 113 spikelets per panicle (ANOVA, $p<0.05$) of the control, and shorter in seed length (ANOVA, $p<0.05$), were observed BKOS6 in M_2 and M_3 generations and showed the stability of photoperiod insensitivity, and an early-flowering (duration from the germination stage to the flowering stage) of 18–22 days earlier than the controls.

3.3. Genomic variation

HAT-RAPD revealed genomic variations in BKOS6. Of ten primers, two arbitrary primers designed OPK10 and OPH15 distinguished genomic DNA between the KDML 105 and

Table 1
Some phenotypic variations found in BKOS6-M_2

Phenotypic variations	BKOS6	KDML 105	Statistic test (analysis of variance, ANOVA)
Culm length (cm)	66.83±4.337	94.22±6.865	($p<0.05$)
Panicle length (cm)	19.83±2.288	24.05±4.006	($p<0.05$)
Spikelet number/panicle	65±12	113±43	($p<0.05$)
Seed size (in mm)			
– Width	2.47±0.145	2.46±0.107	($p<0.05$)
– Length	9.76±0.337	10.30±0.482	
– Thickness	1.87±0.107	1.78±0.151	

Data (average value±SD) was obtained from 20 raw data. "cm" refers to centimeters, and "mm" refers to millimeters.

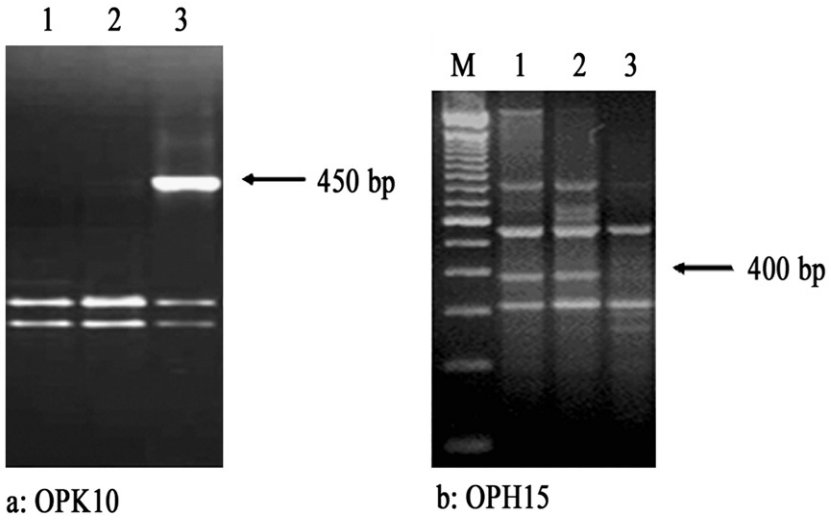

Fig. 5. HAT-RAPD amplification products generated by OPK10 (a) and OPH15 primers (b) using KDML 105 genomic DNA as templates from (1) vac^- control (2) vac^+ control and (3) BKOS6. M is molecular weight marker lane. Arrow indicates the distinguished PCR products.

BKOS6. The DNA fragments at 450 bp and 400 bp were detected by OPK10 and OPH15, respectively (Fig. 5). The nucleotide sequences of the PCR products, designed $BKPK10_{450}$ and $BKPH15_{400}$, and their deduced amino acid sequences were compared with all sequences available in the GenBank database. The sequences and their deduced amino acid were shown in Fig. 6. The sequence analysis revealed that $BKPK10_{450}$ belonged to member of flavonoid 3′hydroxylase of *O. sativa japonica* with the highest identity of 60% whereas $BKPH15_{400}$ was 61% identity to cytochrome P450 of *O. sativa japonica*. The flavanoid 3′hydroxylase is the enzyme that is involved in anthocyanin biosynthetic pathway [18]. Anthocyanin pigments display color ranging from bright red/purple to blue. Color variation of the main red/purple in various tissues [19,20] such as leaf sheath, collar, auricles, ligule, and pericarp, and light brown in starchy endosperm in BKOS6 may be induced from mutation in genes controlling the purple/red to blue. The $BKPH15_{400}$ which is a member of cytochrome P450 may play significant role in biological systems [21,22], such as hormonal regulation, phytoalexin synthesis, as well as, flower petal pigment biosynthesis, may result in the phenotypic variations.

A broad range of the phenotypic variations found in BKOS6, presenting in Table 1, indicated that low-energy ion bombardment could produce a wide mutational spectrum in Thai jasmine rice. A higher mutation rate and wider mutational spectrum with less damage in low-energy ion implanted bio-materials were previously reported [13,23]. However, mechanisms of biological effects caused by ion implantation remain obscure. Based on the proposed four-factors-in-one hypothesis [24,25], in ion beam bombardment of biological organisms, energy deposition, momentum transferring, foreign particle implantation and charge exchange work together on the material and cause combinative effects. Thereafter, the effects should be superior to those achieved by single-factor techniques such as γ-ray radiation [26].

The reliable reason for ion beam-induced mutations should be that energetic ions penetrate through the seed coat and cell

$BKPK10_{450}$

(1) AGAGGCTGCACACGTCGGTACGACAACATGATGAACGGATTCATCAACGAAAGG
AAGGCCGGGGCGCAGCCCGACGGGGTCGTTCCGCTGGCGAGCACCCGGCAACGA
CCTTCTAAGCGTGAACTGCTGGGCGAGGATGCAGGAGGGCAGAAGCTGGACGGC
GACGGCGAAAAGATCACCGAAACTGACATCAACAGCTC

(2) **RGCTRRYDNMMNGFINERKAGAQPDGVVPLASTRQRPSKRELLGEDAGGQKL**
DGDGEKIT

$BKPH15_{400}$

(3) CGTCGGCAAGCTGGCCGCGGCGTCCGGCCAGATGGCTCCCCACGCGTCATGGACA
CGCTCCAGTACGCCATGTTCTGCCTCCTCGTGGTCATGTGCGTTCGGCGAGCGGCT
CGACGAGGCCGACGTGCGCGCCATCGCGCACGGCGCAGCACGACTGGATCGTGTT
TACTTCGCGACGAAGATGAGGGTGTTCGCGTTCTGCTCGAC|GATCACCAAGCATC

(4) **VGKLAAASGQMAPHASWTRSSTPCSASSWSCAFGERLDEADVRAIAHGAARLD**
RVYFATKMRVFAFCSTITKH

Fig. 6. Nucleotide sequence and their deduced amino acids of BKOS6: (1, 2) nucleotide sequence and deduced amino acids of $BKPK10_{450}$ and (3, 4) nucleotide sequence and deduced amino acids of $BKPH15_{400}$. The sequence comparison revealed that $BKPK10_{450}$ was 60% identity to flavonoid 3′hydroxylase and $BKPH15_{400}$ was 61% identity to cytochrome P450 of *Oryza sativa japonica*, respectively. Normal letter: nucleotide sequences. Bold letter: deduced amino acids.

envelop and damage the bases and break sugar phosphate bonds in a nucleotide, the unit of DNA. The base damage can change genetic code, and the release of inorganic phosphate induces strand breaking of DNA. The research on the mutation induced by low-energy ion had been carried out at various level, e.g. in population genetics, single character and single gene levels. Investigation on sequence analysis of *lacZ*-mutation induced by ion beam irradiation in double stranded Mi3mp18DNA, the molecular mechanism of mutation induced by the ion beam has been reported [27]. Yu and Huo [25], also showed that 30 keV N-ion implantation on rice seeds could cause substantial physio-chemical reactions and free radicals in the rice seed. Obvious signal of free radicals was found in the electron spin resonance spectrum of rice seeds implanted with the N-ions, suggesting mutation induction in the rice embryo, resulting in achieving the genetic mutation by the heavy ion beam.

In the aspect of biology, the low-energy ion effects are deduced from the mutation spectra on the cellular and molecular damage induced following the track of penetrating into cells, which lead to DNA damage, chromosome aberration, gene modification and mutation [28]. Relevant fundamental studies will further be carried out.

4. Conclusion

With the appropriate conditions of ion beam bombardment of Thai jasmine rice, a broad mutational spectrum was found in the mutant, BKOS6, carrying the phenotypic variations. The additional fragments, $BKPK10_{450}$ and $BKPH15_{400}$, were detected by PCR reaction in BKOS6. They belong to members of anthocyanin and cytochrome P450, respectively.

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Mutation of *Bacillus licheniformis* using low-energy ion beam bombardment

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Abstract

Anthracnose, caused by the fungus *Colletotrichum* sp., is an important disease affecting a wide range of flowers. To control this fungus various methods including the use of natural antagonists have been used for biological control. In this study, *Bacillus licheniformis*, isolated from hot springs in the Chiang Mai province, was shown to suppress conidia germination of the fungus and reduce symptoms caused by the disease *in planta*. To induce mutations in *B. licheniformis*, a low-energy ion beam was applied to the bacteria using N-ions to bombard the bacteria under vacuum conditions at an energy of 28–50 keV with a fluence range of $1–10\times10^{15}$ ions/cm^2. After this treatment, one mutant was found which had lost its antagonistic property. To try to characterize this mutation, the HAT–RAPD technique was used to produce band patterns for both the bombarded bacteria and the original wild type. From these polymorphic band patterns, differences were found which were induced by the ion beam. These polymorphism bands were subcloned into a pGEM-T vector and sequenced. One-band fragment conserved in the wild type and lost in the mutant bacteria was found to code for the lipase gene. Further research will focus on other genes and gene functions which may be involved in the observed antipathogenicity.

Keywords: *Bacillus licheniformis*; Mutation; Low-energy ion beam; HAT–RAPD

1. Introduction

Anthracnose caused by the fungus *Colletotrichum* sp. is an economically important disease of curcuma in Thailand [1], which is currently controlled by chemical fungicides. Since many of these fungicides are toxic, research has become focused on developing non-chemical alternatives to fungicides for the control of this disease. Already there has been some success with the biological control of diseases in plants using naturally occurring microorganisms. In particular, *Bacillus* sp. have been shown to control anthracnose in many plants such as the avocado [2] and mango [3].

In this study, an induction of mutation by ion beam implantation has been performed, further extending the use of ion beam bombardment in the field of biology and agriculture. Energetic heavy-ion beams have previously been used to bombard biological materials for genetic modification purposes, particularly for the mutagenesis of living organisms including both plants and bacteria [4–6]. The fundamental mechanism used in ion beam bombardment is to the use high-energy charged particles to produce radiation damage. These particles act as a new mutagenic source for genetic modification by directly inducing mutation. In recent years, application of ion beam technologies with microorganism has advanced the development of industrial microbe breeding [7].

The aim of this study is to investigate the antagonistic property of *Bacillus licheniformis* on *Colletotrichum* sp. using a low-energy ion beam to mutate the wild type bacteria. Such a mutation could mutate the gene involved in the antipathogenicity of these bacteria leading to a loss of function which could be further studied. To screen for the genes involved in the antipathogenic ability, a DNA fingerprint technique was used which details the presence and absence of DNA bands to compare between the mutant and wild type bacteria.

2. Experimental

2.1. Plant material

The *Curcuma alismatifolia* Gagnep. (Chiang Mai Pink cultivar) used in this study was kindly provided by the Chiang

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Fig. 1. Anthacnose of curcuma: (A) diseased flower; (B) acervulus and conidia of *Colletotrichum* sp.

Rai Horticulture Research Center, Chiang Rai province, Thailand. The flowers were cultivated individually from bulbs in a greenhouse until the seedlings reached the young bud stage. At full growth (28 days from initial budding) flowers had been chosen for analysis.

2.2. Media and culture conditions

The fungal strain of *Colletotrichum* sp. initially isolated from Curcuma bract was stored at 4 °C and was then routinely subcultured onto potato dextrose agar (PDA). Conidial induction of *Colletotrichum* was performed in nutrient yeast dextrose agar (NYDA) media. All other bacterial strains were cultured on slants of Luria–Bertani (LB) at 4 °C for further study.

2.3. Antagonistic activity in vitro

The assay for antagonism was preformed on a PDA medium using the dual-culture method described by Skidmore and Dickinson [8]. Briefly, the bacteria and pathogenic fungi were inoculated dually on PDA medium in petri dishes 2–2.5 cm apart. The inhibition of actively growing fungus (radial distance growth in cm) by bacteria in the PDA plates was recorded for each sample. The cultures were incubated at room temperature, and growth of the fungus towards and away from the bacterium was allowed for 7 days after incubation. The percentage inhibition of the growth of the fungi was calculated using the following formula:

$$[(R_1 - R_2)/R_1] \times 100,$$

where R_1 was the farthest radial distance growth of the fungus in the direction of the antagonist (a control value) and R_2 was the distance on a line between the inoculation positions of the fungus and bacteria.

2.4. Antagonistic inhibition on conidial germination of Colletotrichum sp.

A mixture of 20 μl of *Colletotrichum* sp. conidial suspension (1×10^6 cells/ml) and 20 μl of bacterial cell cultures was formed at increasing concentrations (1×10^8, 2×10^8, 3×10^8 and 4×10^8 CFU/ml). The mixture was then pipetted onto clean glass slides forming a 0.5-cm-diameter membrane, and slides were then incubated in moist chambers for six and a half hours using a minimum of three replicate slides for each fungal isolate. At the end of the incubation period, a drop of lactophenol was added to each slide to arrest

Table 1
Antagonistic effect of *Bacillus licheniformis* on *Colletotrichum* sp. conidial germination

Treatment	Conidial germination of fungi[a] (%)				
	1×10^8 CFU[b]/ml	2×10^8 CFU[b]/ml	3×10^8 CFU[b]/ml	4×10^8 CFU[b]/ml	Water (control)
B. licheniformis	19.00	0	0	0	–
Control (water)	–	–	–	–	98.33

[a] Average of the three *Colletotrichum* sp. germination tested.
[b] CFU is cell colony-forming units.

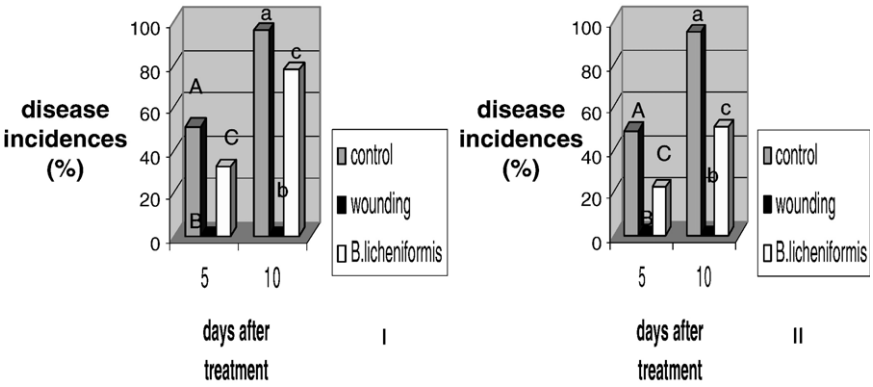

Fig. 2. Mean incidences of disease (% of wounds infected) in curcuma flowers treated with antagonistic bacteria. (I) The flower bracts of curcuma were inoculated by a prick technique with the mixture. (II) The flower bracts of curcuma were inoculated and sprayed every 3 days. The same letters are not significantly different at $p \leq 0.05$.

germination within the drop. The percent germination in each fungal isolate was determined by counting a minimum of 100 conidia from each isolate under the microscope and determining the proportion that had germinated.

2.5. Detection of antagonistic activities in planta

The flower bract of 28-day-old, greenhouse grown curcuma plants was used for the *in planta* assay. Approximately 2×10^8 CFU/ml (were added with 0.001% Tween 20) of bacterial cells and 10^7 cells/ml. of *Colletotrichum* sp. each were prepared for the *in planta* test. The curcuma bracts were first wounded using the prick technique described by May et al. [9]. The experiment was divided into two main components; (1) for the single treatment experiment, a fungal cell suspension was mixed at a ratio of 1:1 with the bacterial cells of interest and a 40-μl aliquot of this suspension was inoculated into the wound. The two controls contained wounded bract with and without inoculation by the fungus cell suspension. (2) In the second component (the serial treatment), the fungal cell suspension was first inoculated into the prick wound and then followed by spraying the bacterial cells onto the wound separately every 3 days for 10 days. The development of spot lesions was recorded daily during the 10 days of the experiment. In all treatments, the bracts were wrapped with a transparent plastic bag after inoculation and secured within the greenhouse to prevent contamination. Each treatment was run with 3 replicates with twenty curcuma bracts used for each replicate.

2.6. Induction of mutation by ion implantation

To prepare the selected antagonistic bacterium *B. licheniformis* for mutation by ion beam bombardment, the bacteria were smeared onto a thin layer of sterile adhesive tape which was attached to a petri dish and then placed inside a sample holder. The holder was capable of sequentially exposing a number of samples to the ion beam, as well as housing the unbombarded control sample. Ion bombardment was carried out using the mass-analyzed heavy-ion-implantation facility at Chiang Mai University [10]. Nitrogen ions (N^+) were chosen for ion bombardment, in the energy range of 28–50 keV. Ions were delivered at fluences of $1–10 \times 10^{15}$ ions/cm^2. Inside the target chamber, the temperature of the target was about 0 °C. The samples were maintained under these conditions for about 1.5–2 h, allowing for system pump-down and ion bombardment.

After bombardment, the newly formed bacteria mutants were cultured and any bacterial mutant that had lost the antagonistic ability was further analyzed (as mentioned in the dual-culture method). Genetic alterations of the bombarded

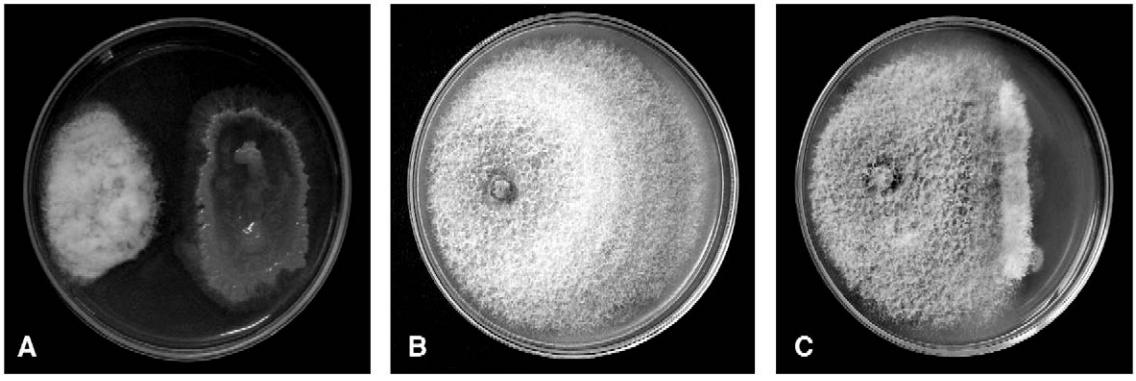

Fig. 3. Antagonistic effect *in vitro* by dual-culture method: (A) *B. licheniformis*; (B) control; (C) mutation (MBL1).

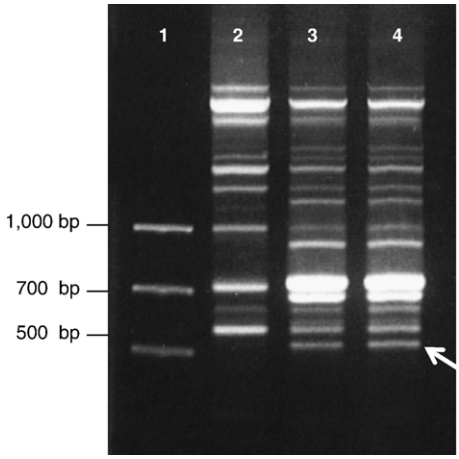

Fig. 4. Amplification products of the bombarded bacterium using primer OPAH 10: (1) marker; (2) bacteria (MBL1) was showed to be losing antagonistic activity; (3) bacteria displayed the same ability for inhibition; (4) control, right arrow indicates polymorphism that subcloned into pGEM-T easy vector.

bacterium were detected by HAT–RAPD [11] in comparison to the wild type.

3. Results

3.1. Isolation of fungal

The natural characteristics of anthracnose in the curcuma flower are shown in Fig. 1. Infected areas become hard, turning brown in color when infected by *Colletotrichum* sp. and acervuli form in the infected area. These acervuli were rounded to elongate and disrupted the outer epidermal cell walls of the host. Conidia were cylindrical to oblong, while spines were sticklike. The fungus was stored for further use in the antagonistic activity testing.

3.2. Antagonistic activity test in vitro and in planta

B. licheniformis was tested using a dual-culture method. This strain reduced the diameter growth of the fungus by more than 75%. When tested, the antagonistic effect of the bacteria on the conidial germination of *Colletotrichum* sp. was evident from the germination rate of 0% in contrast to an average 98% in the control (Table 1) at the time of initial germination (6.5 h). In addition, a swelling of conidia and germ tubes was observed at 6.5 h and then after 24 h of growth. Mycelia swelling with short hyphal cells was seen in every concentration testing.

In planta testing of *B. licheniformis* dramatically reduced flower disease incidence as compared to the infected control. In the second experiment, bacteria were sprayed every 3 days onto the wounded bracts. The isolated *B. licheniformis* was significantly more effective at reducing flower lesions than the control (Fig. 2).

Table 2
The nucleotide sequence of lipase gene compare with the database of NCBI

Bacteria (accession number)	Identity (%)
Bacillus licheniformis	100
AAR84668	76
CAB95850	74
AAA22574	72
AAL36938	66

3.3. Induction of mutation by ion beam implantation

To investigate the similarity of antagonistic ability between the ion beam generated mutants and the wild type a dual culture test was used in which more than 50 colonies were selected at random from the bombarded bacterium. These bacteria initially had the same inhibition ability compared to the control, but after being exposed to the ion beam at an energy of 30 keV with a fluence of 10^{16} ions/cm^2, one isolate of bacterium (MBL1) was found to have lost antagonistic activity (Fig. 3). However, none of the bacteria under vacuum without ion bombardment was obtained (data not shown).

Moreover, the HAT–RAPD fingerprint using primer OPAH10 presented an absence of DNA bands in comparison between the mutant bacteria and wild type bacteria (Fig. 4). Using four other primers (OPAH01, OPAH06, OPAH08 and OPAH13), no difference in the band pattern was found between

D D H R H S F L S I L L I C M L S V V S V F
gat gac cat cgt cat tca ttt tta tct att cta ttg att tgc atg ctg tct gtt gtg tcc gta ttt
S F R P S A A S A A S H N P V V M V H G I
tcg t tc cgg cct tca gca gct tcc gcc gct tcc cac aat ccg gtc gtc atg gtc cac ggc atc
G G A D Y N F I G I K S Y L Q S Q G W T S
ggc gga gcc gat tat aac ttc atc ggc att aaa tcg tat tta caa tct caa ggc tgg aca agc
S E L Y A I N F I D K T G N N I N N A P R L
agt gag ctt tac gcc atc aac ttt atc gat aaa acg gga aat aat ata aac aat gct ccg aga tta
S E Y I K R V L N Q T G A S K V D I V A H
tcc gaa tac atc aag cgt gtg ctg aat caa aca gga gca tca aaa gtc gat att gtc gcc cac
S M G G A N T L Y Y I K N L D G A D K V G
agc atg ggc ggg gcc aat acg ctc tat tat att aaa aat ctg gat ggt gcg gat aaa gtc gga
H V V I
cat gtg gtc atc

Fig. 5. The nucleotide sequence of lipase gene from *B. licheniformis*.

the wild type and MBL1. To characterize the region that displayed an altered band pattern, this polymorphic fragment was subcloned into a pGEM T-vector and subsequently sequenced. When this nucleotide sequence, which contained 393 bp (131 amino acids), was blasted against the NCBI database, it was found to be highly homologous to the lipase gene (Fig. 5) from multiple *Bacillus* strains, such as *Bacillus pumilus* [12], *B. licheniformis* [13], *Bacillus subtilis* [14], *Bacillus* sp. B26 [15] with 76%, 74%, 72% and 66% identities, respectively (Table 2).

4. Discussion and conclusions

In this work, we showed that *B. licheniformis* inhibited *Colletotrichum* sp. *in vitro* and *in planta*. Furthermore, the population dynamics of *B. licheniformis* showed that it is also a good colonizer of curcuma flowers, maintaining high population levels up to 15 days after application (data not shown). This result indicated that *B. licheniformis* was well adapted to the wound environment in curcuma flowers and has considerable potential as a biocontrol organism.

In order to locate the gene involved in the antifungal ability of *B. licheniformis* to *Colletotrichum* sp., modified polymorphism bands using the HAT–RAPD fingerprint technique were subcloned into pGEM-T easy vector and then sequenced. When the sequence of this fragment was compared with those already contained in the database, one of the fragments was found to encode the lipase gene. Lipase catalyzes the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol, and fatty acids, and, under certain conditions, also catalyzes the reverse reaction, esterification, forming glycerides from glycerol and fatty acids. Some lipases are also able to catalyze transesterification and enantioselective hydrolysis reactions [14]. It is also known that lipase is a biocontrol agent against some plant pathologenic fungi produced by different *Bacillus* species [16]. In the present case, lipase A from *Acinetobacter species* SY-01 was used for catalyzing of two acetate isomers of *cis*-2-(bromomethyl)-2-(2,4-dichlorophenyl)-1,3-dioxolane-4-methyl acetate, an intermediate required for the synthesis of Itraconazole which was an anti-fungal drug [17].

Future investigations on the use of *B. licheniformis* may examine different methods for infesting plants with isolates to consider the optimal stage of plant development for antagonistic colonization with the optimal level of inoculum needed to obtain effective disease control. In addition, further new antagonistic microorganisms should be tested for their effectiveness against *Colletotrichum* sp. pathogenicity to curcuma, and integrated strategies for disease control such as those developed for other *Colletotrichum* pathogens [18] could be explored.

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Low-energy ion beam modification of horticultural plants for induction of mutation

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Low-energy ion beam biotechnology, as a newly established and developed highly interdisciplinary technology, has been vigorously applied to modification of horticultural plants for induction of mutations at Chiang Mai University (CMU). Chiang Mai is the horticultural center of Thailand with a magnificent variety of floral resources thanks to its peculiarly favorable environment and climate. Ion beam bombardment induced mutations of flowers have further exploited the horticultural potential. This paper reports recent progress in research and applications of ion beam biotechnology for floral mutation at CMU. Ions at low energies of several tens of keV were implanted into flower seeds, buds and receptacles of various species in vacuum to fluences of an order of 10^{16} ions/cm^2 using self-developed special techniques for the living organism survival. A broad spectrum of mutants has been obtained including changes in phenotypes such as the flower color, color intensity, flower shape, and petal shape and size. DNA fingerprint analysis revealed changes at the DNA level. Physical mechanisms involved in the ion beam induction have been investigated, and however still remained obscure.

Keywords*:* Ion beam, Horticultural plants, Biotechnology, Flowering crops, Mutation

1. Introduction

Mutations are changes in genes. They may occur in any cell at any stage in the cell cycle. They are prime movers of the evolution process [1]. Spontaneous somatic mutations or bud spots are largely responsible for the development of new varieties in ornamental plants [2]. The spontaneous mutation frequency at any one genetic site is usually extremely low, often in the range 10^{-8} – 10^{-10} for each replication of the genome. However the mutation frequencies can be increased by using a variety of mutagenic treatments [3]. Mutagenic radiations are a useful tool to all breeding programs, thus in flowering crops [4]. It is sometime possible to improve a few characters in any otherwise excellent cultivar without altering the remaining genotype, especially in plants which show a high degree of heterzygosity [5].

Apart from the conventional electromagnetic radiations, like X-ray and γ-ray or other particular ionizing radiations such as neutrons, ion beam is now an alternative source of energy to induce mutation [6]. When charged particles are accelerated under vacuum condition and finally bombard the target they deliver their kinetic energy as well as mass and charge [7]. As they densely deposit their energy to the plant tissue, their high linear energy transfer (LET) has an opportunity to create mutants with new characters compared with the conventional radiations [8]. However new mutants reported in many publications in this area were induced from high-energy ion beams (higher than MeV). At Chiang Mai University (CMU), we have recently carried out a research program on low-energy ion beam modification of horticultural plants for induction of mutation. Chiang Mai is the horticultural center of Thailand with a magnificent variety of floral resources thanks to its peculiarly favorable environment and climate. Ion beam bombardment induced mutations of flowers have further exploited the horticultural potential. This paper reports recent development in research and applications of ion beam biotechnology for floral mutation achieved at CMU.

2. Experimental

Three flower species were experimented. In *Dendranthema morifolium*, the variety Reagan Dark was used. As found out from the previous observations in preliminary experiments, both the tips and the internode sections were seriously suffered from dehydration due to being under the vacuum condition in the sample chamber and in almost all cases the meristematic cells both at the tips and at the auxiliary buds had a very low survival rate, alternative methods to avoid the dehydration from the sample were developed. The receptacles were surface sterilized and cultured on to the MS agar media consisting of 0.02 mg/l NAA and 2.0 mg/l Kinetin for 5 days to stimulate growth of the explants. They were moved from the agar media and dipped in sterilized liquid paraffin and were bombarded with fluences of nitrogen ion beam from $1 - 8 \times 10^{16}$ ion/cm^2 at 60 keV. After bombardment they were then sterilized and cultured in the same MS agar media. Adventitious plants were induced to root and potted and evaluated for any kind of mutations.

For *Rosa* hybrids, parafilm 0.5-cm width stripe was used to wind around the internode section to prevent dehydration of the tissue and leaving only a small aperture where the auxiliary bud existed. The buds were bombarded with nitrogen ion beams at energies of 50, 80 and 100 keV to fluences of 1, 5 and 10×10^{16} ion/cm^2. After bombardment, parafilm was removed and the samples were soaked in the water and the buds were budded on *Rosa multiflora* for further evaluation.

With *petunia*, F_1 hybrid seeds of variety Dream Midnight Blue were stuck on one side of a self adhesive patch of 1×1 cm^2. The other side of the patch itself was stuck on a 10-cm Petri dish for ion beam bombardment. The seeds were bombarded with N-ion beams at 60 keV to fluences of 1, 2, 4, 6, 8, 10×10^{16} ions/cm^2. After bombardment the seeds were removed from the adhesive patch using water and germinated at on Watmann Filter paper and later on were transferred to a plug tray and finally to 5-inch pots using 60% coconut coir, 30% rice husk and 10% sand as a media.

In all of the ion implantations, the ion flux was kept around 10^{13} ions/cm^2/sec. This ion flux was about one order lower than standard ones (10^{14} ions/cm^2/sec) as documented [9] and thus should not have particularly additional effects on genetic modification.

3. Results and Discussion

For *Dendranthema morifolium*, it was found that the fluence of 4×10^{16} ion/cm^2 started to induce the survival rate of the receptacle less than 100% and the fluence of 8×10^{16} ion/cm^2 was lethal to the samples (Table 1). This coincided with the freshness evaluation of the explants evaluated at one week after culturing. It can be seen from Table 1 that at 8×10^{16} ion/cm^2 all explants were categorized at level E. The intensity of the damage was lower at lower fluences. Receptacles that received high ion fluence of 6×10^{16} ion/cm^2 produced the least number of adventitious plantlets. Black-out was given to the experimented plants to induce flowering. One color solid mutant, from pink to bronze was observed clearly, indicating that the adventitious plantlet generated from one cell (Fig. 1.). DNA fingerprint also revealed banding difference between the mutants and the control (Fig. 2.). Streaked petals were also found (Table 2).

For the *Rosa* hybrids, after ion beam bombardment the parafilm was removed and the nodal sections were soaked with water, variety Shocking Blue suffered from dehydration more than variety Blue Nile especially at higher ion energy. The good buds were grafted on *Rosa multiflora* and budding success was scored 45 day after budding. There was more bud growth at lower energy such as 50 and 80 keV than that at 100 keV (Fig. 3). After 140 days shoots started to flower when they were about 10-12 cm in height. Three plants of variety Shocking Blue produced flower with a greater number of petal than the control (Fig. 4). However after cutting back a few times the same plants produced normal flower which clearly demonstrated chimera formation of the bombarded buds.

For the *Petunia* hybrids, ion beams at all fluences did not affect on the germination percentage, but growth reduction was observed at high fluence (Fig. 5). Seedlings and the fully grown plants from the irradiated seeds showed deformed leaf shape, leaf chimera and delayed flowering dates. New form of flower shape was achieved and proved to be stable. The stability was confirmed by DNA fingerprint (Table 3, Fig. 6 and 7). It is worth noting that aseptically germinated seeds also gave the similar result.

Low-energy ion beams have clearly induced mutation in those three flowering crops, however the mechanism of the induction has to be elucidated. The penetration depth of the low-energy nitrogen ions into the tissue is much less than the electromagnetic radiation and thus the deposit of energy should be mostly at the near surface region of the tissue. In *petunia* seeds, cross sectioning of the seed reveals that the plumular apex, the area where all the meristematic cells locate, lies almost in the middle of the seed (Fig. 8). However, the mutated characters proved to be due to genetic effect, as shown in Fig. 7 which shows the DNA-fingerprint analytical result, rather than physiological effect as the change is stable through the vegetative propagation. As seeds contain very little of moisture content therefore mutation is unlikely to be caused by indirect effect. In Chrysanthemum, it is likely that the energy of the ions is delivered to the dividing cells of the receptacle therefore the assumption on the deposition of the energy on the chromosome is reasonable. The adventitious plantlet is truly developed from single cell as the mutant is solid and heritable. The effect of low-energy ions at 20 – 30 keV with fluences of 10^{14} – 10^{16} ion/cm^2 has been reported to induce exogenous macromolecule transfer through the cell wall into the cells [10]. This indicates that low-energy ion beams mainly interact with the plant cell envelopes, but a probability of penetration of a very small number of the ions into the deep region where the genetic substance locates inside the cells cannot yet be ruled out.

From technical point of view, bombarding seeds is much simpler than fresh tissues, and a large number of replications can be done but this same manner cannot be achieved with

the stem sections as the target area in the chamber is small. In order to increase the efficiency of the ion bombardment of stem sections which will lose water significantly in vacuum conditions, relevant methods have to be developed to maintain the water content of the tissues during the ion beam treatment process.

4. Conclusion

Low-energy ion beam biotechnology, as a newly established and developed highly interdisciplinary technology, has been vigorously applied to modification of horticultural plants for induction of mutations at Chiang Mai University. A broad spectrum of mutants has been obtained including changes in phenotypes such as the flower color, color intensity, flower shape, and petal shape and size. Mutations at the DNA level have been confirmed. Physical mechanisms involved in the ion beam induction have been discussed, and however still remained obscure.

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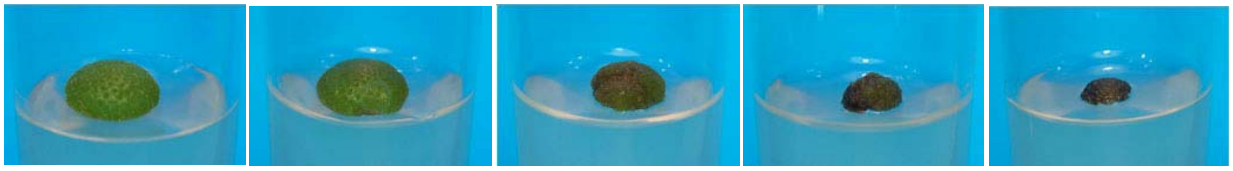
Table 1. The survival of the receptacles of *Dendranthema molifolium* after being 60-keV N-ion bombarded with various fluences and the degree of damage on the explant surface.

Fluence (10^{16} ions/cm^2)	**Replication**	**No. of explants survive**	**No. of explants with different degree of surface damage**					**No. of plantlets collected**
			A	**B**	**C**	**D**	**E**	
0 (Control)	10	10	10	0	0	0	0	15
0 (Vacuum control)	10	10	7	3	0	0	0	143
1	10	10	5	5	0	0	0	223
2	10	10	4	3	2	1	0	113
4	10	8	3	3	2	0	2	227
6	10	4	0	1	0	3	6	52
8	10	0	0	0	0	0	10	-



A = no cell damage

B = 1/3 of the receptacle surface damaged

C = 2/3 damaged

D = more than 2/3 damaged

E = the whole area damaged

Table 2 Number of chrysanthemum plants showing mutation after N-ion beam bombardment at 60 keV to various fluences.

Fluence (10^{16} ions/cm^2)	**Number of plants observed**	**Plants with steak on petal**	**Color mutation**
0 (Control)	15	0	0
0 (Vacuum control)	143	0	0
1	223	2	0
2	113	3	0
4	227	7	1
6	52	2	0
8	-	-	-

Table 3 Germination, malformed plants height, canopy width and flowering date of *petunia* after ion beam bombardment at 60 keV with various fluences. The values are means. L.S.D.: Least significant difference. C.V. %: percent of co-variance. The superscripts a–d are the statistical indicators: means followed by the same superscripts in each column are not significantly different at $P < 0.05$ by DMRT (Duncan Multiple Range Test. D.B. Duncan, Multiple range and multiple F tests. Biometrics 11:1-42, 1955).

Fluence (10^{16} ions/cm^2)	Germination (%)	Malformed plants (%)	Plant height (cm)	Canopy width (cm)	Days from planting to flowering
Natural Control	83	0	10.4^{ab}	32.2^{a}	45.7^{d}
1	93	17.2	9.9^{ab}	25.2^{b}	46.4^{cd}
2	89	7.9	9.8^{ab}	30.2^{a}	48.9^{b}
4	90	6.2	11.3^{a}	30.5^{a}	48.4^{b}
6	90	12.1	10.6^{ab}	32.2^{a}	45.9^{c}
8	88	7.1	9.0^{b}	26.1^{b}	49.4^{bc}
10	89	9.4	10.9^{a}	29.6^{a}	53.3^{a}
L.S.D	-	-	0.6	1.3	0.7
C.V. %	-	-	0.4	0.2	0.2

Figure Captions

Fig. 1. Color mutation of chrysanthemum induced by ion beam. (a) Control. (b) Mutant.

Fig. 2. DNA fingerprints of mutant and the control of Chrysanthemum. (M) Marker, (C) control, (MC) Mutant. White arrows show the band from each mutant that is different from that of the control.

Fig. 3. Wilting of the rose buds 14 days after budding on the root stock after ion beam bombardment at (a) 50 keV, (b) 80 keV, and (c) 100 keV.

Fig. 4. Mutants of rose variety Shocking Blue induced by ion beam. (a) Control. (b) Bombarded with a fluence of 1×10^{16} ions/cm^2 at 80 keV. (c) Bombarded with a fluence of 1×10^{16} ions/cm^2 at 100 keV.

Fig. 5. Germination of ion beam bombarded *petunia* seeds. (a) Control. (b) Bombarded with a fluence of 6×10^{16} ions/cm^2 at 60 keV.

Fig. 6. Shape and color of flower and leaves of *petunia* from ion beam bombarded seeds. (a) Control. (b) and (c) 1×10^{16} ions/cm^2. (d) 8×10^{16} ions/cm^2. (e) 10×10^{16} ions/cm^2. (f) 6×10^{16} ions/cm^2. (g) Control. (h) 6×10^{16} ions/cm^2. (i) 10×10^{16} ions/cm^2.

Fig. 7. DNA fingerprints of mutant and the control of *petunia*. (M) Marker. White arrows show band from each mutant that is different from that of the control. (Lane 1: control, Lanes 2 – 9: mutants)

Fig. 8. Longitudinal section of a *petunia* seed. The size of the seed is about 0.5 mm or less.

Figure 1

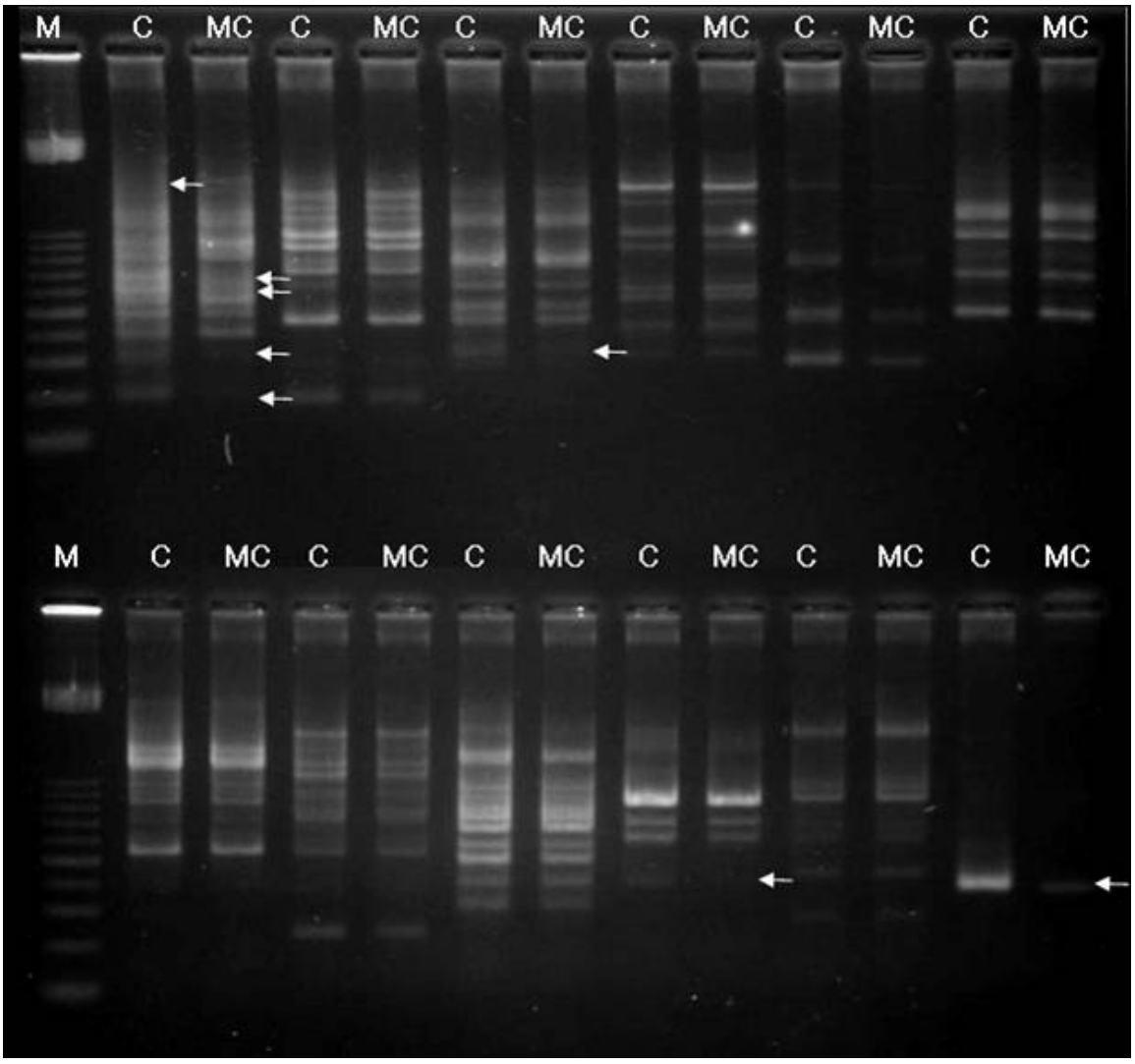


Figure 2

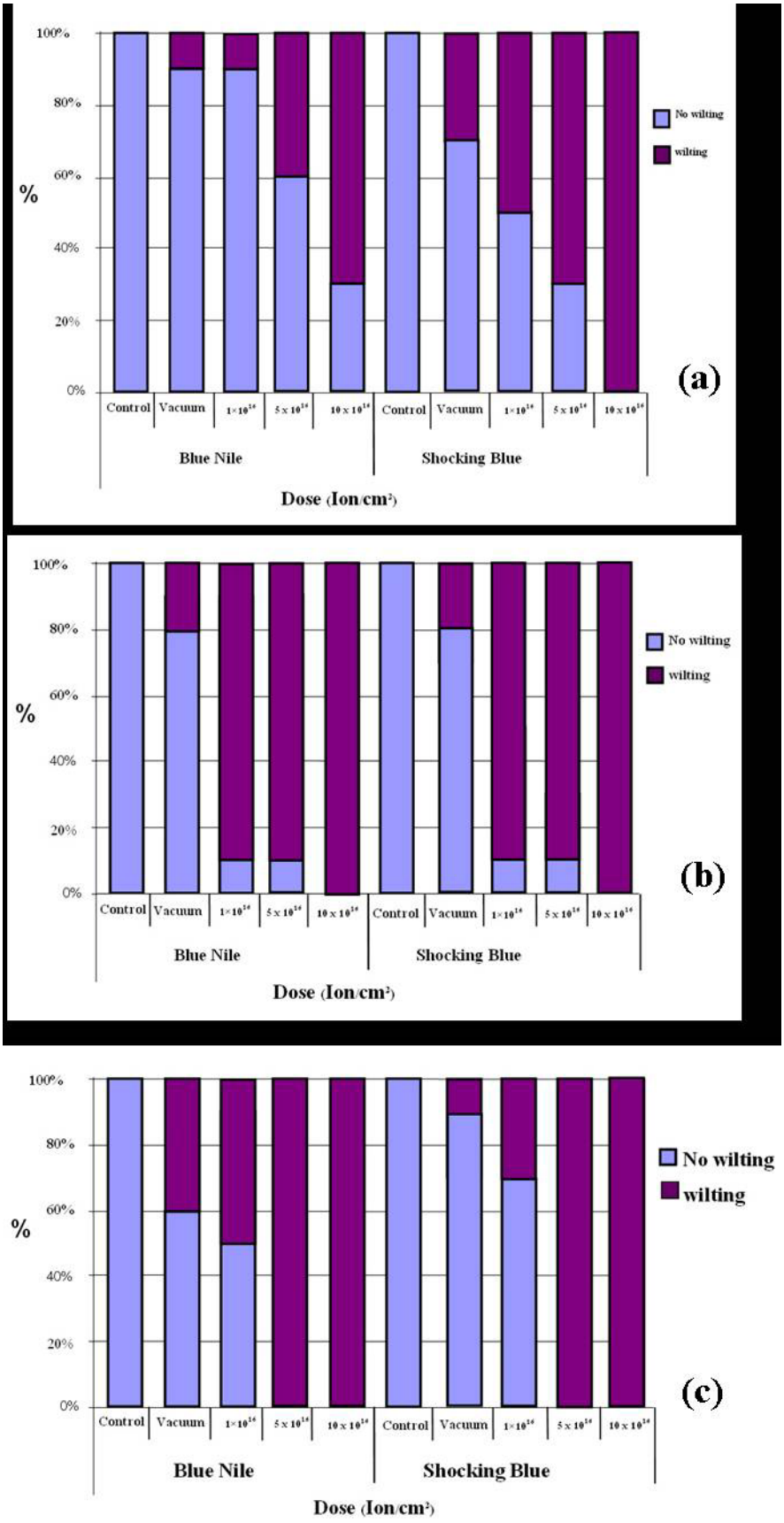

100%
80%
60%
40%
20%
0%
%
No wilting
wilting
(a)
Control
Vacuum
1×10¹⁴
5×10¹⁴
10×10¹⁴
Blue Nile
Shocking Blue
Dose (Ion/cm²)
(b)
(c)

Figure 3

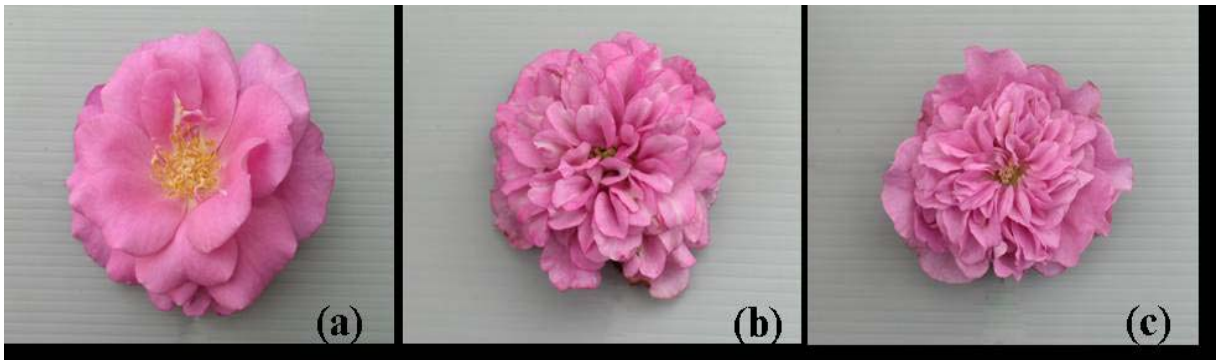


Figure 4

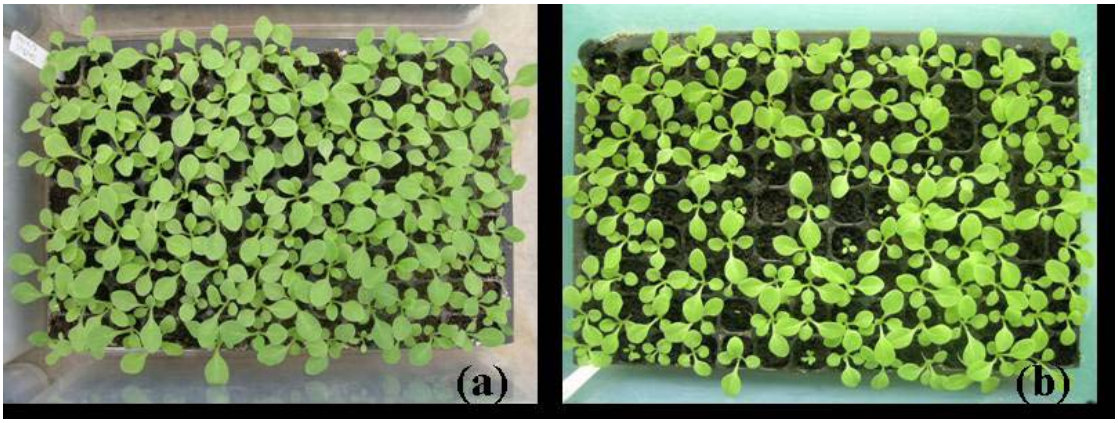


Figure 5

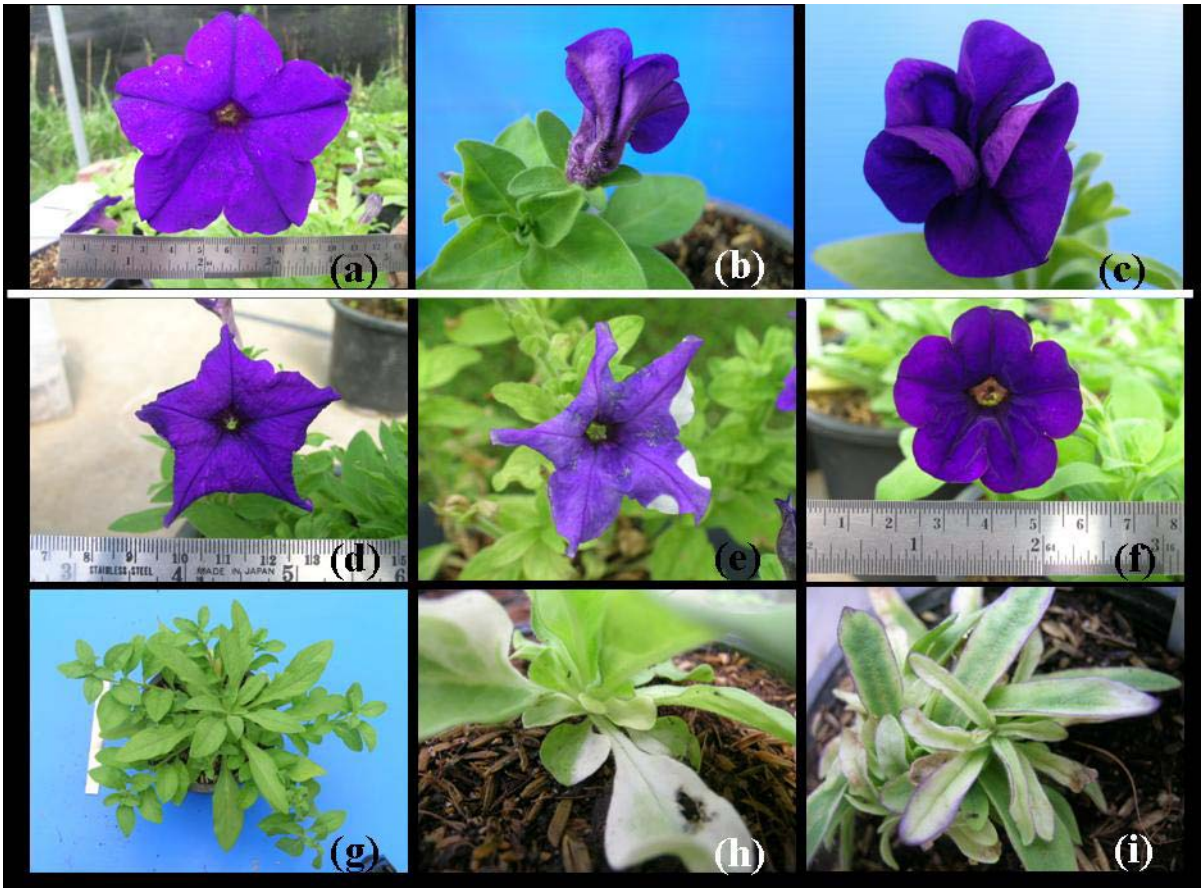


Figure 6

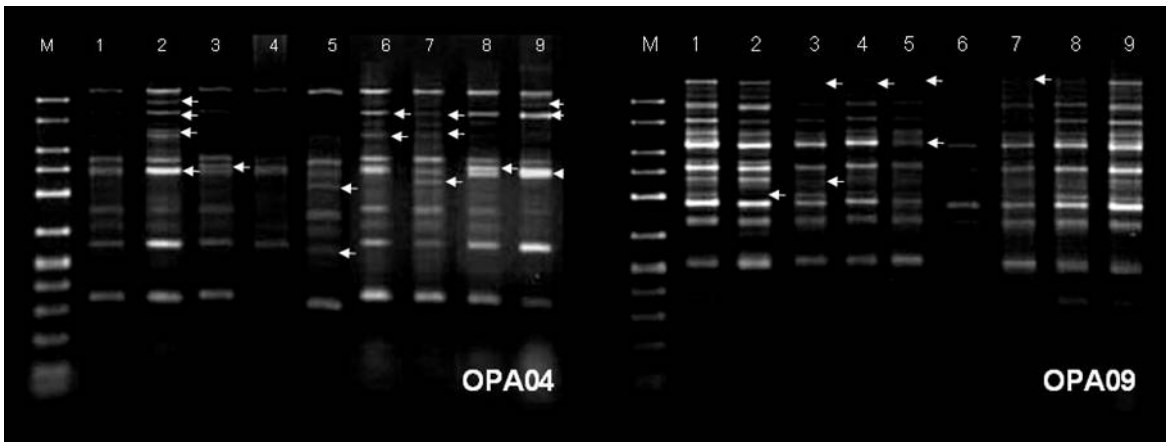


Figure 7

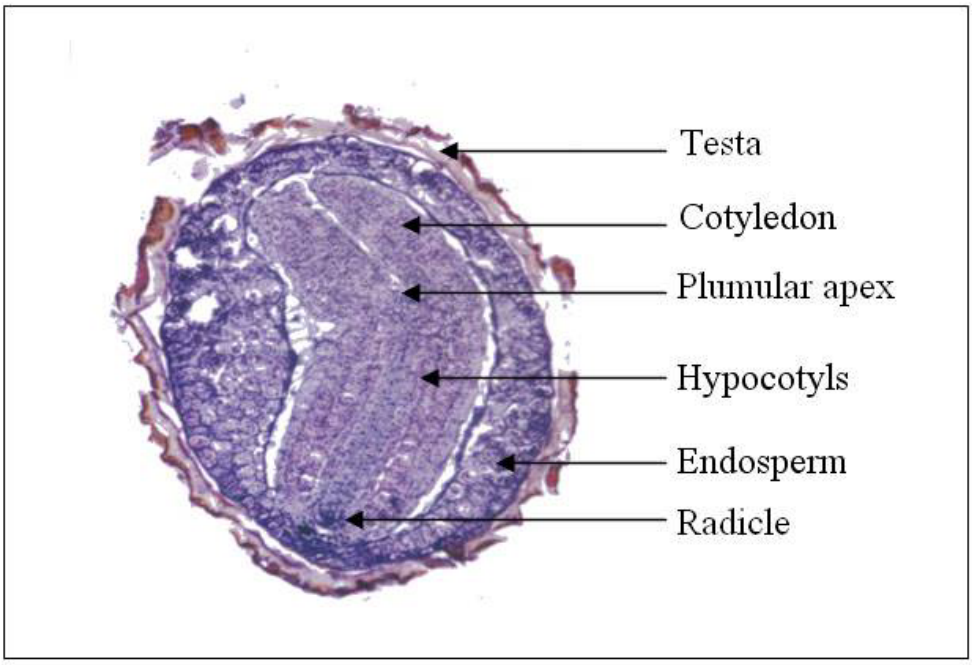

Testa
Cotyledon
Plumular apex
Hypocotyls
Endosperm
Radicle

Figure 9

Very Low-Energy and Low-Fluence Ion Beam Bombardment of Naked Plasmid DNA

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Ion beam bombardment of biological organisms has been recently applied to mutation breeding of both agricultural and horticultural plants. In order to explore relevant mechanisms, this study employed low-energy ion beams to bombard naked plasmid DNA. The study aimed at simulation of the final stage of the process of the ion beam bombardment of real cells to check whether and how very low-energy and low-fluence of ions can induce mutation. Argon and nitrogen ions at 5 keV and 2.5 keV respectively bombarded naked plasmid DNA pGFP to very low fluences, an order of 10^{13} ions/cm^2. Subsequently, DNA states were analyzed using electrophoresis. Results provided evidences that the very low-energy low-fluence ion bombardment indeed altered the DNA structure from supercoil to short linear fragments through multiple double strand breaks and thus induced mutation, which was confirmed by transfer of the bombarded DNA into bacteria *E. coli* and subsequent expression of the marker gene.

PACS codes: 61.80.Jh, 87.53.Ay

Keyword: low-energy ion beam bombardment, naked DNA, plasmid, mutation, electrophoresis

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1. Introduction

Low-energy ion beam bombardment of biological organisms has recently been successfully applied to mutation breeding of both agricultural and horticultural plants [1]. Mutation can be introduced to DNA as a result of enzymatic processing of DNA lesions of post-bombarding replication. However, the mechanisms of low-energy ion bombardment-induced mutations are not well clarified at the molecular level [2]. Many experiments using tens of keV ion beams have achieved mutations. Two types of mechanisms have been suggested for the mutation, namely direct and indirect effects of ion beam bombardment [3]. The direct effect refers to implanted ions directly interacting with DNA to cause changes in the DNA structures. The indirect effects involve ion implantation induced productions of x-ray, secondary electrons, heating and free radicals. This research was focused on the direct effect. The range of such low-energy ions in plant cell materials has been predicted to be several-fold that calculated for compressed solid of the cell materials [4]. Thus, it is statistically possible for a very small portion of ions able to penetrate through the cell materials including the cell envelope and cell inner substance that cover the cell nucleus to reach DNA. In this case, both energy and fluence of these ions must be very low. A question raised is whether the few very low energy ions can still induce mutation for DNA. A good way to study this issue is to use low-energy and low-fluence ions to directly bombard naked DNA in vitro to simulate the final-step interaction between the ions and DNA. There have been some investigations done on various low-energy radiations including low-energy ion bombardment of DNA [5-10]. The results showed that low-energy ions could produce plasmid DNA strand breaks. However, our investigation is the first attempt to use very low energy nitrogen ions, which are the ion species most popularly applied for ion beam mutation practice and also the abundant element in DNA, and compare effects from N ions with inert and heavy Ar ions.

2. Experiment

2.1 DNA preparation and bombardment

An initial sample of plasmid pGFP (3344 base pairs) was purchased from Clontech. The sample was replicated following transformation into *Escherichia coli* (*E. coli*) and subsequently extracted and purified using a QIAGEN® Plasmid Purification kit according to the manufacturer's protocol. The plasmid DNA was dissolved in sterile, de-ionized water resulting in a plasmid concentration of 1 μg/μl. The solution was divided into aliquots and later diluted in water as necessary. Aliquots of 1-μl plasmid DNA solution (containing 100 μg DNA unless otherwise indicated) was deposited on sample pots of a sample holder. The holder, as shown in Fig. 1, was designed and made in special considerations. It was made from glass for bio-cleanness, and a set of glass tubes, instead of a dish which was found easily cracked by beam heating. On the tubes, pots, each with 5 mm in diameter and 5 mm in depth, were glass-worked in separation to prevent DNA molecules from jumping out due to beam sputtering effect. The DNA samples in the pots were then dried by heating to 60 ºC for 5 minute before bombarding. Nitrogen ions at 2.5 keV and argon ions at 5 keV (both having almost the same ranges in most materials) to fluencies of 3, 6, 9×10^{13} ions/cm^2 bombarded the naked plasmid DNA with the bioengineering-specialized ion beam line [11]. In each ion beam bombardment, one condition was normally applied to three samples and a group of three samples which were covered with a stainless steel plate in the neighbor of the ion bombarded samples were used as the vacuum or internal control.

2.2 Gel electrophoresis

After bombardment, the samples of both control and bombardment were then individually recovered in 10 μl of de-ionized water for dilution and divided two parts for analysis. One part was stored at –20 ºC for transfer into *E. coli* and others were then added to with 2 μl of gel loading buffer (0.25% (w/v) bromophenol blue, 40% (w/v) sucrose). The

samples added with gel and also the natural or solution control and digested control samples were then loaded onto a gel electrophoresis apparatus [6]. This gel was run at a constant voltage (100V cm^{-1}) for about one hour. Images of the gels were captured using UV-transilluminator (for viewing DNA in agarose gels stained with ethidium bromide) and digital camera (for capturing). Fluorescence intensity plots were obtained using the Scion Image for Windows. There were totally four types of samples analyzed in electrophoresis: (1) ion bombarded, (2) vacuum or internal control, (3) natural or solution control, and (4) digested control. The internal control samples were subjected to the same preparation, vacuum and collection procedures as the bombarded samples but not subjected to bombardment. The solution control consisted of the same quantity of plasmid as the internal controls but it was not subjected to the deposition, vacuum and collection procedures. The digested control consisted of a sample of pGFP digested with restriction enzyme EcoRI (Sigma) in order to act as a marker for full length linear plasmid. The intensity corresponding to each form in the electrophoresis was quantified by integrating the area under the corresponding peak [6,12,13]. Curve fitting was carried out using the Microsoft Office Excel 2007.

2.3 Selection of DNA mutation

The ion-bombarded DNA sample which was stored at –20 ºC was transferred into *E. coli* JM109 competent cells. And the transformation mixture, containing 200 μg/ml IPTG (isopropyl *β*-D-thiogalactoside), was plated on plates. After overnight incubation at 37 ºC, white plaques were picked out and plated on plates again to check for their purity under ultra violet (UV) light.

3. Results and Discussion

The results from the electrophoresis analysis are shown in Fig. 2. It is known that when a single-strand break (SSB) is induced, DNA converts into a relaxed form, and when a double strand break (DSB) or multiple DSBs are produced, DNA converts to a linear full-length form or fragments. From the figures, it is clearly seen that upon the very low-energy low-fluence ion bombardment both relaxed and linear forms are produced and hence SSB and DSB indeed occur. It is noticed that in the vacuum controls, the relaxed form is dominantly produced, indicating vacuum effect on DNA SSB. The changes in the amounts of the DNA forms as increasing the ion fluence are found related to ion species. As increasing the ion fluence, the amount of the original DNA supercoiled form decreases for the N-ion bombardment case but does not much change for the Ar-ion bombardment case; the amount of the relaxed form is almost stable for the N-ion case but slightly increasing for the Ar-ion bombardment; the amount of the linear form increases for the N-ion case more than for the Ar-ion case (Fig. 3). This comparison indicates that nitrogen ions, even with lower energy than that of argon ions, are more effective in producing double strand breaks and thus more capable to induce GFP gene mutation than argon ions. This result seems to be conflict with common knowledge that predicts higher-energy and heavier ions able to produce more damage than lower-energy and lighter ions. Whether more physics and biology are involved is being further investigated. One hypothesis is that because DNA contains much nitrogen at the nitrogenous bases, externally introduced nitrogen will have intimate interaction with the original nitrogen so that more effects can be produced. It can be seen that the large reduction in the supercoiled form is not balanced by increases in the relaxed and full-length linear forms. These facts suggest that the relaxed and full-length linear forms are not only the transient products and they must be further converted to a form that is not detected [5]. Other reasons include that these undetected fragments caused by the multiple DSBs may move

more rapidly than full length linear strands and may be lost due to migration out of studied area of the gel [6].

The result of DNA transfer in *E. coli* showed that green (non-mutant) and white (mutant) colonies were produced. The white colonies were picked out and plated on plates again to check for their purity as shown in Fig. 4. The appearance of white colonies that are the evidence of the GFP gene damaged and thus not functioning confirms that low-energy ion beam bombardment indeed induced DNA mutation. Our gene sequencing showed that the sequences of the GFP gene in the mutants induced by both Ar-ion and N-ion bombardments were similar to that of the GFP in the control. This means that the GFP gene is not mutated. Therefore, the mutation can only be attributed to the Lac promoter, because GFP is expressed from the Lac promoter as a fusion with several additional amino acids, including the first five amino acids of the lacZ protein.

Note that the ion fluence was in the order of 10^{13} ions/cm^2, about three orders lower than that applied for normal ion bombardment induction of mutation. This is corresponding to, on average, only one ion reaching DNA per 1000 incident ions. This low fluence means about 1/10 ions impinging on DNA at per 1 nm^2. It is well known that the DNA chain is about 2 - 3 nm wide and one nucleotide unit is 0.33 nm long [14], or about 1-nm^2 area for a nucleotide unit. So, on average, every 10 units are bombarded by only one ion. This low probability of impingement has already caused significant changes in the DNA structure, demonstrating how sensitive and effective the low-energy ions are capable of inducing mutation.

4. Conclusion

The effect of very-low-energy and low-fluence ion beam bombardment of naked DNA was investigated for exploring mechanisms involved in low-energy ion beam induced

mutation. It is concluded that low-energy low-fluence ion beams can indeed produce DNA damage in the forms of SSB, DSB and multiple DSB, which are the bases of mutation of biological organisms. Lighter active nitrogen ions are found more effective in induction of mutation than heavier inert argon ions, probably due to more biological effects. The study confirms that one of the physical mechanisms in ion beam mutation is a small portion of incident ions capable of penetrating the materials covering the nucleus to directly interact with DNA and thus cause mutation.

Acknowledgements

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Figure captions

Figure 1. The glass sample holder used for holding naked plasmid DNA samples in ion bombardment.

Figure 2. Examples of electrophoresis results. The samples were separated on 1.4% agarose gel and visualized by ethidium bromide staining. Keys: S-supercoiled, R-relaxed, L-linear, M-marker, Sc-solution control, Dc-EcoRI digested control, Ic-internal control, Ir-irradiated with fluences of 3 $\times 10^{13}$ (Ir1), 6 $\times$ 10^{13} (Ir2), and 9 $\times$ 10^{13} (Ir3) ions/cm^2. (a) N-ion bombardment. (b) Quantification of various forms of plasmid DNA after N-ion bombardment. (c) Ar-ion bombardment. (d) Quantification of various forms of plasmid DNA after Ar-ion bombardment. In each electrophoresis analysis, two samples were used for each ion beam condition.

Figure 3. A comparison of the linear-form plasmid DNA between Ar-ion and N-ion bombardments. The data are means of many sets of the electrophoresis results.

Figure 4. UV observation of plasmid containing green fluorescence protein (pGFP) transferred into *E. coli.* White colony indicates pGFP damaged (not functioning) and thus DNA mutated, while green colony is non-mutant.

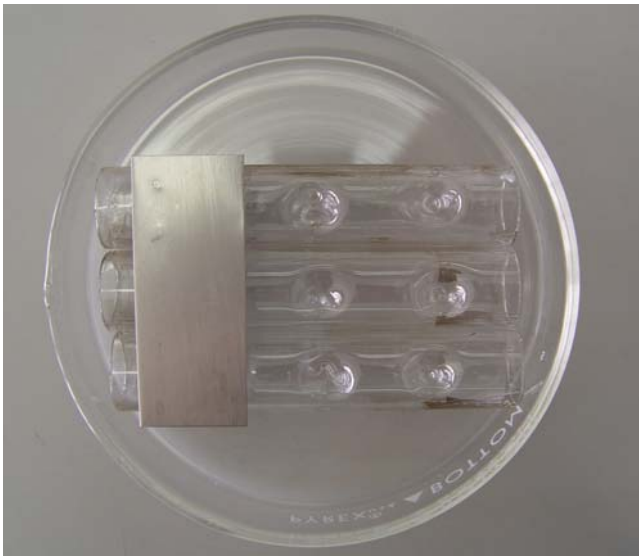
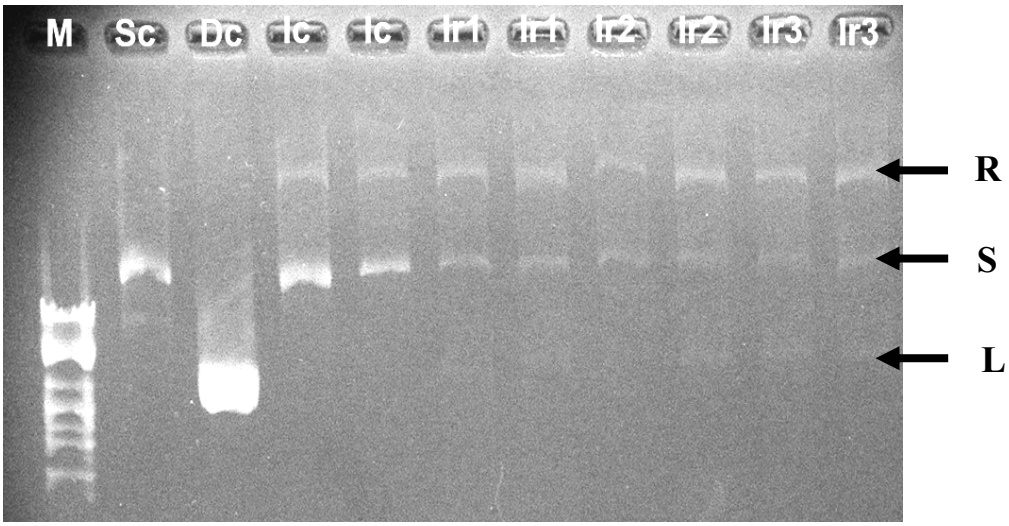
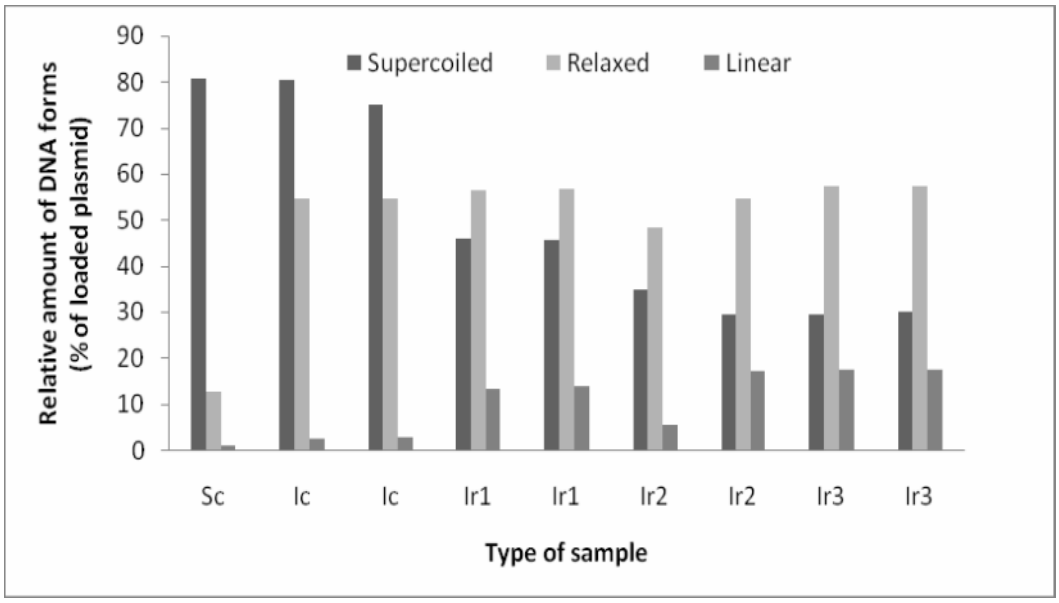


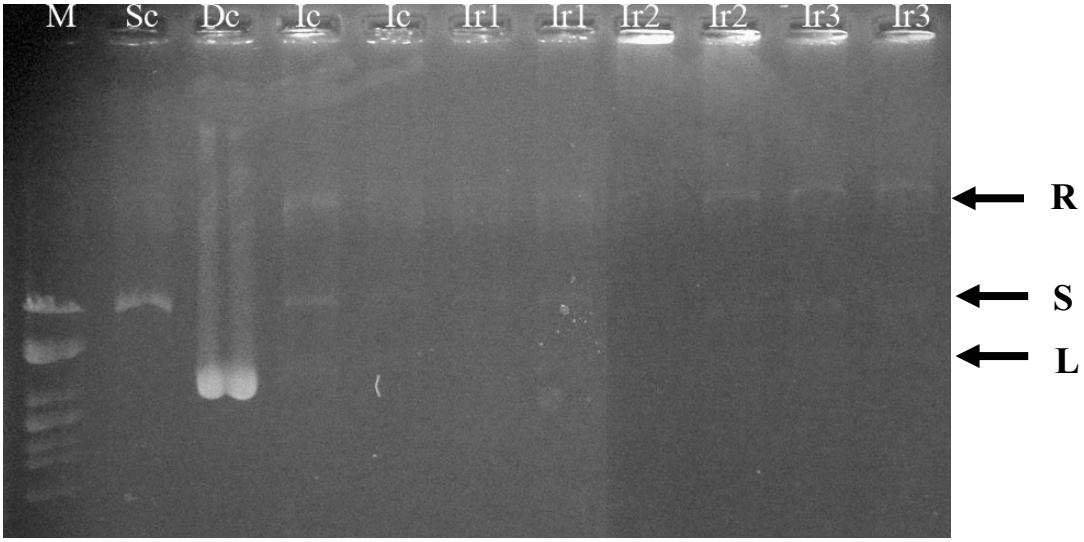
Figure 1


M
Sc
Dc
Ic
Ic
Ir1
Ir1
Ir2
Ir2
Ir3
Ir3
R
S
L

(a)


Relative amount of DNA forms (% of loaded plasmid)
90
80
70
60
50
40
30
20
10
0
Supercoiled
Relaxed
Linear
Sc
Ic
Ic
Ir1
Ir1
Ir2
Ir2
Ir3
Ir3
Type of sample

(b)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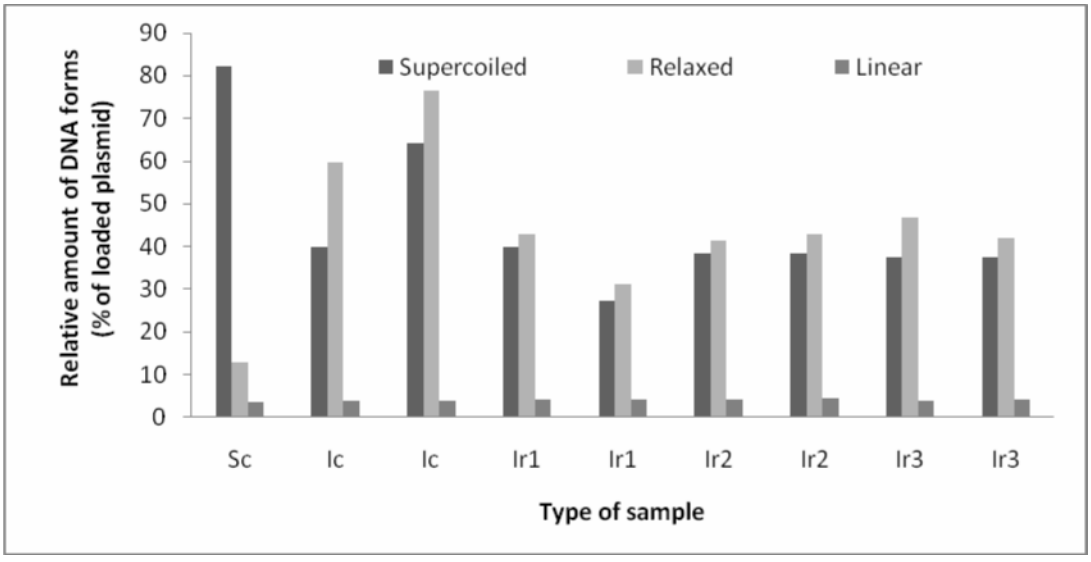
Ic
Ic
Ir1
Ir1
Ir2
Ir2
Ir3
Ir3
R
S
L

(c)


Relative amount of DNA forms (% of loaded plasmid)
90
80
70
60
50
40
30
20
10
0
Supercoiled
Relaxed
Linear
Sc
Ic
Ic
Ir1
Ir1
Ir2
Ir2
Ir3
Ir3
Type of sample

(d)

Figure 2

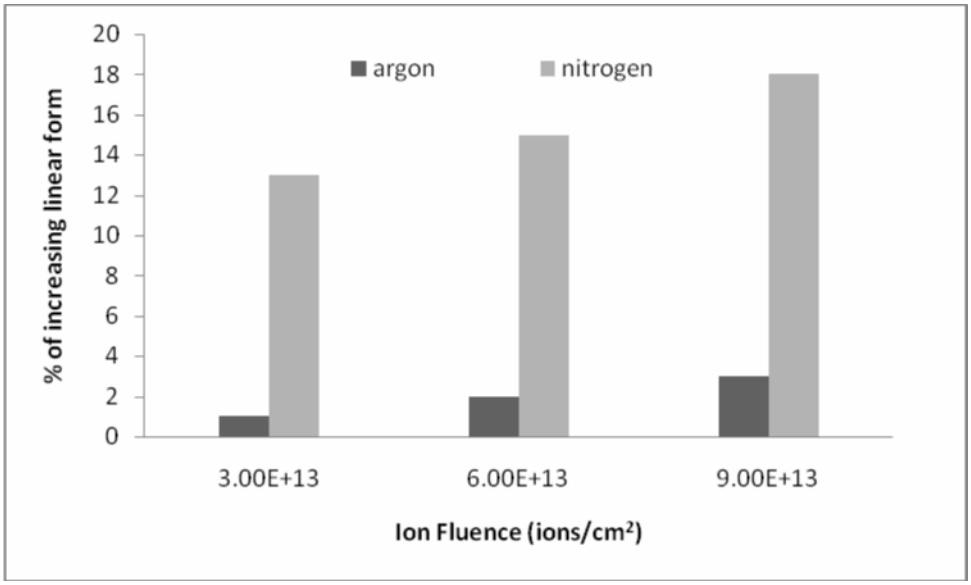


Figure 3

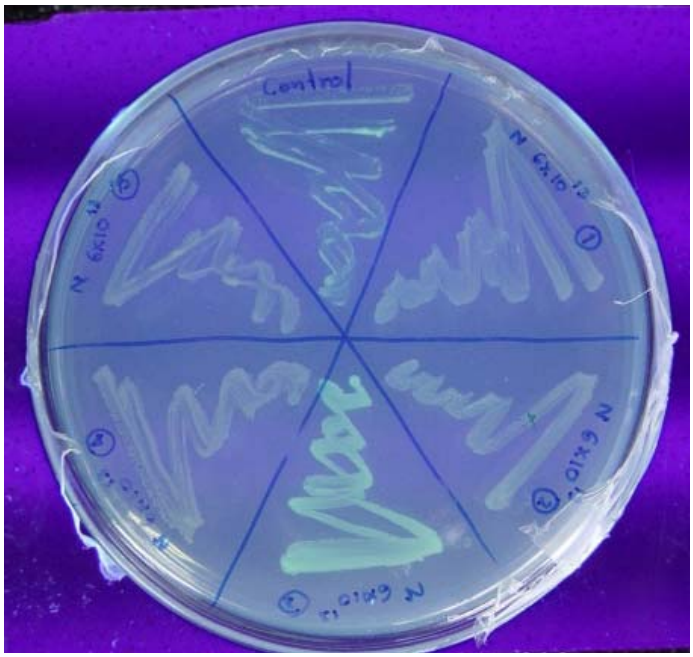


Figure 4