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

Ruttaporn Chundet[1], [2]Robert W. Cutler, [3]Somboon Anuntalabhochai

[1]Division of Biotechnology, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand
[2]Box 15 Prasing Post Chiang Mai, Thailand 50205
[3]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

Using a low-energy N^+ / N_2^+ ion beam, a mutant variety of Thai jasmine rice (*Oryza sativa* L. cv. KDML105) was created with distinctive black seeds, short-in-stature and photoperiod insensitive. To characterize the biochemical origin of the black seed color phenotype, flavonoid and anthocyanin accumulation levels were measured as was the expression of the genes involved in the anthocyanin biosynthesis pathway. Anthocyanin synthase, an enzyme not expressed in the original variety, was found to be expressed in all mutant tissues in addition to the two enzymes *F3'H* and *F3'5'H* which initiate the alternative color pathways. The expression of *MYC or Ra*, a known anthocyanin upregulator, in the mutant is proposed to be caused by the inactivation of a repressor gene present in the original variety which was inactivated in the mutant by the ion beam bombardment. The increased production of anthocyanin, a known antioxidant, and the additional growing season due to the high-light insensitivity mark this mutant as a possible new improved crop variety for Thai rice cultivation.

Keywords: Low energy ion-beam, *Oryza sativa* L. cv. KDML105, *F3'H, F3'5'H, DFR, MYC.*

INTRODUCTION

To characterize a potentially economically important mutant variety of Thai jasmine rice (*Oryza sativa* L. cv. KDML105) created using low-energy ion beam bombardment, we examined the morphological and physiological features, flavonoid and anthocyanin accumulation levels, and explored the expression of genes involved in anthocyanin biosynthesis. The progenitor of this mutant variety of Thai jasmine rice called Khao Hom Mali 105 (KDML105) is widely valued due to its long grain, appealing flavor and good texture, which marks it as one of the key varieties for export from Thailand (Wongpornchai et al. 2004). Although this variety is the reportedly highest quality rice available (Leelayuthsoontorn and Thipayarat, 2006), it has several characteristics which could be improved such as photo sensitivity which limits growth to short daylight seasons, long stalks which can be damaged by high winds, relatively low yield as compared to other rice varieties and a lack of resistance to pathogens (Ronald, 1997).

In an effort to create additional varieties of jasmine rice with novel characteristics, seeds were bombarded with low-energy N^+ / N_2^+ ions in vacuum as a mechanism to induce mutation. Such ion beams have been characterized as having a wide mutation spectrum with lower damage to living tissue and a higher mutation rate in comparison to other mutation methods used in plants (Anuntalabhochai et al., 2004; Morishita et al. 2003). The mutant described herein had a modified anthocyanin biochemical pathway leading to an increased accumulation of anthocyanin in various tissues of the mutant jasmine rice. Anthocyanins are water-soluble pigments produced in many vascular plants which accumulate in vacuoles (Marty, 1999), and are involved in a broad range of biological functions (Dixon and Steele, 1999; Winkle-Shirley, 2001). The first enzymes in the anthocyanin pathway are chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone-3-hydroxylase (F3H), which produce chalcones, flavanones and dihydroflavonols, respectively. From these anthocyanins 3-O-glycosides are synthesized from dihydroflavonols by the consecutive reactions catalyzed by dihydroflavonol 4-reductase (*DFR*), anthocyanin synthase (*ANS*) and

UDP-glucose flavonol 3-O-glucosyltransferase (Dixon and Palva, 1995). The expression of anthocyanins biosynthesis genes is regulated at the transcription level and consequently, the pigmentation pattern must be specified by expression of the regulatory genes (Lin-Wang et al., 2010). Therefore both differential expression of structural genes and the modification of regulatory genes could be responsible for modified anthocyanin expression patterns.

MATERIALS AND METHODS

Ion beam bombardment

To prepare the Thai jasmine rice seeds for bombardment, about 4,800 seed coats were carefully peeled to avoid damage to the embryo tissues. All of the peeled seeds were then placed into the sample holder. The rice seeds were positioned so that their embryonic end was exposed to the ion beam line. The nitrogen ion beam composed of both atomic (N) and molecular (N_2) ions was used to bombard the seeds at an accelerating voltage of 60 kV where the energy of the nitrogen ions was 60 keV with fluences of 4 x 10^{16} ions cm^{-2} using the protocol described by Anuntalabhochai et.al., 2004.

Scanning electron microscopy

To image the surface of the bombarded rice, a scanning electron microscope (SEM) was used where the bombarded and control rice seeds were fixed in glutaraldehyde, dehydrated through an alcohol-acetone series, dried in a critical-point drying apparatus, mounted on stubs and coated with gold in a sputter coater (Maiti, 1994). The specimens were observed and photographed with a JEOL 5800 LV SEM operating at a 15-kV accelerating voltage.

Plant materials

The seeds of Thai jasmine rice (*Oryza sativa* indica KDML105) used in this study were kindly provided by the Agronomy Department, Agriculture Faculty, Chiang Mai University, Thailand. After being bombarded, the seeds were kept moist overnight, after which the seeds were planted in potting soil, and allowed to grow for three to four weeks until attaining the rice seedling growth stage. From these rice seedlings, which were around 15cm in height, samples were selected and transplanted to plastic pots for two months. The cultivations were carried out during the July to December season. A mutant from the bombarded seed sample which had distinctive black seeds and short-in-stature was selected for analysis.

Preparation of rice extract and anthocyanin analysis

Leaves, roots, auricles and seeds from the M5 mutant and control were harvested and stored at −80°C. Each sample was ground to a fine powder in liquid N_2 and any anthocyanin present was extracted using 1% HCl in methanol or 70% (w/v) acetone containing 0.1% (w/v) ascorbate, respectively, for 12 h at 4°C in the dark. The total anthocyanin solutions were then extracted using Folch partitioning (Folch et al., 1951) and the anthocyanin absorbance peak was measured at 530 to 540 nm.

Analysis of gene expression (RT-PCR)

Total RNA was isolated from the seedlings, multiple body parts of mature M5 mutants and controls using the manufacturer's recommended Trizol reagent protocol (Invitrogen). For each RNA sample, absorption at 260 nm was measured and the RNA concentration calculated as A_{260} × 40 (µg mL^{-1}) × dilution factors. The quality of each RNA sample was checked using agarose gel electrophoresis. From this RNA pool, cDNA was synthesized using the First Strand cDNA Synthesis Kit (Fermentas). To test for the presence of the six mRNA transcripts involved in the anthocyanin biochemical pathway (*Ra*, *F3'H, F3'5'H, DFR, ANS* and *Actin*), primer sequences (Table 1.) were designed to amplify internal segments of these genes.

RESULTS

Effect of ion beam bombardment on the rice embryo cell surface

Due to a high linear energy transfer, ion beam technology has been used to increase the mutation frequency in a wide spectrum of plant species (Yu et al., 1991). The mutation mechanism is through ablation of the cell surface by the positively charged nitrogen ions which perforate the seed allowing cascade ions to penetrate the cell without causing extensive damage to the interior of the cell (Vilaithong et al., 2000; Yu et al., 2002). The mutant jasmine rice seeds generated by bombardment with the low-energy N^+ / N_2^+ ion beam show a characteristic sputtering of the embryo cell envelope (Figure 1). From this image, it is clear that the surface topography has been significantly altered and the treated cell surfaces are now much rougher with several surface intrusions visible in the SEM.

Mutant jasmine rice morphological characteristics

The mutant jasmine rice presented here exhibited several

Table 1. Primer sequences used in RT-PCR

MYC, Ra	Forward	5'-ATGGCTCAGAATCATGAGAGGGTG-3'
	Reverse	5'-TCAGCACTTACCAGCAATTTTC-3'
F3'H	Forward	5'-GGTGATCGGCGCCTCGAGAATC-3'
	Reverse	5'-GGCATGTGTGGACATGGACCC-5'
F3'5'H	Forward	5'-CTACAAGATGCGTTTCGTGTATGC-3'
	Reverse	5'-CAAAACAAACACACTTCATTCATC-3'
DFR	Forward	5'-CGATTGTCTTGGAGACGAAG-3'
	Reverse	5'-GACCCCACGAATCAGAAGAAGG-3'
ANS	Forward	5'-CCAGCCTCCCTTCATCCAATCC-3'
	Reverse	5'-ATTCGAGCTCGGTACCCGGGG-3'
Actin	Forward	5'-CTTTGATTTCTCATAAGGTGCC-3'
	Reverse	5'-CTAAATCCCTTAACGAGGATCC-3'

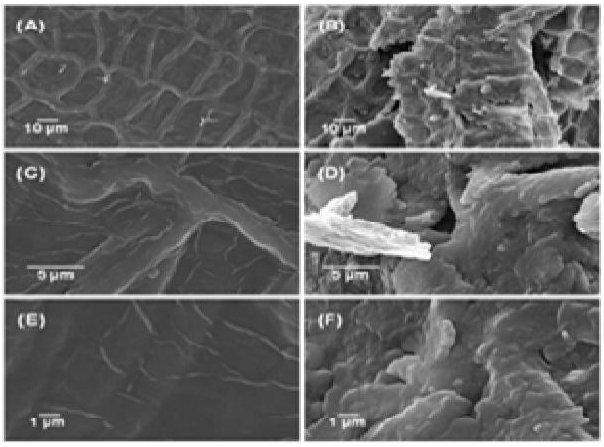

Figure 1. The morphological effect of ion-beam bombardment on seed embryo cells of jasmine rice. The six figures contain Scanning Electron Microscope (SEM) images of untreated embryo cell surfaces of KDML105 at x1,000 (*A*), x5,000 (*C*) and x10,000 (*E*) times magnification, and embryo cell surfaces bombarded with N^+ / N_2^+ ions at x1,000 (*B*), x5,000 (*D*) and x10,000 (*F*) times magnification. The treatment conditions were an applied voltage of 60 keV with a dose of $2x10^{16}$ ions cm^{-2}.

striking morphological changes from that of the wild-type variety. These changes include a decreased height of approximately 80 to 90 cm from that of the wild-type which grows to 1.2-1.5 meters, an insensitivity to high light intensity which enables it to grow in the middle of the Thai summer (March to June), and a deep purple color in multiple tissues of the plant. Figure 2*A* and Figure 2*B* present the appearance of the normal wild-type (WT) rice plant and the deep purple mutant variety respectively. As can be seen in these images, the mutant variety can be easily distinguished from the wild-type by both the color of the rice sheath and the plant stalks. The purple accumulation occurs in the stalk (Figure 2*C*), leaf (Figure 2*D*), and in the immature rice seeds where there is a slight accumulation of anthocyanin as compared to the WT (Figure 2*E*). This becomes significantly more pronounced in the mature seeds both in the husk (Figure 2*F*) and after being husked (Figure 2*G*) as compared to the progenitor variety. In addition, the buildup of purple color in the cells is clearly visible in the mutant auricle (Figure 2*I*) versus the wild-type (Figure 2*H*). Interestingly, the only tissue of the mutant variety which does not show a purple signature is in the young roots, but in older established roots the purple color is clearly observed (Figure 2*K*).

Accumulation of anthocyanin

The presence of anthocyanin was tested in the leaves,

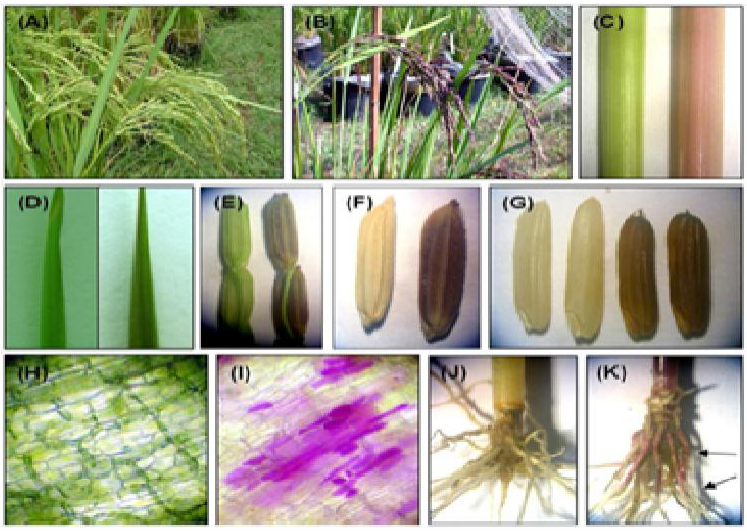

Figure 2. Morphological mutants in KDML105 jasmine rice induced with a low-energy N^+ / N_2^+ ion-beam. Figs (*A*) and (*B*) illustrate the wild-type (WT) plant versus the mutant (BKOS) phenotype. The mutant has a purple color in the plant stem (*C*), Leaf (*D*), immature-seeds (*E*), husked (*F*) and de-husked mature seeds (*G*), in the normal auricle (*H*) versus mutant auricle (*I*) which shows the accumulation of purple inside the cells, and the normal root (*J*) versus the mutant roots structures (*K*). Note the lack of purple accumulation in the young roots versus older roots of the mutant BKOS phenotype.

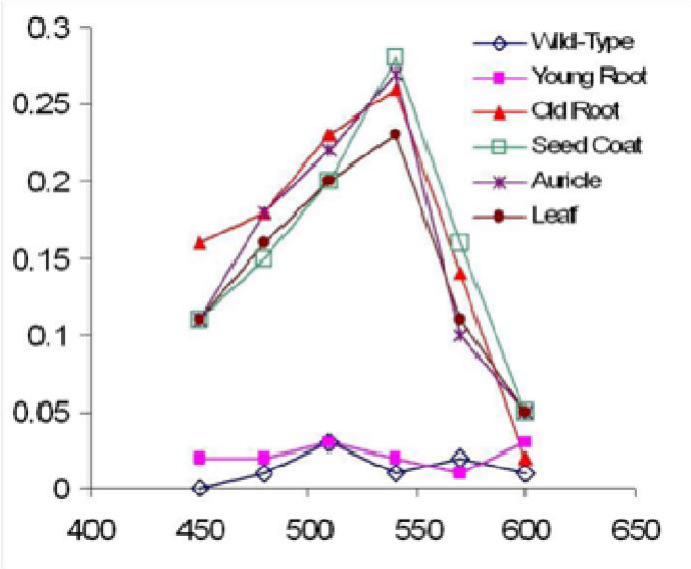

Figure 3. Anthocyanin levels in various body tissues.

young and old roots, auricles and seeds from the M5 mutant and control. In each case, the measured absorbance peaks for the different tissues (Figure 3) showed that the purple color accumulation was accompanied by the presence of a chemical with the correct absorbance peak at 530 to 540 nm.

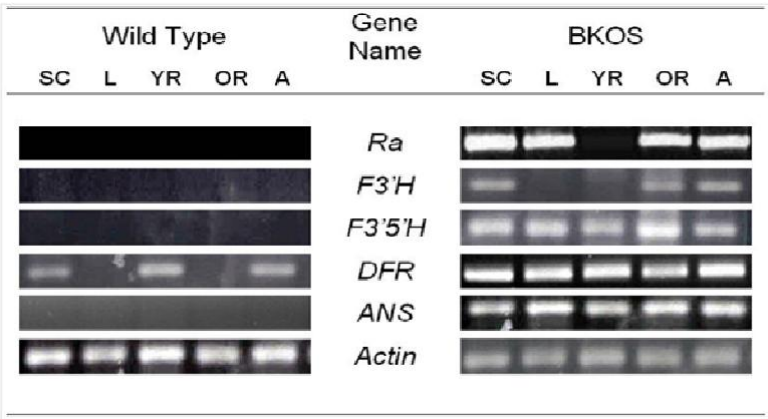

Figure 4. Semi-quantitative RT-PCR analysis used to detect the expression of genes in the anthocyanin biosynthesis pathway. Expression was tested in tissues of the seed coat (SC), leaf (L), young root (YR), old root (OR) and auricle (A) for both the wild-type and mutant BKOS jasmine rice.

Biochemical regulation of anthocyanin production

The differentially expressed proteins in the anthocyanin biochemical pathway were determined in the mutant versus the wild type. The expression levels of four core enzymes of anthocyanin biochemical pathway (*F3'H, F3'5'H, DFR,* and *ANS*), a known regulator of anthocyanin production MYC (*Ra*) and an internal standard control (*Actin*) were measured. As shown in Figure 5, both *DFR* and *ANS* are critical steps in the anthocyanin pathway, and DFR was found to be expressed in some tissues of the wild type and all tissues of the mutant (Figure. 4), while *ANS* was not expressed in the wild type at all and was highly expressed in all mutant tissues. Although the proteins *F3'H* and *F3'5'H* are not required for activation of the anthocyanin pathway, both were active in the mutant type and not in the wild type. The expression of protein *Ra* which is a known activator of the anthocyanin pathway (Schwechheimer and Zourelidou, 1998) was found to be differentially expressed in the wild type versus the mutant.

Discussion

The use of low-energy ion beams to induce mutation in cereal crops has proven to be an effective method of mutation due to a high linear energy transfer and relative biological effectiveness as composed to mutation using gamma rays. During ion beam implantation, in addition to energy absorption (as is deposited with gamma-ray and X-ray radiation), there is also mass deposition and charge exchange (Yu and Shao, 1994; Shao and Yu, 1997). This mass and charge deposition enables a low dose of irradiation to damage the double chain of DNA causing large-scale deletions (Zengliang et al., 1991) in addition to point mutations (Yang et al., 1997). To capitalize on this ability to mutate plants while retaining viability, nitrogen ions were used to bombard Thai Jasmine rice seeds and a mutant with a bright purple phenotype due to increased levels of anthocyanin was created.

The buildup of anthrocyanin in the mutant Thai jasmine rice KDML105 due to the activation of the anthocyanin biochemical pathway is most likely due to the activation of the *Ra* gene which is active in the mutant jasmine rice and is known to activate anthocyanin production in Maize (Bovy et al., 2002). Since ion beam bombardment predominantly leads to loss of function mutations, the most likely explanation for the activation of *MYC or Ra* in the mutant is due to the knockout of a repressor gene for *Ra* as shown at the top of Figure 5. The expression of *ANS* in the mutant variety is the second critical requirement for anthocyanin production, since it is not expressed in the original variety and without *ANS* the leucoanthocyanidins cannot be converted to the colored cyanin products. The protein product dihydrokaempferol is not present in the wild type (data not shown), this points to a breakdown of the initiation of the biochemical pathway which is why the *Ra* activator was chosen to be tested.

Horticultural approaches to improve the nutritional quality of crops provide an inexpensive complement to medical programs and nutritional supplementation to prevent human disease. In particular anthocyanins are known to be effective antioxidants capable of free radical scavenging (Rice-Evans, 2001; Einbond et al., 2004). Besides antioxidant activity, anthocyanins are considered important substances in cancer prevention as they have been shown to inhibit the growth of cancer cells in multiple tissues (Geeta et al., 2006; Manach et el., 2004). To capitalize on these beneficial properties, the increased

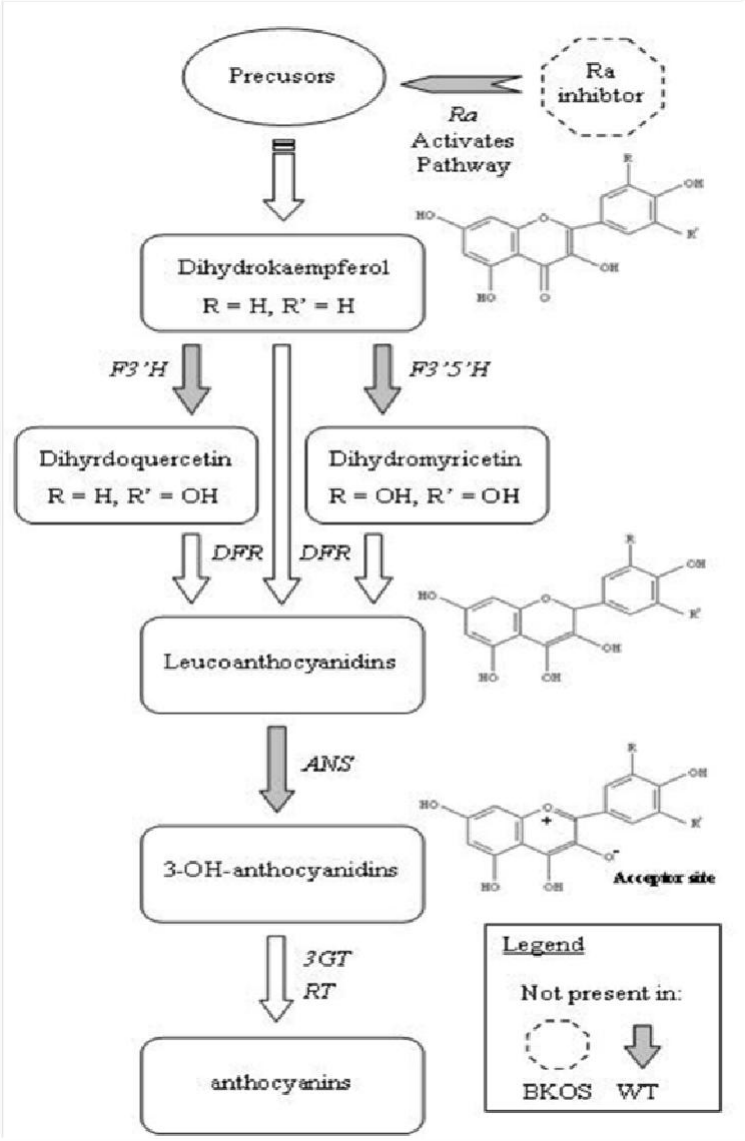

Figure 5. Anthocyanin biosynthesis pathway. Dihydrokaempferol is the base product for the orange color pathway. Two additional pathways proceed using the same biochemical steps after dihydrokaempferol is modified using *F3'H* (red color) or *F3'5'H* (blue color) by adding extra OH groups at resides R and/or R'. Proteins shaded gray are not expressed in the WT and the predicted *Ra* repressor protein is not expressed in BKOS.

production of anthocyanins has become a highly sought trait in plants as tomato (Butelli et al., 2008) and Shiraz grape berries (Boss et al., 1996). Although the anthocyanin biosynthetic pathway has been completely elucidated, attempts to modify anthocyanin biosynthesis have met with varying degrees of success (Peer et al., 2001), showing that multiple regulatory proteins may act synergistically (Verhoeyen et al., 2002; Turnbull et al., 2004). The increased anthocyanin production in a staple crop such as rice could provide an effective means to provide the benefit of anthocyanins to a wide range of economically disadvantaged people otherwise unable to afford the costs of nutritional supplementation. The mutant Thai jasmine rice described here has the potential to be just such a staple crop which could provide another direction for the growing interest in the development of agronomically important food crops with optimized levels and composition of anthocyanins.

*This research was conducted using funding from the following sources: The Thailand Research Fund (TRF (*code **MRG5180017***) and the National Research Council of Thailand (NRCT).*

REFERENCES

Anuntalabhochai S, Chandej R, Phanchaisri B, Yu LD, Vilaithong T, Brown IG (2004). Mutation Induction In Thai Purple Rice By Low-Energy Ion Beam. Proceedings of the Ninth Asia Pacific Physics Conference (9th APPC), Vietnam, pp.1-6.

Boss PK, Davies C, Robinson SP (1996). Analysis of the Expression of Anthocyanin Pathway Genes in Developing Vitis vinifera L. cv Shiraz Grape Berries and the Implications for Pathway Regulation. Plant Physiol., 111: 1059-1066.

Bovy A, de Vos R, Kemper M, Schijlen E, Pertejo MA, Muir S, Collins G, Robinson S, Verhoeyen M, Hughes S, Santos-Buelga C, van Tunen A (2002). High-Flavonol Tomatoes Resulting from the Heterologous Expression of the Maize Transcription Factor Genes LC and C1, Plant Cell, 14, 2509-2526.

Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EGWM, Hall RD, Bovy AG, Luo J, Martin C (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat. Biotechnol., 26: 1301-1308.

Dixon RA, Palva NL (1995). Stress-induced phenylpropanoid metabolism. Plant Cell, 7: 1085-1097.

Dixon RA, Steele CL (1999). Flavonoids and isoflavonoids-a gold mine for metabolic engineering. Trends Plant Sci., 4: 394-400.

Einbond LS, Reynertson KA, Luo X-D, Basile MJ, Kennelly EJ (2004). Anthocyanin antioxidants from edible fruits. Food Chem., 84: 23-28.

Folch J, Less M, Sloane-Stanley GHB (1951). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226: 497-509.

Geeta L, Minnie M, Cuiwei Z (2006). Anthocyanin-Rich Extracts Inhibit Multiple Biomarkers of Colon Cancer in Rats. Nutr. Cancer, 54: 84-93.

Leelayuthsoontorn P and Thipayarat A (2006). Textural and morphological changes of Jasmine rice under various elevated cooking conditions. Food Chem, 96: 606-613.

Lin-Wang K, Bolitho K, Grafton K (2010). An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. BMC Plant Biol., 10: 1-17.

Maiti RK, Hernandez-Pineiro JL and Valdez-Marroquin M (1994). Seed ultrastructure and germination of some species of Cactaceae. Phyton., 55: 97-105.

Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L (2004). Polyphenols: Food sources and bioavailability. Am. J. Clin. Nutr., 79(5): 727-747.

Marty F (1999). Plant Vacuoles. Plant Cell, 11: 587-599

Morishita T, Yamaguchi H, Degi K, Shikazono N, Hase Y, Tanka A, Abe T (2003). Dose response and mutation induction by ion beam irradiation in buckwheat. Nucl. Instrum. Meth., 206: 565-569.

Peer WA, Brown DE, Tague BW, Muday GK, Taiz L, Murphy AS (2001). Flavonoid Accumulation Patterns of Transparent Testa Mutants of Arabidopsis. Plant Physiol., 126: 536-548.

Rice-Evans C (2001). Flavonoid Antioxidants. Curr. Med. Chem., 8: 797-807.

Ronald PC (1997). Molecular basis of disease resistance in rice. Plant Mol. Biol., 35: 179 186.

Schwechheimer C, Zourelidou M, Bevan MW (1998). Plant Transcription Factor Studies. Annu. Rev. Plant Phys., 49: 127-50.

Shao CL and Yu ZL (1997). Mass deposition in tyrosine irradiated by a N^+ ion. Radiat. Phys. Chem., 50: 595-599.

Turnbull JJ, Nakajima J-i, Welford RWD (2004). Mechanistic Studies on Three 2-Oxoglutarate-dependent Oxygenases of Flavonoid Biosynthesis. J. Biol. Chem., 279(2): 1206-1216.

Verhoeyen ME, Bovy A, Collins G, Muir S, Robinson S, deVos CHR, Colliver S (2002). Increasing antioxidant levels in tomatoes through modification of the flavonoid biosynthetic pathway. J. Exp. Bot., 53(377): 2099-2106.

Vilaithong T, Yu LD, Alisi C, Phanchaisri B, Apavatjrut P, Anuntalabhochai S (2000). A study of low-energy ion beam effects on outer plant cell structure for exogenous macromolecule transferring Surf. Coat Tech., 128/129: 133-138. .

Winkle-Shirley B (2001). Flavonoid biosynthesis: A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol., 126: 485-493.

Wongpornchai S, Dumri K, Jongkaewwattana S, Siri B (2004). Effects of drying methods and storage time on the aroma and milling quality of rice (Oryza sativa L.) cv. Khao Dawk Mali 105. Food Chem, 87: 407-414.

Yang JB, Wu LJ, Li L (1997). Sequence analysis of lacZ–mutations induced by ion beam irradiation in double-stranded M13mp18DNA. Sci. China ser. C., 40: 107-112.

Yu LD, Phanchaisri B, Apavatjrut P (2002). Some investigations of ion bombardment effects on plant cell wall surfaces. Surf. Coat Tech., 158159: 146-150.

Yu ZL, Deng J, He JJ (1991). Mutation breeding by ion implantation. Nucl. Instr. Meth. Phys. Res., B 59/60: 705-708.

Yu ZL, Shao CL (1994). Dose effect of the tyrosine sample implanted by a low energy N^+ ion beam. Radiat. Phys. Chem., 43: 349-351.

Zengliang Y, Jianguo D, Jianjun H (1991). "Mutation breeding by ion implantation. Nucl. Instr. Meth. Phys. Res., B 59/60: 705-708.