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Mutation induction of EMS and 60Co γ irradiation in vitro cultured seedlings of red pulp pitaya (Stenocereus) and ISSR analyzing of mutant

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Abstract

Background

Pitaya is a new type of fruit tree developed vigorously in recent years in China. The current variety characteristics of pitaya can not meet the needs of market development. In order to speed up the breeding process and increase the variation frequency, the red pulp pitaya variety 'Zi Honglong' seedlings in vitro were used as experimental materials, which were treated with different dose of ethyl methanesulfonate (EMS) and ^{60}Co γ irradiation, respectively.

Results

The results indicated that the appropriate concentration for the EMS mutagenesis was 3.71%-3.95%, and the optimum time was 8.26–9.32 h. The feasible irradiation dose range for ^{60}Co γ mutation was 38.5–42.4 Gy. The dose of mutagens was inversely proportional to the survival and differentiation rate of seedlings. By observing the morphological characteristics of mutagenic materials, 14 mutants were screened out. According to the ISSR analysis, a total of 67 bands were amplified from the 9 primers, among which 71.64% polymorphism was obtained. Compared with the control, there was a specific band in No. 5 mutant detected by primer M06, but its functional characteristics needed further research and verification. The genetic distances (GD) among the tested materials were 0.0120 to 0.4169 analyzed by NTSYS pc 2.1. The UPGMA clustering analysis demonstrated that 15 germplasm could be divided into 6 groups at a cutpoint of genetic distance 0.11, and among them No. 5, 6, 10, 12 and 14 warranted separate categorization, which showed that they had more differences in tested germplasm.

Conclusions

It concluded that pitaya mutant might be produced regardless of the mutagenic dose high or low, and a higher dose could increase the frequency of variation. In this study, critical dose and half lethal dose can be used as the appropriate dose for mutation breeding of pitaya. Additional, molecular analysis showed that there was a certain difference between the mutant plants and the control. It needs to be further verified whether this difference was a physiological variation or a real variation due to the randomness of mutation, so as to lay a foundation for better carrying out mutation breeding of pitaya.

Background

Mutation breeding is one of the effective methods of screening new varieties. In particular, the combination of mutation technology and culture technology *in vitro*, which can broaden mutation spectrum, improve mutation frequency, and keep clone variation (Predieri, 2001; Shang et al. 2014). Moreover, the whole process is carried out under sterile conditions, thus opening up a greater space for mutation screening. According to the data on the official website of FAO/IAEA in 2016 (Wu et al. 2016),

breeders in all countries have cultivated 55 new varieties of deciduous fruit trees by mutation technology, including varieties of 2 jujubes, 6 peaches, 13 apples, 1 apricot, 1 plum, 8 pears, 1 grape, 21 cherries and 2 pomegranates.

Pitaya, a member of cactus family, is a perennial, climbing and succulent plant, and also a new type of tropical and subtropical fruit tree developed widely in recent years in China (Deng et al. 2019). But there are very few reports on breeding of pitaya at home and abroad (Deng et al. 2011). Therefore, the study took seedlings *in vitro* of red pulp pitaya as materials, which treated with different dose of EMS and ^{60}Co γ, respectively, then screened the optimal mutagen dosage and mutant plants, which would lay the foundation for screening mutants with beneficial characters and cultivating new resources of pitaya fruit with excellent comprehensive characters.

Results

Effect of the EMS concentration and treatment time on the survival of seedlings

It can be seen from table 1, the survival rate of seedlings decreased with the increase of EMS concentration and treatment time. When the time was constant, a straight line regression equation could be fitted according to the relationship between EMS concentration and the survival rate. The lowest correlation coefficient (R^2 = 0.7542) among the EMS concentration and survival rate occurred at the treatment time of 3 h, while the highest correlation (R^2 = 0.9916) occurred at treatment time of 9 h. According to the linear regression equation $Y = 205.44x + 41.902$, when the EMS concentration was 3.71%, 50% of the pitaya seedlings survived, while the survival rate decreased to 40% at an EMS concentration of 3.95% (Table 2). when the EMS concentration was constant, the data could be fitted by a straight line regression equation according to the relationship between the treatment time and the survival rates. the lower EMS concentration, the lower correlation between the treatment times and survival rates. The highest correlation coefficient (R^2 = 0.9873) was obtained at EMS concentration of 3.8%. According to the linear regression equation $Y = -9.4300x + 127.90$, the survival rate reached 50% at the treatment time of 8.26 h, while it decreased to 40% at the treatment time of 9.32 h (Table 3). It concluded that the suitable EMS concentration for pitaya seedlings ranged from 3.71-3.95%, and the appropriate treatment time ranged from 8.26-9.32 h.

Effect of subculture time on the survival of seedlings treated by EMS

It can be seen from the table 4, the survival rate of seedlings was the lowest when they were directly treated by EMS after being separated from their mother plants. When seedlings subcultured for 15-30 d, the average survival rate was marked increase. Thus, it concluded that the resistance of seedlings to EMS increased with the extension of subculture time, and if the transgenerational adaptation time was too short, EMS might have more damaging effect on the seedlings, which makes recovery growth more difficult.

Effect of EMS concentration and treatment time on the differentiation of seedlings

As shown in figure 1, the differentiation ability of seedlings degraded significantly with the increase of EMS concentration and treatment time. When EMS concentration was 3.5% and the treatment time was 9 h, the differentiation rate of seedlings was only 46.6%. When EMS concentration was greater than or equal to 3.8% and the treatment time was more than 12 h, the differentiation capacity was very weak (4.7%). When EMS concentration equaled to 4.0%, buds cannot be differentiated.

Effect of ^{60}Co γ irradiation on the survival and differentiation of seedlings

As seen from table 5, it had no effect on survival rate, and little effect on differentiation rate when the radiation dose less than or equal to 10 Gy. The survival and differentiation rate of seedlings decreased significantly with the increase of radiation intensity when the dose was higher than 15 Gy. According to the relations between radiation doses and survival rate, which fitted the linear regression equation as shown in figure 2, and then calculated that the half lethal dose (LD50) of pitaya seedling *in vitro* was 38.5Gy, and the critical lethal dose (LD40) was 42.4Gy.

Screening and ISSR analyzing of mutant plants

By observing the morphological characteristics of seedlings treated with EMS and ^{60}Co γ, 14 mutants were screened out, materials with non-mutagen as control. The specific characteristics can be seen from table 6 and figure 3. According to the ISSR analysis, 9 primers (Table 7), with good polymorphism and stable amplification, were screened out from 38 primers to amplify mutant materials, and a total of 67 bands were amplified, 48 of which were polymorphic bands, and the proportion of polymorphic bands was 71.6%, which indicated that the variation of test materials was relatively rich. Each primer can amplify 3-14 bands, with an average of 5.33 polymorphic bands (Table 8). Among them, primer M08 amplified the most number of bands with 14, and primer M866 amplified the highest ratio of polymorphic bands with 100%, and the lowest ratio of polymorphic bands was amplified by M06, with 45.5%, but a specific band was found on electrophoresis channel 5 (Figure 4). The amplified bands ranged from 260 bp to 2000 bp, most of which were from 500 bp to 1200 bp (Figure 5 and Figure 6).

Genetic distance and cluster analysis

Based on the ISSR data analysis, the genetic distance (GD) of mutant plants ranged from 0.0120-0.4169 (Table 9). Among them, the smallest genetic distance was between No.1 and No.3, GD = 0.012, which indicated that the genetic background of the two was similar, and the variation of No.3 was smaller compared with the control (No.1). The largest genetic distance was No.2 and No.5, GD = 0.4169, which could be seen from that there was a large variation between them. Cluster analysis (Figure 7) shows that the control and 14 mutants can be divided into 6 groups, when GD = 0.11, among which No. 5, 6, 10, 12 and 14 materials can be separated into one group, which showed that there was a great variation among No.5, 6, 10, 12 and 14 in compared with the control.

Discussion

Different plant species and different parts of the same plant have different sensitivity to mutagens. In general, stem tips, blades and calluses can be used as mutagenic material *in vitro*, but it is very important that the mutagen can penetrate the tissues adequately and the mutagenic material can continue to grow and develop to form a new individual eventually (Ahloowalia and Maluszynski, 2001; Stefano, 2001; Wang et al. 2011; Serrat et al. 2014). The appropriate dose is one of the key factors for the success of mutation breeding whether it is chemical or physical mutation (Cui et al. 2011). Increasing the dose in a certain range can improve the mutation rate and expand the mutation spectrum of plant materials, but it can also reduce the survival rate when using too much higher dose, resulting in the increase of bad mutation characters. Critical dose and half lethal dose are generally used to determine the appropriate dose, but there is also a trend to select two more doses within the range of 20% difference between the upper and lower half lethal doses as the appropriate dose to produce more beneficial mutations (Li et al. 1999).

Before this study, there was only one report on the mutation of pitaya using stems and buds in field. However, it was difficult to control the field conditions and there was also no continuous observation and study. In addition, the leaves of pitaya degenerate into spines, and the variation of morphological characteristics is less than other plants, so there are fewer morphological types that can be marked (Peng, 2007; Liu et al. 2010). On the basis of morphological screening, molecular markers can more objectively reflect the differences between different mutants. Studies have shown that it is difficult to identify point mutations or deletions of minimal fragments by RAPD (Gustavo and Peter, 1998). ISSR primers are repetitive sequences, which are not affected by natural selection or less, and it is the fastest in the genome, and the variation is easy to retain. Therefore, ISSR markers with high polymorphism can effectively reveal the differences between individuals with very similar genetic relationship.

Conclusion

The study took red pulp pitaya 'Zi Honglong' seedlings *in vitro* as experimental materials, and selected the suitable EMS concentration and the optimum dose of ^{60}Co γ irradiation. The conclusion was very different from the result of mutagenesis using stems or buds of adult pitaya plant (Deng et al., 2011), which may be related to the sedlings in vitro were more sensitive to the mutagens and were more likely to mutate. ISSR molecular detection showed that a total of 67 bands were amplified with 9 better primers from 15 samples, 48 of which were polymorphic bands, the proportion of polymorphic bands was 71.64%. it was significantly higher than the results of RAPD and ISSR labeling pitaya resources by Junqueira et al. (2010), Zhang et al. (2013), Wang et al. (2013). From the PCR results of all primers, M08 amplified the most bands, and m866 had the highest polymorphism ratio (100%), while M06, although the polymorphism ratio was relatively low, found a specific band around 380 bp in Lane 5, and its functional characteristics needed further study and verification. What's more, from the genetic distance and cluster analysis, we can see that the average genetic distance of 15 resources of Pitaya is 0.2836, which showed that the mutation rate was high. However, the genetic distance between No. 3 and No. 1

(the control) was the smallest, which indicated that the variation of No. 3 mutant was the smallest, which was consistent with the result of cluster analysis.

Although the mutants were screened by EMS and radiation mutagenesis in the study, due to the randomness of mutation breeding, the difference between the mutant individuals and the control may be physiological or real variation. Therefore, it is necessary to further study, observing the growth, field performance and stability of mutant individuals in the future, so as to lay a foundation for better carrying out mutation breeding of pitaya.

Methods

The original materials were from the pitaya variety ‘Zihonglong’, which was independently cultivated by my institution. The sampling point was located in the demonstration park of Longping Town, Luodian county (106°45' E, 25°26' N, a.s.l 385 m). Then seedlings in vitro were obtained by stem tissue culture in the College of Horticulture, Sichuan Agricultural University (102°58' E,29°58' N□a.s.l 630 m).

EMS treatment. Referring to the methods of Deng et al. (2011) and Cui et al. (2011) and making some improvements. Mother liquors containing different EMS concentrations (2.8%, 3.0%, 3.2%, 3.5%, 3.8%, 4.0%) were prepared, and filter sterilized under aseptic conditions. Phosphate buffer [0.01M, pH 7.0] was prepared by autoclaved sterilization and then cooled to room temperature The EMS mother liquors were transfer into a sterile cylindrical bottles and bring to volume by phosphate buffer to prepare different concentration gradient EMS working liquid under aseptic condition. (EMS has a half-life of 25.9 h at 30 ℃). According to the times of subculture in the transfer medium (0, 7, 15, and 30 d, respectively), the seedlings were divided into four groups. Then, the seedlings were placed into sterilized solutions containing different concentration of EMS and shaken for 3, 6, 9, and 12 h, respectively. After the treatment, washing the seedlings with sterile water at least five times, and drying the surface water by blotting, then culturing on the subculture medium MS + 6-BA 1.0 mg/L + NAA 0.2 mg/L + AC 0.05%+ CCC 0.5 mg/L for 45 d to promote growth and differentiation, Seedlings without EMS treatment took as control.

^{60}Co γ irradiation treatment. According to the radiation related researches of other plant seedlings *in vitro* (Liu et al. 2010; Li et al. 2014) and pitaya plant (Deng et al. 2011), the seedlings of red pulp pitaya cultured on subculture medium for 30 days were irradiated with ^{60}Co γ in Jinnong Irradiation Center of Guizhou Academy of Agricultural Sciences. The dosage was set as 5 Gy, 10 Gy, 15 Gy, 20 Gy, 25 Gy, 30 Gy, 35 Gy, 40 Gy and 45 Gy. The dose rate was 1 Gy/min, and the corresponding irradiation time was 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min and 45 min, respectively. 120 sterile seedlings per treatment, 3 repetitions. After irradiation, seedlings were subcultured on the medium MS + 6-BA 1.0 mg/L + NAA 0.2 mg/L + AC 0.05%+ CCC 0.5 mg/L for 45 d in the culture room, and observed the survival and differentiation. Seedlings without radiation took as control.

Screening and molecular detection of mutant. By observing the morphological variation of mutagenic materials, mutants would be screened out. Seedlings without mutation were used as control. Molecular detection of mutants was analyzed by inter simple sequence repeats (ISSR), referring to the established ISSR marking system by Yuan et al. (2013) and Zhang et al. (2013). Primers were synthesized by Chengdu Qingke Zixi Biotechnology Co., Ltd (No. 1666, Sect. 2, Chenglong Avenue, Chengdu Economic and Technological Development Zone, Longquanyi District, Sichuan, China).

Statistical analysis

Data were statistically analyzed using t-test. Differences were considered significant if $p < 0.05$.

Abbreviations

6-BA
6-benzyladenine
2, 4-D
2, 4-Dichlprpphenoxyacetic acid
AC
Activaed charcoa
MS
Murashige and skoog
NAA
1-naphthlcetic acid
CCC
Chlormequat chloride
EMS
Ethylmethane sulfonatc
L
Liter
M
Mol/L
Mg
Milligram
d
Day
h
Hour
min
Minute
s
Second

℃
Centigrade
%
Percentage
bp
Base pair
pH
Potential of hydrogen
NO.
Number
Gy
Gray
DPS
Data processing system
ISSR
Inter simple sequence repeat
DNA
Deoxyribonucleic acid
dNTP
Deoxy-Ribonucleoside triphosphate
GS
Genetic similarity coefficient
UPGMA
Unweighted pair group method analysis

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors Contributions

R.J.D. and J. X. F. performed the experiments, analyzed the data, and drafted the manuscript. Y.Q.W. conceived the study, designed experiment, retouched and revised the paper. T. L. and J.F.J helped with sampling, participated in its design and coordination, and helped draft the manuscript. All authors have read and approved the final manuscript.

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References

Ahloowalia BS, Maluszynski M (2001) Induction mutation-A new paradigm in plant breeding [J]. Euphytica, 118: 167-173

Cui GR, Zhang ZX, Zhang CY, Hu NB, Sui YH, Li JQ (2011) Mutation induction of EMS in vitro cultured oncidium and RAPD analyzing of regenerated plantlets [J]. Chinese Journal of Tropical Crops, 32 (2): 261-268

Deng RJ, Fan JX, Cai YQ. Gao AH, Yuan QF (2011) Effects of ^{60}Co-γ irradiation and EMS treatment on survival rate and seeding growth of pitaya (*Hylocereus spp*·) [J]. Acta Laser Biology Sinica, 20 (1): 15-20

Deng RJ, Fan JX, Wang YQ, LiuT, Jin JF (2019) Researches of callus induction, proliferation and differentiation using stem from adult Hylocereus undatus plants [J]. Southwest China Journal of Agricultural Sciences, 32 (3): 595-603

Gustavo CA, Peter MC (1998) DNA marker protocols, applications and overview [M]. New York: Wiley -Vch Press: 55

Junqueira KP, Faleiro FG, Bellon G, Junqueira NTV, Fonseca KG, Lima CA, Santos EC (2010) Pitaya accesses genetic variability with different production levels through RAPD markers [J]. Revista Brasilleira de Fruticultura, 32 (3): 840-846

Li H, Wu LF, Song DJ, Wu ZL, Yu YJ (1999) RAPD analysis of hereditary and variation of maize rice developed by ion beam mediated transferring maize dna into rice[J]. Acta Laser Biology Sinica, 8 (4): 261-265

Li N, Qu YT, Chen F (2014) Study on the Tissue culture seedlings of Hosta ventricosa by radiation breeding [J]. Forestry Science Technology, 39 (5): 7-8

Liu LQ, Liu JL, Zhang J, Gen H, Zhang YP, Shen HX, Gao XH, Yao YC (2010) Mutagenic effects on tissue culture plants of ornamental crabapple induced by 60 Co-γ irradiation [J]. Scientia Agricultura Sinica, 43 (20): 4255-4264

Peng B, Xu QG, Li HL, Liu HM (2007) Advances in chemical mutation breeding of crops[J]. Crop Research, 21 (5): 517-519, 524

Serrat X, Esteban R, Guibourt N, Moysset L, Nogues S, Lalanne E (2014) EMS mutagenesis in mature seed-derived rice *calli* as a new method for rapidly obtaining TILLING mutant populations [J]. Plant Methods, 10 (1): 5-12

Shang XL (2014) Research progress on radiation breeding methods of fruit trees [J]. Agricultural Science and Technology Communication, (4): 131-133

Stefano P (2001) Mutation induction and tissue culture in improving fruits [J]. Plant Cell, Tissue and Organ Culture, 64: 185-210

Wang F, Xu B, Yang ZL, Ling YH, He GH, Chen S, Qin MJ, Sang XC (2011) Identification and analysis of EMS- induced dwarf mutants in rice [J]. Acta Agriculturae Nucleatae Sinica, 25 (2): 197-201

Wang B, Zheng W, Song S, Li XZ, Cai YQ (2013) Identification of Zihonglong pitaya and Its big-fruit sport line by ISSR[J]. Southwest China Journal of Agricultural Sciences, 26 (4): 1614-1617

Yuan YF, Chen MX, Chen QX, Zhuang WD, Zheng JS (2013) Investigate and ISSR Analysis of Pitaya Germplasm Resources in Fujian Province[J]. Chinese Agricultural Science Bulletin, 29 (34): 216-220

Zhang BX, Fan FH, Qiao G, Song S, Wen XB, Liu T (2013) DNA fingerprint construction and genetic relationship analysis of dragon fruit germplasm in Guizhou by ISSR markers [J]. Journal of Fruit Science, 30 (4): 573-577

Wu X, Qi KJ, Yin H and Zhang SL (2016) Applying of the Induced Mutation Techniques in the Breeding of Deciduous Fruit Tree Varieties [J]. Acta Horticulturae Sinica, 43 (9): 1633-1652

Tables

Table 1 Effects of EMS concentration and treatment time on the seedling survival rate

Concentration of EMS (%)	Treatment time of EMS (h)	No. of EMS treatment	No. of survival from EMS treatment	Survival rate (%)
2.8	3	180	180	100aA
	6	180	170	94.4aB
	9	180	161	89.4 bA
	12	180	92	51.1cA
3.0	3	180	180	100aA
	6	180	157	87.2bB
	9	180	138	76.7cB
	12	180	66	36.7dB
3.2	3	180	178	98.9.0aA
	6	180	146	81.1bB
	9	180	130	72.2cB
	12	180	47	26.1dC
3.5	3	180	180	100aA
	6	180	141	78.3bBC
	9	180	109	60.6cC
	12	180	38	21.1dCD
3.8	3	180	168	93.3aA
	6	180	127	70.6bC
	9	180	85	47.2cD
	12	180	19	10.5dE
4.0	3	180	161	89.4aAB
	6	180	128	71.1bBC
	9	180	66	36.7cE
	12	180	10	5.6dF

Note: Lowercase and uppercase letters within a column denote significant differences (P <0.05) in the survival rate at EMS concentrations and treatment times.

Table 2 Regression analysis between the EMS concentration and the seedling survival rate

Treatment time of EMS (h)	Regression equation	Correlation coefficient R^2	Median lethal concentration (%)	Critical lethal concentration(%)
3	Y=-4.2879x+113.04	0.7542	14.70	17.03
6	Y=-20.224x+149.12	0.9540**	4.90	5.40
9	Y=-41.902x+205.44	0.9916**	3.71	3.95
12	Y=-35.256x+144.58	0.9532**	2.68	2.97

Note: ** indicates a statistically significant difference at the 0.01 level.

Table 3 Regression analysis between the EMS treatment time and the seedling survival rate

Concentration of EMS(%)	Regression equation	Correlation coefficient R^2	median lethal time (h)	Critical lethal time (h)
2.8	Y=-5.0767x+121.85	0.7860	14.15	16.12
3.0	Y=-6.6967x+125.45	0.8934*	11.27	12.76
3.2	Y=-7.6633x+127.50	0.8978*	10.11	11.42
3.5	Y=-8.4700x+128.60	0.9686**	9.28	10.46
3.8	Y=-9.4300x+127.90	0.9873**	8.26	9.32
4.0	Y=-10.013x+127.80	0.9700**	7.77	8.77

Note: ** indicates a statistically significant difference at the 0.01 level.

Table 4 Effects of subculture time on the seedling survival rate

Subculture time of pitaya seedlings (d)	Concentration of EMS (%)	Treatment time of EMS (h)	No. of seedlings with EMS	No. of survived seedlings	Survival rate (%)
0	3.0	9	180	80	44.4aD
	3.5	9	180	19	32.8bCD
	4.0	9	180	8	12.1cBC
7	3.0	9	180	96	53.3aC
	3.5	9	180	68	37.8bC
	4.0	9	180	32	17.8cB
15	3.0	9	180	125	69.4aAB
	3.5	9	180	102	56.7bAB
	4.0	9	180	62	34.4cA
30	3.0	9	180	138	76.7aA
	3.5	9	180	109	60.6bA
	4.0	9	180	66	36.7cA

Note: Different superscripts within a column denote significant differences (P <0.05) in the seedling survival rate at different subculture times.

Table 5 Effect of radiation dosage on survive and differentiation of seedlings

Radiation dosage (Gy)	No. of seedlings with irradiation	No. of survived seedlings	No. of differentiated seedlings	Survival rate (%)	Differentiation rate (%)
0	120	120	120	100a	100a
5	120	120	117	100a	97.5a
10	120	120	113	100a	94.2ab
15	120	116	106	96.7a	88.5b
20	120	111	96	92.8ab	80.3c
25	120	103	83	85.7b	69.1d
30	120	90	66	75.3c	54.7e
35	120	75	46	62.3d	38.3f
40	120	56	24	46.8e	20.4g
45	120	35	8	29.2f	6.7h

Note: Different superscripts within a column denote the significant differences of survival rate and differentiation rate under ^{60}Co γ treatment (p<0.05).

Table 6 Screening of mutants from treatment of EMS and ^{60}Co γ irradiation

No. of materials	Sources
1	Control without treatment of EMS and ^{60}Co γ irradiation
2	Treated with 4.0% EMS for 12 h. Morphological variation of stem, spire and red color.
3	Treated with 0.4% EMS for 6 h. New bud with red tip and morphological variation of stem.
4	Treated with 3.0% EMS for 9 h. Morphological variation of stem.
5	Treated with 3.8% EMS for 9 h. New bud with red tip and morphological variation of stem.
6	Treated with 0.7% EMS for 3 h. Morphological variation of stem and tip without spines.
7	Treated with 4.0% EMS for 6 h. Morphological variation of stem, , spire and red color.
8	Treated with 0.6% EMS for 9 h. Morphological variation of stem
9	Treated with 3.0% EMS for 12 h. New bud with red tip and morphological variation of stem.
10	Treated with 3.5% EMS for 9 h. New bud with red tip and morphological variation of stem.
11	Treated with 2.8% EMS for 12 h. Morphological variation of stem.
12	Treated with 20 Gy ^{60}Co γ irradiation. Morphological variation of stem.
13	Treated with 35Gy ^{60}Co γ irradiation. Morphological variation of stem
14	Treated with 35Gy ^{60}Co γ irradiation. New bud with red tip and morphological variation of stem.
15	Treated with 38.5Gy ^{60}Co γ irradiation. New bud with red tip and morphological variation of stem.

Table 7 Sequences and annealing temperatures of primers used for PCR amplification

Primers	Sequence /5’-3’	annealing temperature /℃
M06	AGCAGCAGCAGCY	50.5
M08	AGCAGCAGCAGCAY	54.0
807	AGAGAGAGAGAGAGAGT	54.0
812	GAGAGAGAGAGAGAGAA	50.5
845	CTCTCTCTCTCTCTCTRG	50.5
848	CACACACACACACACARG	46.5
853	TCTCTCTCTCTCTCTCRT	50.5
866	CTCCTCCTCCTCCTCCTC	57.0
876	GATAGATAGACAGACA	46.5

Table 8 ISSR primers and their diversity analysis

Primers	NO. of amplified bands	NO. of polymorphic bands	Ratio of polymorphic band
M06	11	5	45.45
M08	14	10	71.43
807	8	6	75.00
812	5	3	60.00
845	5	3	60.00
848	3	2	66.67
853	4	3	75.00
866	9	9	100.00
876	8	7	87.50
Sum	67	48	71.64
Average	7.44	5.33	71.64

Table 9 Genetics distance of samples of 15 materials selected from EMS and ^{60}Co γ induction

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.0000														
2	0.0474	0.0000													
3	0.0120	0.0600	0.0000												
4	0.0497	0.0730	0.0377	0.0000											
5	0.3597	0.4169	0.3477	0.3353	0.0000										
6	0.1514	0.2064	0.1393	0.1587	0.3445	0.0000									
7	0.0488	0.0974	0.0621	0.0757	0.3597	0.1831	0.0000								
8	0.0880	0.1380	0.1027	0.1177	0.2831	0.1711	0.0880	0.0000							
9	0.0359	0.0838	0.0485	0.0615	0.3715	0.1949	0.0606	0.0998	0.0000						
10	0.2349	0.2899	0.2228	0.2105	0.3227	0.2842	0.2349	0.1921	0.2466	0.0000					
11	0.0359	0.0838	0.0238	0.0362	0.3387	0.1631	0.0606	0.0998	0.0235	0.2159	0.0000				
12	0.2219	0.2452	0.2416	0.1975	0.2452	0.2712	0.2219	0.1791	0.2029	0.3547	0.2029	0.0000			
13	0.0621	0.1113	0.0760	0.0636	0.2831	0.1711	0.0621	0.1027	0.0738	0.1622	0.0738	0.2098	0.0000		
14	0.1147	0.1654	0.1301	0.1459	0.3149	0.1711	0.1147	0.1301	0.0998	0.2546	0.0998	0.2098	0.1027	0.0000	
15	0.0474	0.0953	0.0600	0.0730	0.3185	0.2064	0.0721	0.0853	0.0350	0.1975	0.0350	0.1845	0.0600	0.0853	0.0000

Figures

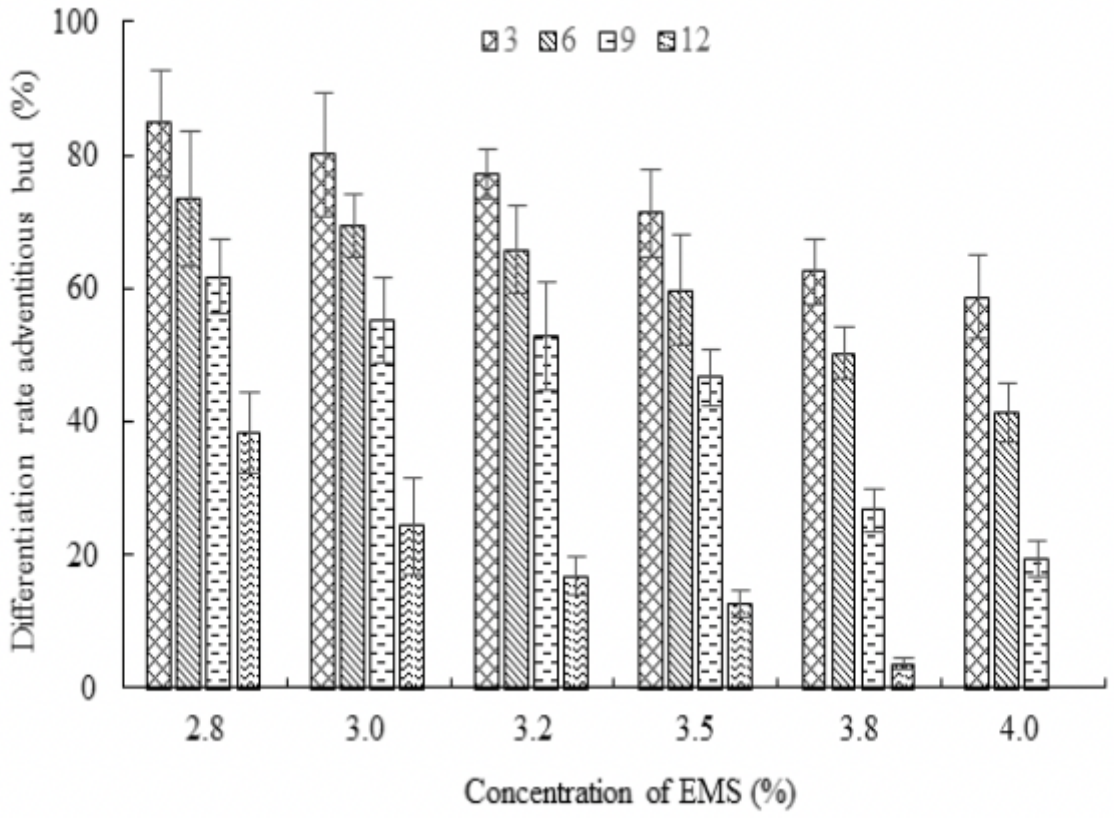

Figure 1

The effects of the EMS concentration on the differentiation of seedlings

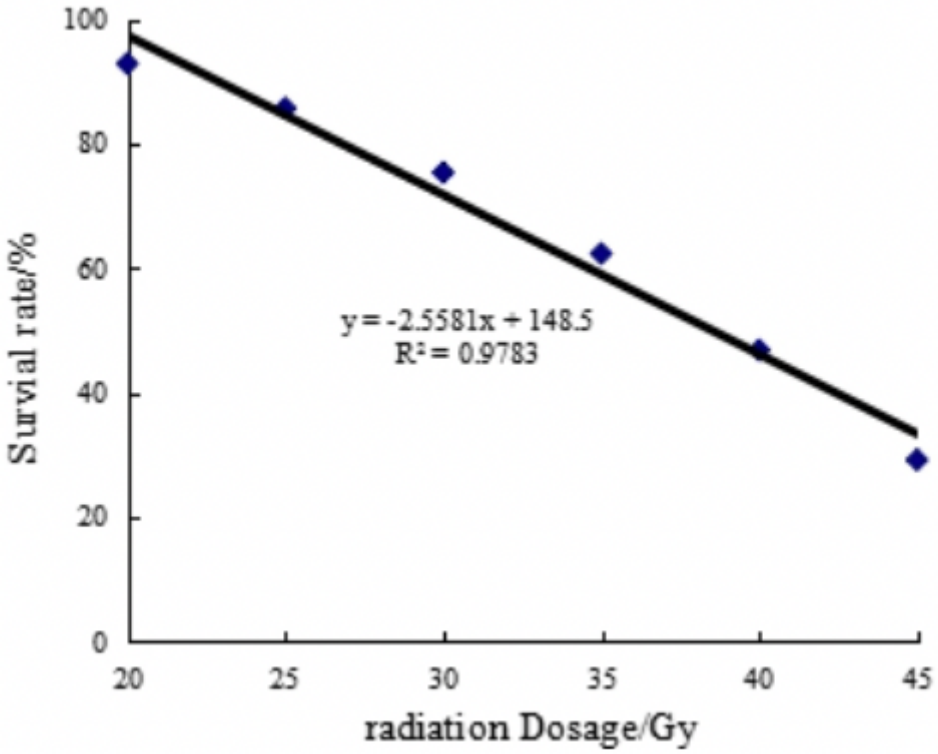

Figure 2

Regression analysis between radiation intensity and survival rate

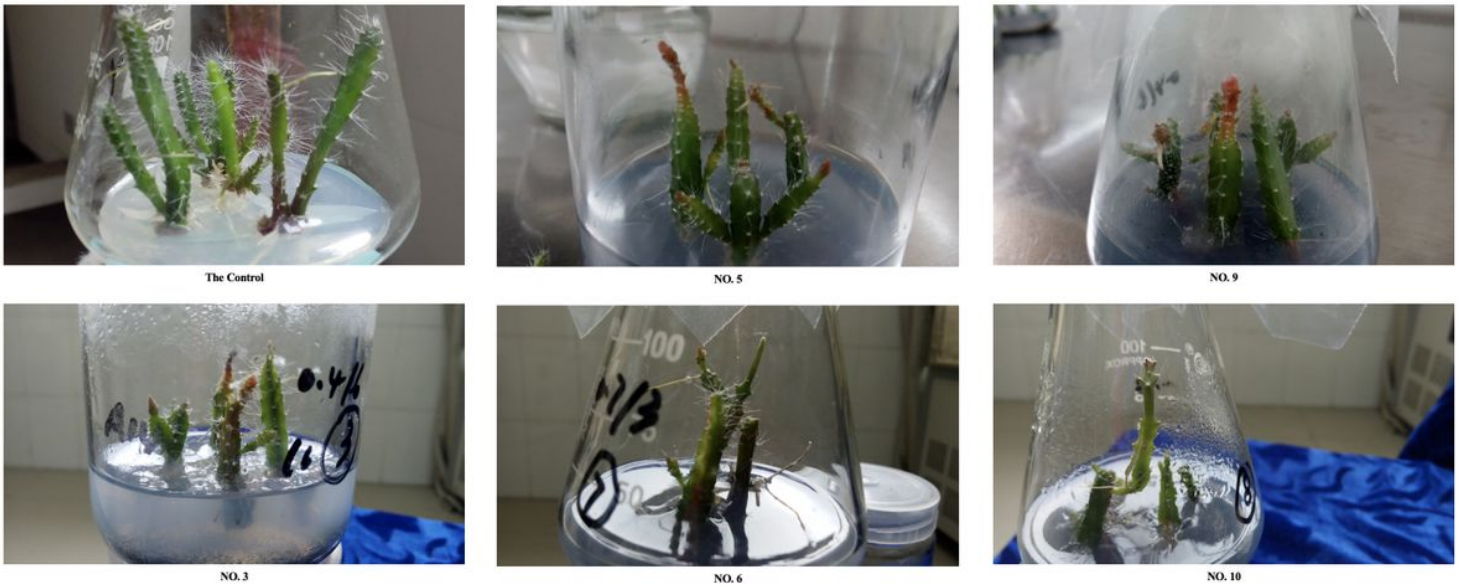

Figure 3

The control and part of mutants

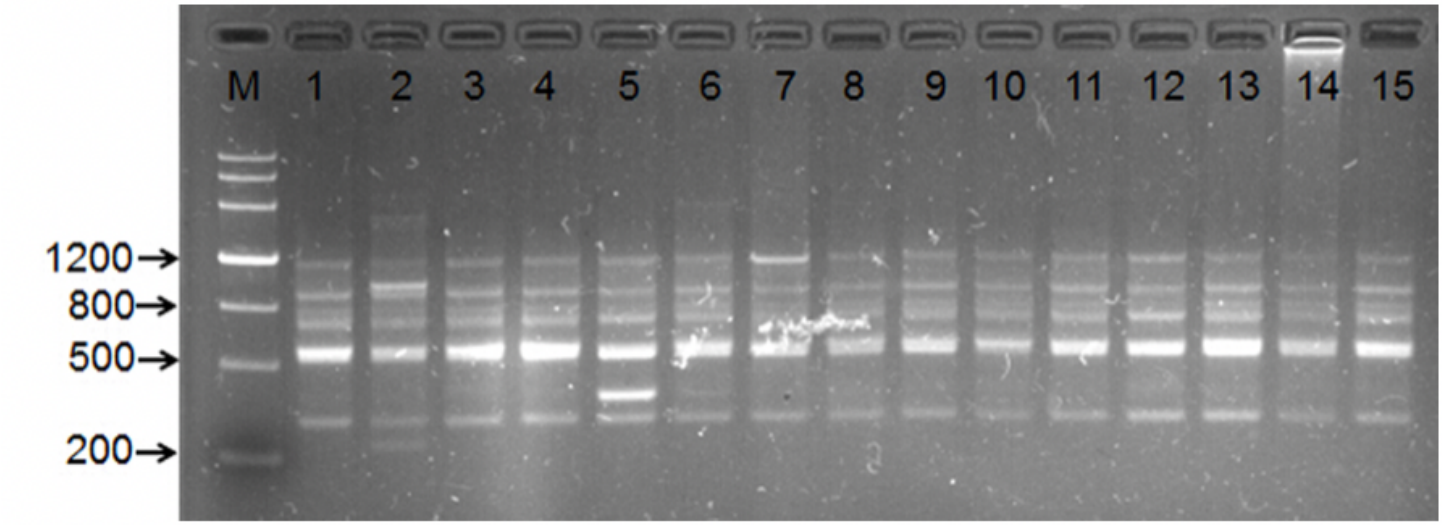

Figure 4

Electrophoresis pattern with primer M06 Lane M. DL2000 Marker; Abbr. of samples of other lanes seen in Table 7. The same below.

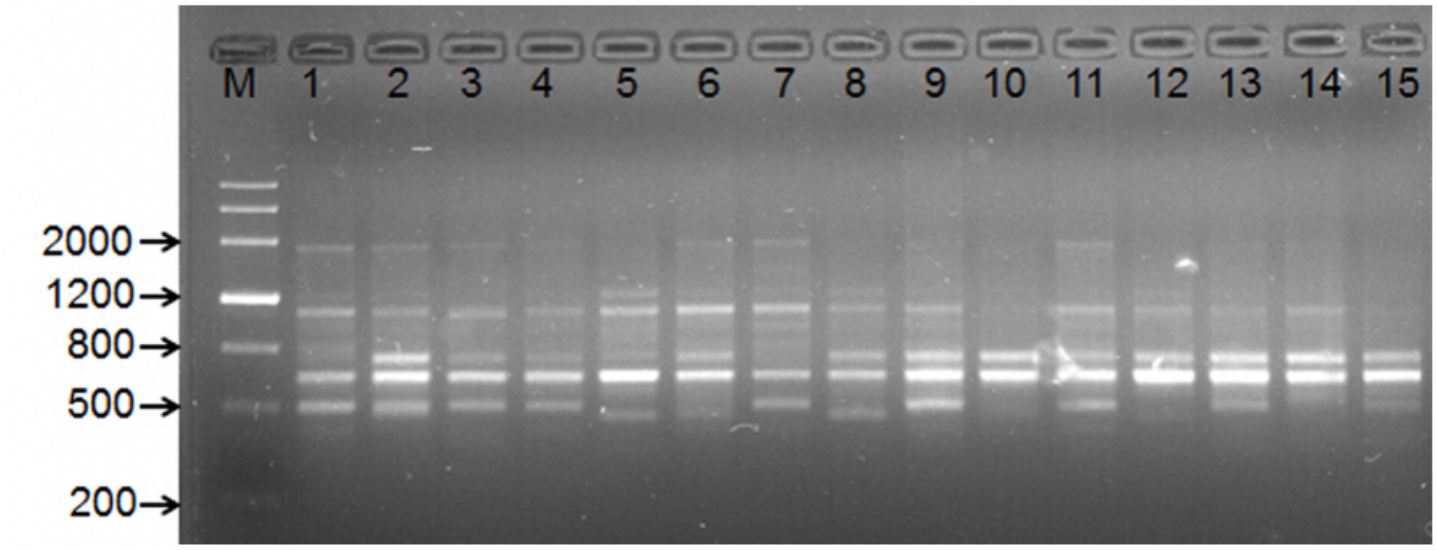

Figure 5

Electrophoresis pattern with primer 876

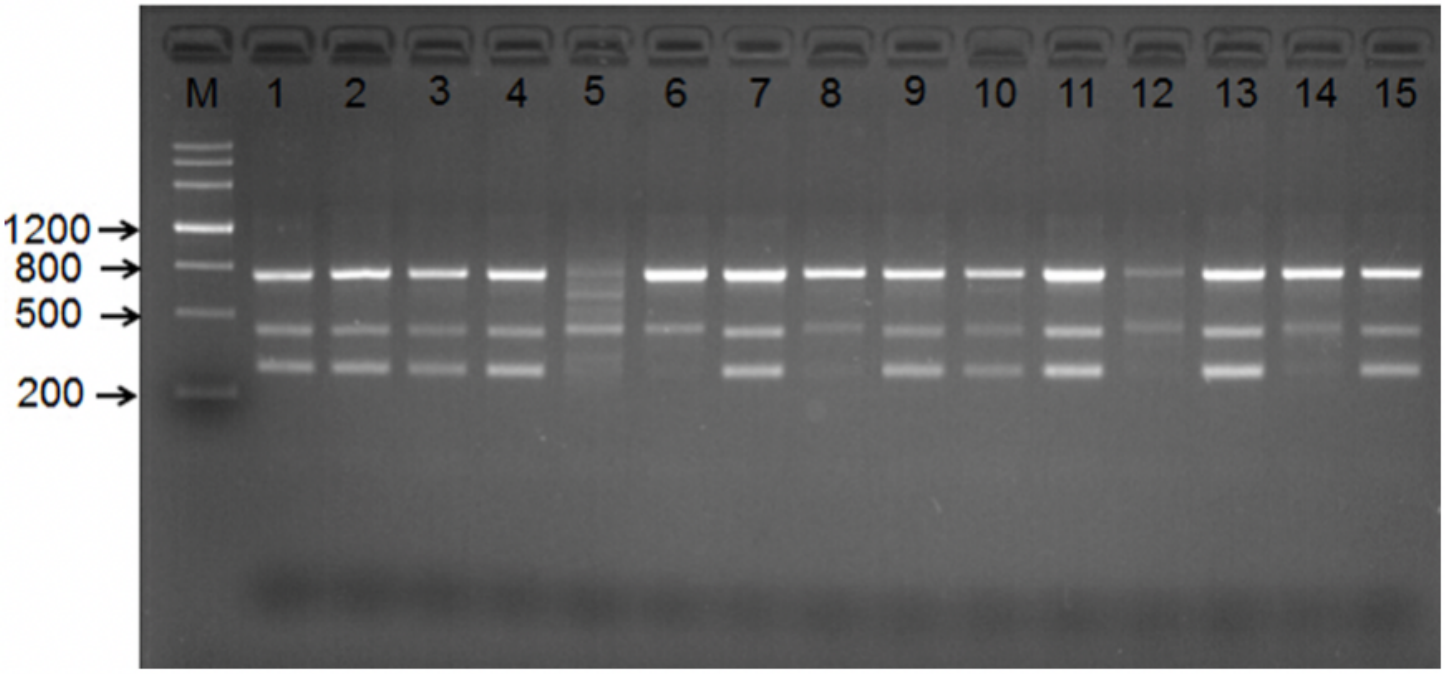

Figure 6

Electrophoresis pattern with primer 845

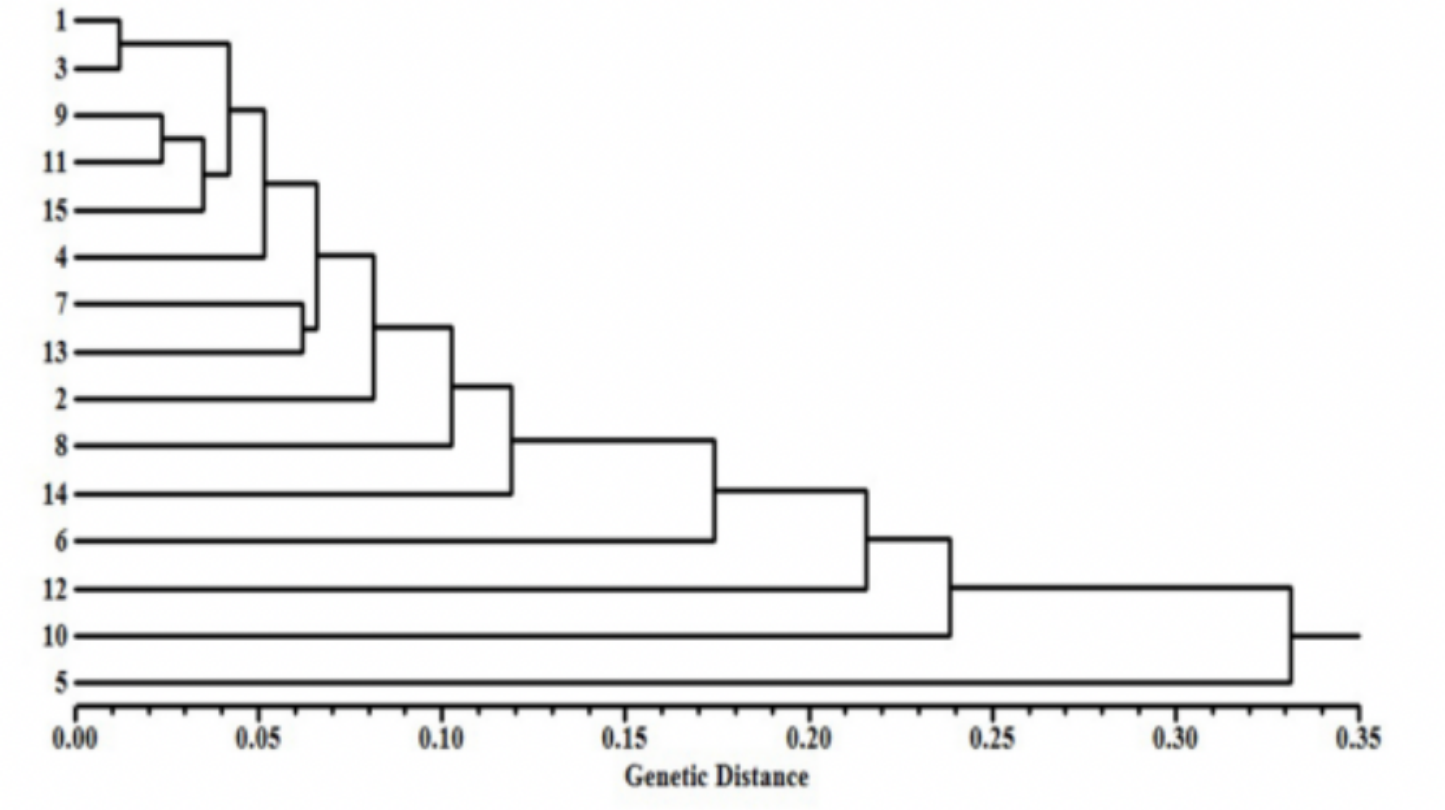

Figure 7

ISSR cluster analysis of 14 mutants and 1 control sample