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Selection of Azetidine-2-carboxylic Acid Resistant Cell Lines by *In vitro* Mutagenesis in Rice (*Oryza sativa* L.)

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Abstract

Resistant cell lines to azetidine-2-carboxylic acid (AZCA) were selected through rice embryo culture after mutagenic treatment of callus irradiated with 30, 50, 70, 90 and 120 Gy. The optimum AZCA concentration for the selection of resistant cell lines was 3 or 4 mM AZCA considering LD_{50} and the fresh weight of callus. Survival rate of the AZCA resistant callus showed remarkable increase in the callus irradiated with 50 and 70 Gy. Regeneration rate of the AZCA resistant callus was much lower on the whole. Ninety and 120 Gy increased the regeneration rate for calli selected from 3 and 4 mM AZCA, respectively. Based on fresh weight, survival rate and regeneration for selection of the AZCA resistant cell line, 50-90 Gy was considered as the optimum range of gamma irradiation. Irradiated calli selected from AZCA were more tolerant to NaCl than those from non-irradiated calli. It suggests that elevated resistance to osmotic stress resulted from mutagenic treatment. The level of free proline content in the AZCA resistant cell line was increased up to 3.5 times compared with that in the control. Proline content in the regenerant derived from the AZCA resistant cell line also increased to 1.7 times that from the control plants regenerated from callus grown in AZCA free medium. Selection of proline overproducing cell lines by *in vitro* mutagenesis was successful and seems to be useful for improvement of stress tolerance in this crop.

Key words: Embryo culture, Gamma ray, *in vitro* mutagenesis, Proline overproduction, Rice

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Introduction

Many environmental stresses, such as drought, salinity and cold, are serious factors limiting the growth and productivity of rice. When plant cells are exposed to these stresses, proline accumulation is a widespread phenomenon. Under stress conditions free proline increase plays a key role for osmotic adjustment in a large number of plant species (Delauney and Verma 1993). This amino acid is widely believed to function as a protector or stabilizer of enzymes or membrane structures that are sensitive to dehydration or ionically induced damage (Iyer and Caplan 1998). Proline accumulation in plant tissues has been suggested to result from (a) a decrease in proline degradation, (b) an increase in proline biosynthesis, (c) a decrease in protein synthesis or proline utilization and (d) hydrolysis of proteins (Yoshiba et al. 1997; Charest and Phan 1990). Although there are many controversial issues with proline accumulation and stress resistance, it is believed that high levels of proline can be beneficial to stressed plants (Igarashi et al. 1997; Delauney and Verma, 1993; Smirnoff and Cumbes 1989).

Mutants resistant to proline analogs were considered as a useful material for studying proline biosynthesis and saline, drought, and cold resistance in higher plants (Miflin et al. 1983). Various mutants resistant to proline analogs were selected in rice (Hasegawa and Mori 1986), barley (Kueh and Bright, 1982), carrot (Cella et al. 1982), and *Arabidopsis* (Verbruggen et al. 1996). Azetidine-2-carboxylic acid (AZCA), a proline analog, is a natural product found in some species of the *Liliaceae*, which inhibited cell growth irreversibly by competition with proline for incorporation into protein (Cella et al. 1982). This incorporation presumably leads to altered protein conformation and function, and accounts for the cytotoxic effects of AZCA (Lodato et al. 1984). In many cases, mechanism of resistance to amino acid analogs has been shown to be due to the insensitivity to

feedback inhibition. Mutants with altered feedback mechanism express more resistant to feedback inhibition and this results in greatly elevated free proline accumulation. The decrease of uptake inhibitors by a change in cell membrane permeability and inactivation of inhibitors have also been reported as mechanisms of amino acid analog resistance. However, in the latter cases, any amino acids are not increased *in vivo* plants.

Mutation techniques in combination with tissue culture provide a powerful technology to improve crop species (Ahloowalia and Maluszynski 2001). *In vitro* culture not only provides relative uniform and large populations of cells and tissues in a disease-free situation for irradiation, but also it is possible to irradiate a very large number, and also to further separate the desired mutated cell lines from ones in a short time (Maluszynski et al. 1995). *In vitro* culture constitutes a useful tool for rapidly and economically evaluating tolerance in plants via addition of sodium chloride, amino acid analogs, herbicides, antibiotic to the medium or exposure of cell to heat, cold and freezing (Watanabe et al. 2000; Maluszynski et al. 1995). *In vitro* radiation induced mutagenesis can produce or enlarge desired genetic variation that are mixed with somaclonal variation. It can elevate selection efficiency for stress resistant cell lines on selection media.

In the present paper, the selection of AZCA resistant cell lines by *in vitro* mutagenesis for proline overproduction rice was reported.

Materials and Methods

Embryo culture

Callus was initiated from the embryo culture of hulled seed of *Oryza sativa* L. *Japonica* cv. Donganbyeo. The seeds were sterilized in 75% ethanol for 30 sec and followed by immersing in the solution of 10% sodium hypochlorite with 2-3 drops of tween-40 for 30 min. The embryos of seeds were rinsed 3-4 times with sterile distilled water and placed on N_6 basal medium containing 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 g/L myo-inositol, 0.25 g/L L-proline, 1 g/L casein hydrolysate, 30 g/L sucrose, and 0.4% (W/V) phytagel in sterile petri dishes. The medium was adjusted to pH 5.8 and autoclaved for 20 min at 1.1 kg/cm^2 pressure at 121°C. The petri dishes were maintained for 30 days at 25±1°C in continuous darkness.

Screening of optimum AZCA concentration

Induced callus was divided into 30 pieces (ϕ 1-2 mm) with sterile razor and transferred to the N_6 basal medium containing 1.5, 2, 3 and 4 mM AZCA concentrations. Thirty callus pieces were plated per each concentration with 10 replications and the data of survival rate and fresh weight were obtained 40 days after culture. Based on survival rate and fresh weight, the optimum AZCA concentration was assessed as 3 or 4 mM.

Gamma ray treatment and selection of AZCA resistant cell lines

Effect of gamma rays on growth of callus was investigated 40 days after irradiation. Callus was irradiated with 30, 50, 70, 90 and 120 Gy of gamma rays. Selection of AZCA resistant cell lines was conducted on selection medium containing 3 or 4 mM AZCA.

Regeneration of AZCA resistant cell lines

AZCA resistant callus was transferred to the Murashige and Skoog (MS) (1962) medium containing 0.2 mg/L indoleacetic acid (IAA), 3 mg/L kinetin, and 30 g/L sucrose solidified with 0.5% (W/V) phytagel. The medium was adjusted to pH 5.8 and autoclaved for 20 min at 121°C. Regeneration rates were obtained 30 days after transfer to regeneration medium as the percentage of green plantlet to the plated callus. The plantlets were grown further in the bottle containing half strength MS medium without hormone.

Selection of resistant regenerants

Regenerants from resistant callus were grown in solution containing half strength MS salts and 2 mM AZCA to determine their resistance. The shoots of regenerants were removed except 5 cm in length and roots were removed thoroughly at the 2-3 leaf stage seedlings for comparison of their revival ability. AZCA resistant regenerants were selected after 2 weeks.

NaCl tolerance of AZCA resistant cell lines

After growth for 40 days in the presence of AZCA, resistant calli were selected based on their fresh weight and survival rate. And then, calli derived from control, calli selected with non-irradiation, and calli selected with irradiation were transferred to N_6 medium containing 170 mM NaCl. These caused the lowering of the osmotic potential of the plant environment. Resistance was verified 40 days after culture based on fresh weight and survivals of calli.

Proline analysis

Proline content was measured by a modified method of Bates et al. (1973). Proline was extracted from 0.1 g of fresh leaf with 5 mL of MCW buffer (methanol : chloroform : water,

12 : 5 : 1). The extract was centrifuged at 10,000 rpm for 10 min at 4°C and 125 μL of the supernatant was transferred to a new tube and diluted with 875 μL water. Ninhydrin solution of 1.5 mL was added to the diluted sample. The mixture was heated in a boiling water bath for 1 hour, and then incubated at room temperature for 30 min. The absorbance was read at 520 nm. Proline content was calculated from a proline standard curve.

Results

Selection of AZCA resistant cell lines

Rice callus obtained from the embryo of *japonica* cv. Donganbyeo was cultured on N_6 basal medium containing 2 mg/L 2,4-D. The callus was transferred onto N_6 medium supplemented with various AZCA concentrations for 40 days. When calli were transferred to the medium with various concentration of AZCA, fresh weights and survival rates of the calli were decreased (Table 1). However, the fresh weights of calli were slightly higher than those of the control calli on the medium containing 1.5 mM AZCA. Fresh weights and survival rates of calli were less than 50% of those from control calli at 3 and 4 mM AZCA. The fresh weights of calli at 3 and 4 mM were 46.6 % and 26.6 % compared to those of the control, respectively. Callus at concentration of 1.5 and 2 mM AZCA was healthy and yellow. Callus on the 3 and 4 mM AZCA medium lost their colors gradually and turned yellow. Therefore, optimum AZCA concentration for the selection of AZCA resistant cell lines was 3 or 4 mM AZCA considering both LD_{50} and fresh weights of calli.

To test the effect of gamma rays on growth of calli, the calli induced from cv. Donganbyeo and were irradiated with gamma rays of 30, 50, 70, 90 and 120 Gy doses, then the fresh weights of calli cultured in N_6 medium were measured 40 days after irradiation. As gamma ray doses increased, the fresh weight of calli per clone was decreased gradually and LD_{50}-LD_{70} of calli was about 50-70 Gy (Figure 2).

After irradiated with gamma rays, calli induced from Donganbyeo were plated on the selective medium containing 3 or 4 mM AZCA for selection of AZCA resistant cell lines. Compared with the control, survival rates of calli irradiated with up to 70 Gy dose were higher than those of control calli. Calli irradiated with 50 Gy showed highest survival rate in both 3 and 4 mM AZCA selective medium (Table 2).

In contrast to survival rates, increase of fresh weight was observed partially (Figure 3). The fresh weights of calli irradiated with 50 and 70 Gy were higher than those of the control in both 3 and 4 mM. However, the fresh weight of calli at 30, 90 and 120 Gy was lower than the control. The survival rates exhibited the highest level at 50 and 70 Gy in both 3 and 4

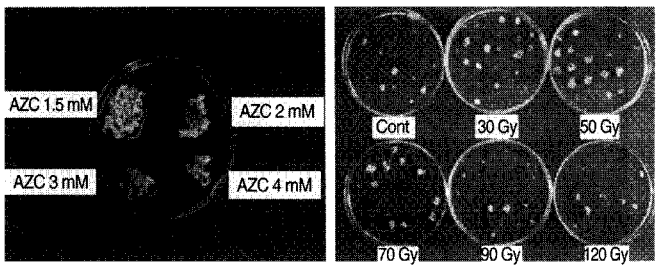

Figure 1. Determination of the optimum AZCA concentration for the selection of resistant cell lines (left) and selection of AZCA resistant cell lines after gamma-ray irradiation in 4 mM AZCA medium (right).

Table 1. Survival rates and fresh weights of calli on AZCA-containing medium 40 days after treatment. Thirty callus pieces (ϕ 1-2 mm) were plated per each concentration with 10 replications.

Items	AZCA concentration (mM)				
	0	1.5	2	3	4
Fresh weight (mg±SE)	150±24	160±22	110±34	70±14	40±1
Survival rate (%)	76.3	79.6	59.3	45.3	37.6

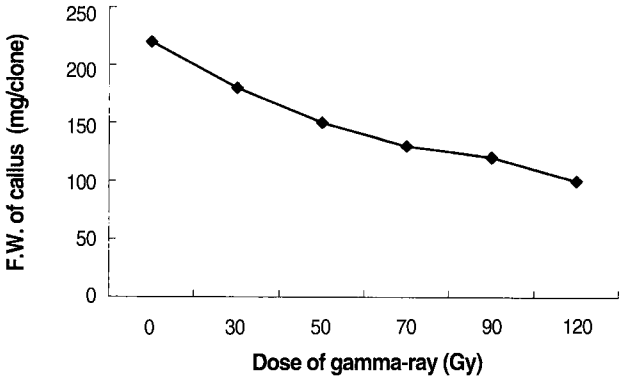

Figure 2. Effect of gamma rays on growth of callus 40 days after irradiation.

Table 2. Survival rates of callus irradiated with various radiation doses on AZCA selective medium. Data were obtained 40 days after irradiation.

Radiation dose (Gy)	3 mM AZCA		4 mM AZCA	
	No. of plated calli	Survival rate (%)	No. of plated calli	Survival rate (%)
0	270	17.7bc[a]	210	5.7b
30	240	22.9bc	240	8.8b
50	240	37.5a	210	23.3a
70	210	28.6ab	210	19.5a
90	240	13.3c	240	5.4b
120	270	18.1bc	270	7.0b

[a]Duncan's multiple range test at 5% level.

mM. Similar results were obtained at 50 and 70 Gy for the fresh weight of calli.

Regeneration rates of calli were investigated on regeneration medium 30 days after inoculation (Table 3). Regeneration rates of calli were very low on the whole. Among them, 90 and 120 Gy increased the regeneration rates for calli selected from 3 and 4 mM AZCA, respectively. As compared to the control, the regeneration rates of calli were increased to 10.4 and 3.7 times at these doses.

Selection of AZCA resistant regenerants

AZCA resistant regenerants were selected from 2 mM AZCA solution 2 weeks after excision (Figure 4). Shoots were removed except 5 cm in length and roots were removed thoroughly. Shoots and roots of AZCA resistant regenerants survived in this solution and growth was maintained with vigor during 2 weeks of evaluation in comparison with the control plants that showed retarded growth or dead.

Resistant regenerants showed shoot revival and transpiration due to water absorption of roots 6 hrs after excision. Shoots revived almost 1 cm everyday and roots appeared a day after removal. The mean number of roots was 4 for resistant regenerants after 2 weeks. Control plants also showed shoot revival and transpiration. However, when it grew to 1-2 cm in length, no revival of roots and growth of the shoot ceased, withered and then died.

Cross resistance of calli selected

To verify cross resistance of calli selected from 4 mM AZCA, the calli derived from control, selected calli with non-irradiation (calli Ⅰ), and selected calli with irradiation (calli Ⅱ) were plated to each N_6 medium containing 170 mM NaCl which caused the lowering of the osmotic potential of the plant growth environment (Table 4).

As compared with the control, the survival of calli Ⅰ was decreased by 33% , while it was increased to 27% of the control in calli Ⅱ. The 170 mM NaCl medium that caused osmotic stress and ion imbalance resulted in low survival rates. There was a significant increase in fresh weight of call Ⅰ and calli Ⅱ. Although survivals of calli Ⅰ were lower than those of the control, fresh weight was increased to 29% of the control. Fresh weight of calli Ⅱ was about 53% higher than that of the control. Calli irradiated with gamma ray were higher than the control in

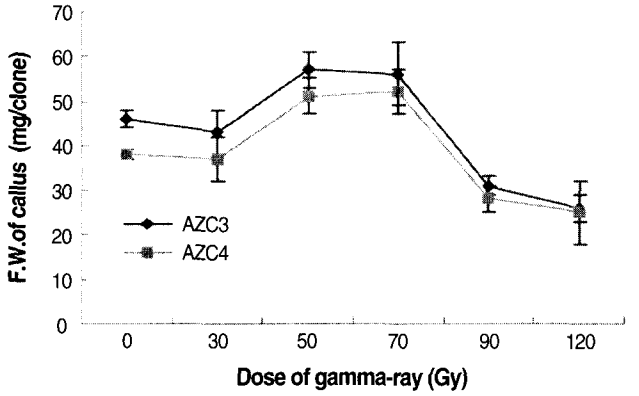

Figure 3. Fresh weights of calli irradiated with various gamma rays. Data were obtained 40 days after plating.

Table 3. Regeneration rates of AZCA resistant callus irradiated with various radiation doses 30 days on regeneration medium.

Radiation dose (Gy)	3 mM AZCA		4 mM AZCA	
	No. of transferred calli	No. of regenerant (%)	No. of transferred calli	No. of regenerant (%)
0	3300	16(0.5)cd[a]	1470	18(1.2)b
30	3600	1(0.03)cd	2490	21(0.8)b
50	1020	6(0.6)cd	1620	30(1.9)b
70	5280	69(1.3)bc	2280	19(0.8)b
90	2520	133(5.2)a	990	26(2.6)b
120	5100	118(2.3)b	2250	102(4.5)a

[a]Duncan's multiple range test at 5% level.

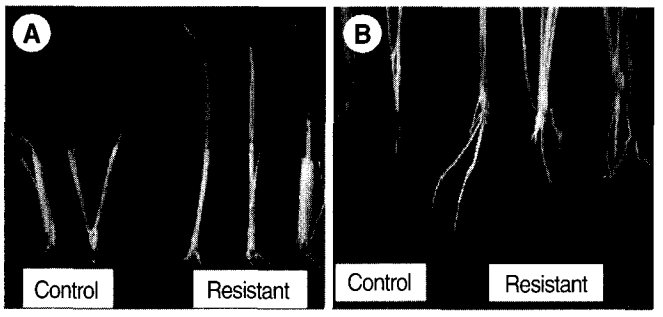

Figure 4. Selection of AZCA resistant regenerants grown for 2 weeks in solution containing 2 mM AZCA after excision. Shoots were removed except for 5 cm in length and roots were removed thoroughly for comparison of revival ability. A; Shoot revival 2 weeks after excision, B; Root revival 2 weeks after removal.

Table 4. Survivals and fresh weights of AZCA resistant calli on 170 mM NaCl containing medium, surveyed 40 days after culture.

Calli[a]	No. of plated calli	No. of survivals (%)	Fresh aweight (mg±SE)
Control	300	73(24)	170±10
Calli Ⅰ	300	49(16)	220±20[a]
Calli Ⅱ	300	93(31)	260±20[b]

[a]Calli Ⅰ : AZCA resistant calli selected from non-irradiated callus.
Calli Ⅱ : AZCA resistant calli selected from irradiated callus.
[b]Significance at $p < 0.05$ level of t-test
[c]Significance at $p < 0.01$ level of t-test

both survival and fresh weight.

Proline analysis

Proline contents were analyzed for resistant calli and regenerants. Proline content in callus is shown in Figure 5A. The level of free proline content of resistant callus was 2.8 times higher than that of the control. Although fresh weight of calli irradiated with 120 Gy was lower than that of the control, and remarkable increase in proline content was observed. It was the highest among the treatments with different doses (data not shown). Proline content in calli irradiated with 30 Gy was higher than that of calli irradiated with 50 Gy showing the highest fresh weight (data not shown). Hence, different proline contents demonstrated that there were variations between selected calli.

To verify proline increase, resistant regenerants and control plants were grown at the same soil conditions without AZCA treatment. Proline content of regenerants revealed that regenerants from calli irradiated with 50 and 70 Gy had the highest contents among analyzed regenerants. There were little, if any, increases of proline contents in regenerants from calli irradiated with 30, 90 and 120 Gy (data not shown). However, regenerants with gamma rays had higher proline contents than the control. Proline content in the regenerants was 1.8 times higher than that of the control (Figure 5B), but increase of proline in the regenerants was lower than that in calli.

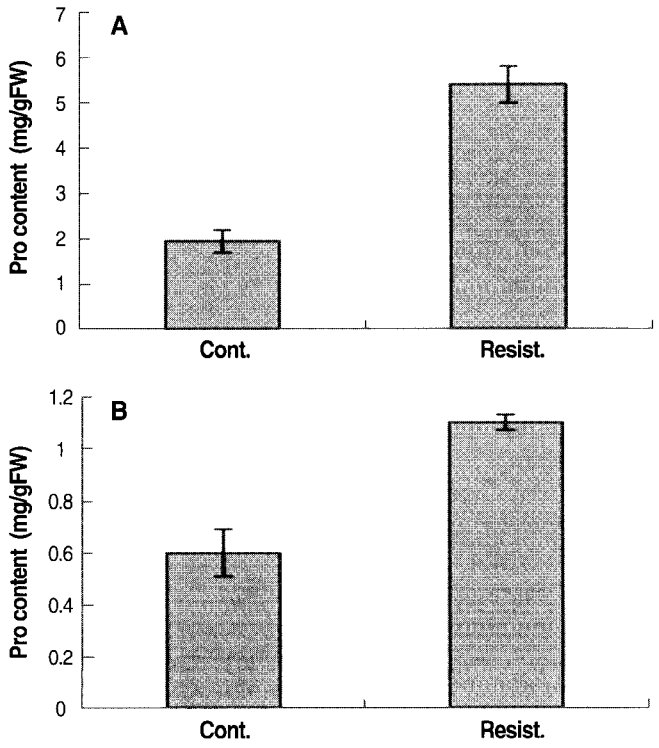

Figure 5. Proline content of AZCA resistant cell line (A) and resistant regenerant (B). Values represent means±SE (n=9).

Discussion

The potential of somaclonal variation to generate new, desirable traits for breeders has been advocated (Larkin and Scowcroft 1981). Since the highest level of somaclonal variation occurs in plants in particular after an intermediate callus phase (Yamagish et al. 1996; Piccioni et al. 1997), somaclonal variation is a problem in breeding and propagation techniques that involve adventitious regeneration. To overcome the problem of somaclonal variation, Maluszynski et al. (1995) mentioned the use of mutation induction technique with mutagenic treatment for crop improvement. And the use of gamma irradiation can also induce physiological and biochemical changes resulting in faster vegetative growth and early flowering (Al-Oudat 1990). In this paper, the selection of AZCA resistant cell lines through rice embryo culture after mutagenic treatment of callus with gamma rays was reported.

Survival rate and fresh weight of callus were decreased with increasing AZCA concentrations and/or radiation doses. It was suggested that AZCA was toxic to cells because incorporating into protein in place of proline resulted in altered protein conformation and function. These results indicated that selection of AZCA resistant cell lines seem to be dependent on a combination between the radiation dose used and the AZCA selection pressure. The optimum AZCA concentration for the selection of resistant cell lines was 3 or 4 mM AZCA considering LD_{50} and fresh weight of callus. Survival rate and fresh weight of calli were remarkably increased in the calli irradiated with 50 and 70 Gy. It showed that certain doses of gamma rays as a mutagen were effective to increase AZCA resistance. An increase of plant dry weight due to irradiation has been reported by Baker et al. (1976) for corn. Increase in root length and number and dry weight of *in vitro* growth by gamma irradiation was reported by Charbaji and Nabulsi (1999) in grapevine. Regeneration rates of the calli were much lower on the whole. Regeneration frequency decreased in long-term callus culture and chromosomal aberrations frequently resulted in loss of regenerative potential. Ahloowalia and Maluszynski (2001) reported that callus culture requires much lower doses (2 to 5 Gy) than stem cutting or seeds with relatively higher doses (15 to 20 Gy) that they turn necrotic or lose their regenerative capacity. Calli from AZCA-free medium had a lower regenerate rate than that from the AZCA selective medium. It was supposed that callus regeneration was not affected by AZCA. Although regeneration rate was low, 90 and 120 Gy increased the regeneration of calli selected. It is proposed that these doses were effective to regenerate callus for selection of AZCA

resistant cell lines. As for fresh weight, it was found that a survival rate and regeneration for selection of AZCA resistant cell line, 50-90 Gy was the optimum range of gamma irradiation.

It was demonstrated that regenerants from resistant calli showed resistance to AZCA. At a solution containing 2 mM AZCA resistant regenerants revived shoots and roots; whereas control plants died in the end. This implies that resistance was transmitted from callus to regenerant. Verbruggen et al. (1996) reported that AZCA resistance was afforded by an impairment of AZCA uptake. Hence, further investigations are required on whether resistance of regenerants resulted from genetic or physiological change. NaCl resistance of calli selected from AZCA showed the increase in resistance to osmotic stress by gamma ray treatment. Irradiated calli selected from 4 mM AZCA were increased in proline content (Figure 5A) that was osmoprotectant for plants under a stress environment, suggesting that elevated proline content resulted in resistance to NaCl that caused severe inhibition to plant growth. Compared with the control, irradiated calli were increased in survival and fresh weight, whereas non-irradiated calli decreased in survival. It is supposed that increased resistance resulted from gamma ray irradiation and irradiated calli were mutated in relation to osmotic stress. Increasing content of free proline in the calli suggested a modified biosynthetic pathway for proline synthesis that allowed overproduction of the amino acid. Dörffling et al. (1997) reported selection of hydroxyproline (Hyp), a proline analog, resistant cell line and Hyp-resistnat lines showed improvement of frost tolerance with elevated proline. Van swaaij et al. (1987, 1986) also used Hyp as a selection agent to select for proline overproducing lines in potato. The selected cell lines were more resistant to salt and freezing stress. Their regenerants contained increased levels of proline and showed more frost tolerance than the wild type. Lodato et al. (1984) has shown that two mutant cell lines in Chinese hamster lung fibroblasts were resistant to AZCA because of altered activity or regulator properties of the proline biosynthetic enzyme pyrroline-5-carboxylate synthetase (P5CS). The activity of P5CS was increased 30-fold in AZCA resistant cell lines. More proline is available to compete with AZCA for incorporation of protein, thus making the cells resistant to the toxic effects of the analog. Hong et al. (2000) recently reported that the removal of the end-product feedback inhibition of the enzyme in proline biosynthesis, P5CS resulted in increased proline accumulation in transgenic tobacco. They also showed that elevated proline content reduced the free radical damage induced by osmotic stress. Accordingly, it may be suggested that the mutation by *in vitro* mutagenesis affects one of the enzymes involved in the biosynthesis of proline and it probably is the rate limiting enzyme P5CS.

Although the protective role of proline in the tolerance of plants to many environmental stresses has been the matter of controversial debate, proline overproduction may be beneficial to stressed plants and the selection of proline overproducing mutants by *in vitro* mutagenesis seems to be a useful tool for the improvement of stress tolerance in this crop.

Acknowledgements

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References

Ahloowalia BS, Maluszynski M (2001) Induced mutations - A new paradigm in plant breeding. Euphytica 118: 167-173

Al-Oudat M (1990) Effect of low dose gamma irradiation on onion yield. Ann Biol 6: 61-67

Baker AM, Ashour NE, Elbasyouni SZ, Saed AM (1976) Response of the photosynthetic apparatus of corn (*Zea mays*) to presowing seed treatment with gamma rays and ammonium molybdate. Environ Exp Bot 16: 217-222

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water stress studies. Plant and Soil 39: 205-207

Cella R, Parisi B, Nielsen E (1982) Characterization of a carrot cell line resistant to azetidine-2-carboxylic acid. Plant Sci Lett 24: 125-135

Charbaji T, Nabulsi I (1999) Effect of low doses of gamma irradiation on *in vitro* growth of grapevine. Plant Cell Tiss Org Cult 57: 129-132

Charest C, Phan CT (1990) Cold acclimation of wheat (*Triticum aestivum*): Properties of enzymes involved in proline metabolism. Physiol Plant 80: 159-168

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FI (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sinica 18: 659-668

Delauney AJ, Verma DPS (1993) Proline biosynthesis and osmoregulation in plants. Plant J 4: 215-223

Dorffling K, Dorffling H, Lesselich G, Luck E, Zimmermann C, Melz G, Jurgens HU (1997) Heritable improvement of frost tolerance in winter wheat by *in vitro*-selection of hydroxyproline-resistant proline overproducing mutants. Euphytica 93: 1-10

Hasegawa H, Mori S (1986) Non-proline accumulating rice mutants resistant to hydroxy-L-proline. Theol Appl Genet 72: 226-230

Hong Z, Lakkineni K, Zhang Z, Verma DPS (2000) Removal of feedback inhibition of Δ^1-pyrroline-5-carboxylate synthetase

results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol 122: 1129-1136

Igarashi Y, Yoshiba I, Yamaguchi-Shinozaki K, Wada K, Shinozaki K (1997) Characterization of the gene for delta1-pyrroline-5-carboxylate synthetase and correlation between the statement of the gene and salt tolerance in *Oryza sativa* L. Plant Mol Biol 33: 857-865

Iyer S, Caplan A (1998) Products of proline catabolism can induce osmotically regulated genes in rice. Plant Physiol 116: 203-211

Kueh JHS, Bright SWJ (1982) Biochemical and genetical analysis of three proline accumulating barley mutants. Plant Sci Lett 27: 233-241

Larkin PJ, Scowcroft WR (1981) Somaclonal variation - a novel source of variability from cell cultures for plant improvement. Theol Appl Genet 60: 197-214

Lodato RF, Smith RJ, Valle DL, Crane K (1984) Mutant cell lines resistant to azetidine-2-carboxylic acid: alterations in the synthesis of proline from glutamic acid. J Cell Physiol 119: 137-143

Maluszynski M, Ahloowalia BS, Sigurbjornsson B (1995) Application of *in vivo* and *in vitro* mutation techniques for crop improvement. Euphytica 85: 303-315

Miflin BJ, Bright SW, Rognes SE, Kueh JHS (1983) Amino acids, nutrition and stress: the role of biochemical mutants in solving problems of crop quality. In Kosuge T, Meredith CP, Hollaender A (eds), Genetic engineering of plants - an agricultural perspective, pp. 391-414. Plenum Press, London

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497

Piccioni E, Barcaccia G, Falcinelli M, Standard A (1997) Estimating somaclonal variation in axillary branching propagation and indirect somatic embryogenesis by RAPD fingerprinting. Int J Plant Sci 158: 556-562

Smirnoff N, Cumbes QJ (1989) Hydroxyl radical scavenging activity of compatible solute. Phytochemistry 28: 1057-1060

Van Swaaij AC, Nijdam H, Jacobsen E, Feenstra WJ (1987) Increased frost tolerance and amino acid content in leaves, tubers and leaf callus of regenerated hydroxyproline resistant potato clones. Euphytica 36: 369-380

Van Swaaij AC, Jacobsen E, Kiel JAKW, Feenstra WJ (1986) Selection, characterization, and regeneration of hydroxyproline-resistant cell lines of *Solanum tuberosum*: tolerance to NaCl and freezing stress. Physiol Plant 68: 359-366

Verbruggen N, Borstlap AC, Jacobs M, Van Montagu M, Messens E (1996) The *raz1* mutant of *Arabidopsis thaliana* lacks the activity of a high-affinity amino acid transporter. Planta 200: 247-253

Watanabe S, Kojima K, Ide Y, Sasaki S (2000) Effects of saline and osmotic stress on proline and sugar accumulation in *Populus euphratica in vitro*. Plant Cell Tiss Org Cult 63: 199-206

Yamagishi M, Otani M, Shimada T (1996) A comparison of somaclonal variation in rice plants derived and not derived from protoplast. Plant Breed 115: 289-294

Yoshiba Y, Kiyosue T, Nakashima K, Yamaguch-Shinozaki K, Shinozaki K (1997) Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol 38: 1095-1102