Selection of hydroxyproline-resistant proline-accumulating mutants of cauliflower (*brassica oleracea* var. *botrytis*)

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Summary

A procedure is described by which hydroxyproline-resistant lines could be selected from regenerating curd tissue of cauliflower. Mutagenesis was by N-nitroso-N-ethylurea, supplied as a drop of 0.3 mM solution on each 3 mm diameter curd piece. The mutagen generated numerous morphological and pigment mutations without significantly affecting shoot regeneration from explants. Thirty one resistant shoots were recovered from more than six thousand explants mutagenised on regeneration medium supplemented with 3 mM hydroxyproline, while none was obtained from a similar number of non-mutagenised controls. Out of twenty-three resistant shoots which survived subculture, only one showed consistently elevated levels of endogenous proline. During early shoot culture passages, proline levels were 3.6–4.7 times higher than controls, but this was reduced to 1.6 times after 10–12 culture passages in the absence of hydroxyproline. Possible reasons for this decline are discussed. Leaf strip assays suggest resistant shoots may be chimeras and current efforts are directed towards regenerating solid mutants from resistant sectors. These will then be evaluated for any alteration in frost tolerance.

Abbreviations: IBA – Indole-3-butyric-acid, NEU – N-nitroso-N-ethylurea

Introduction

The accumulation of free proline in response to environmental stress has been observed in plants (Aspinall & Paleg, 1981, Larher et al., 1982) and bacteria (Tempest et al., 1970). The significance of this accumulation in plants has been attributed to the suggested ability of proline to act as a protective agent for cytoplasmic enzymes (Aspinall & Paleg, 1981).

High proline content has been related to frost tolerance in a number of plant species (Aspinall & Paleg, 1981). In potato, leaf proline content and frost tolerance are correlated and exogenous application of proline increases frost tolerance (Van Swaaij et al., 1985). One way to obtain a proline-accumulating mutant is to select for proline analogue resistance. The mechanism thought to confer amino acid analogue resistance involves a mutation which results in an enzyme becoming feedback-insensitive resulting in

over-production of the corresponding amino acid (Dix, 1986). This approach was first applied to plants by Widholm (1972a, 1972b), who isolated tryptophan-producing lines of tobacco by selecting for resistance to 5-methyltryptophan. Amino acid analogue-resistant mutants which overproduce the specific amino acid have been found in bacteria (Czonka, 1981; Sugiura & Kisumi, 1985) as well as plants (Widholm, 1976; Kueh & Bright, 1982; Van Swaaij et al., 1986; Mori et al., 1989). It has been reported that free proline-accumulating cell lines resistant to a proline analogue show increased resistance to stresses such as salt (Riccardi et al., 1983) and freezing (Van Swaaij et al., 1986, 1987). Van Swaaij et al. (1986) selected hydroxyproline-resistant potato callus which overproduced proline. Proline accumulation and increased frost tolerance were exhibited in the leaves of plants regenerated from this callus.

The present report describes studies with cauliflower in which *in vitro* mutagenesis and hydroxyproline resistance selection were used to obtain proline-accumulating shoots, as a strategy for improving the frost tolerance of this crop.

Materials and methods

Plant material

Four cauliflower (*Brassica oleracea* var. *botrytis*) F1 hybrid cultivars were used; 'Plana', a summer heading cultivar, 'Arbon' and 'Dova', two autumn heading cultivars, and 'Arcade', an overwintering cultivar. Plants were greenhouse-grown (15–20° C, with supplementary lighting during winter) and curd was freshly cut or stored for up to two weeks at 4° C. Florets, 3–4 cm in length, were surface-sterilised by soaking in 10% *Domestos* (a commercial disinfectant containing 5% calcium hypochlorite) for 10 minutes and rinsing three times in sterile distilled water. Pieces of curd approximately 3 mm in diameter were cut from the surface and placed onto medium in 9 cm diameter plastic Petri dishes (5–20 per dish).

Regeneration of shoots from curd

Pieces of curd were placed on regeneration medium stage I (Table 1). Dishes were sealed with parafilm and incubated in the culture room (23–25° C, 16 hour photoperiod, 2,000–3,000 Lux). Curd explants became green after about 1 week and shoots were separated as soon as they appeared, to prevent crowding. After 4–5 weeks on stage I medium, shoots were transferred to stage II (Table 1) and could be maintained on this indefinitely. To induce rooting, shoots were cut and transferred to either stage III medium (Table 1) or hormone-free RM medium (Murashige & Skoog, 1962) salts with 30 gl^{-1} sucrose, 6 gl^{-1} agar, pH 5.7.

Mutagenesis with N-nitroso-N-ethylurea (NEU)

Precautions taken in handling NEU and disposing of waste are as outlined in McCabe et al. (1990). A 0.3 mM solution was made with distilled water and dropped with a sterile Pasteur pipette onto each piece of curd (one drop per piece) on stage I medium. Controls were treated with sterile distilled water. Plates were sealed with parafilm and placed on a shelf lined

Table 1. Media used for culture of cauliflower curd. Murashige & Skoog (1962) plant salt mixture with the following additions

Thiamine		0.4 mgl^{-1}	
Adenine sulphate		80 mgl^{-1}	
Sodium dihydrogen orthophosphate		170 mgl^{-1}	
Sucrose		30 gl^{-1}	

	Stage I	Stage II	Stage III
IBA mgl^{-1}	1	2	2
Kinetin mgl^{-1}	2	4	0
Agar gl^{-1}	4	6	6

pH adjusted to 5.7 before autoclaving.

with absorbent paper in the culture room. NEU was not washed out.

Selection for hydroxyproline-resistant shoots

Curd pieces were placed on stage I medium supplemented with hydroxyproline. When a survivor was identified (on NEU-treated plates) the piece of curd on which it arose was transferred to fresh medium with the same level of hydroxyproline. It was maintained on hydroxyproline for approximately 5 weeks to ensure that all non-resistant cells had been killed. It was then transferred to stage II medium without hydroxyproline for 3–4 weeks and then either to stage III medium or to RM.

Estimation of free proline content

Proline levels were measured by the method of Bates et al. (1973). Fully expanded leaves were selected from shoot cultures and homogenised in 3% sulfosalicylic acid. The filtered homogenate was allowed to react with acid ninhydrin and glacial acetic acid for one hour at 100° C. The reaction mixture was extracted with toluene. The chromophore containing toluene was aspirated from the aqueous phase and the absorbance read at 520 nm. The proline concentration was determined from a standard curve and calculated on a fesh weight basis.

Table 2. The survival of curd, both freshly cut and stored at 4° C for two weeks, on a range of hydroxyproline concentrations. There were 16 pieces in each dish and at least 4 dishes of each hydroxyproline concentration for each treatment and variety

mM Hyp.	% Survival					
	Plana		Arbon		Dova	
	Fresh	2 weeks	Fresh	2 weeks	Fresh	2 weeks
30	0	0	0	0	0	0
10	0	0	0	0	0	0
3	0	0	0	0	0	0
1	1.5	4.5	3.2	7.8	0	1.6
0.3	100	100	100	100	100	100
0.1	100	100	100	100	100	100
0	100	100	100	100	100	100

Results

Mutagenic effect of NEU

Three weeks after NEU treatment, fifty leaves were selected at random from shoots from each of ten batches of NEU and distilled water-treated curd. These leaves were scored for abnormalities. 41.6% of leaves from NEU-treated curd showed abnormalities in chlorophyll content or distribution and 26.2% showed morphological abnormalities. Less than 2% of leaves from distilled water-treated curd showed abnormalities.

NEU at this concentration did not have a significant effect on the number of shoots regenerated.

Selective level of hydroxyproline

Curd pieces were scored for survival (possession of chlorophyll) after three weeks on stage I medium supplemented with a range of hydroxyproline concentrations. In three cultivars tested (Plana, Arbon and Dova), there were no survivors on 3–30 mM hydroxyproline (Table 2). The lowest concentration giving 100% explant death (3 mM) was subsequently chosen for selection.

Duration of exposure to hydroxyproline

Curd tissue which was exposed to hydroxyproline for one week showed almost complete survival after three subsequent weeks on hydroxyproline-free medium, indicating that regeneration is merely suppressed by a short exposure to hydroxyproline. As the duration of exposure to hydroxyproline increased, so did the time taken for recovery. Pieces of curd 3 mm in diameter exposed for two weeks showed no sign of recovery after three weeks on hydroxyproline-free medium, but 7.5% survived after five weeks. A three week exposure to 3 mM hydroxyproline caused complete killing in 3 mm pieces, but larger pieces survived for longer and 17.5% recovered after five weeks on hydroxyproline-free medium. Therefore 3 mm pieces of curd, the size used in mutagenesis, must remain on hydroxyproline for at least three weeks to ensure complete killing.

Selection of hydroxyproline-resistant shoots

Twenty curd pieces were put into each Petri dish containing regeneration medium I supplemented with 3 mM hydroxyproline. Curd tissue was treated with either NEU or sterile distilled water. Dishes were checked for survivors initially one week after mutagenesis treatment and then weekly. The numbers of shoots apparent after 4 weeks are shown in Table 3. Twenty-three of the 31 hydroxyproline-resistant shoots survived subculture on stage II medium, but only two of them showed consistently higher levels of proline in initial tests (after one subculture). In later tests (after 5–6 and 10–12 subcultures), only one of them had consistently higher levels. These results are shown in Table 4.

Discussion

Results show that a low concentration of NEU (0.3 mM) applied to curd tissue and not washed out,

Table 3. Effect of NEU on shoot formation from curd pieces on medium supplemented with 3 mM hydroxyproline. The numbers of shoots which were produced from each treatment are shown

Treatment	Number of curd pieces		Number of shoots	
variety	NEU	H_2O	NEU	H_2O
Plana	160	220	2	0
Arbon	1600	1620	8	0
Dova	2880	2400	10	0
Arcade	1560	1440	11	0

Table 4. Proline levels in shoot cultures of hydroxyproline. resistant line of the variety Plana. Values are means of 5 shoot cultures (1st subculure) or 8 sublines (5–6 subcultures, 10–12 subcultures), and errors are the standard error in the mean. Controls are means of 4 or 6 independently maintained lines

No. of subculture	μmol. proline g^{-1} of leaf fresh weight		Accumulation*
	Control	Mutant	
1	251.1 ± 47.7	902.2 ± 117.8	3.6
5–6	130 ± 35.5	607.5 ± 101.2	4.7
10–12	189.6 ± 27.4	310.2 ± 28.3	1.6

* Factor by which mutant exceeds control.

does not significantly reduce the numbers of shoots produced but does cause mutations in the genes controlling chlorophyll biosynthesis and plant morphology.

Curd tissue exposed to hydroxyproline concentrations ranging from 3 mM to 30 mM does not survive after three weeks. A small percentage of those on 1 mM survive, however. In all three varieties tested, a higher percentage survive on 1 mM if the curd has been exposed to 4° C for two weeks prior to testing. It is possible that this low temperature pre-treatment induces proline accumulation in cells of the curd which protects the tissue from hydroxyproline.

Hydroxyproline at a concentration of 3 mM proved a suitable level for selecting resistant shoots, and 31 were recovered from a total of more than six thousand curd pieces. The importance of the mutagenesis treatment is indicated by the complete absence of resistant shoots in a similar number of non-mutagenised controls. The eight hydroxyproline-resistant shoots which did not survive subculture may have acquired mutations in genes crucial for survival. Some failed to form roots in culture, others simply did not grow.

Of the 23 shoots which survived, only one showed consistently higher levels of proline. It is possible that some mechanism other than proline accumulation for hydroxyproline resistance is in action in the other lines. Hasegawa & Mori (1986) reported the isolation of three rice mutants resistant to hydroxyproline but not proline-accumulating. Their resistance was found to be controlled by a single recessive nuclear gene. Resistance to amino acid analogues may also be due to a mutation causing decreased uptake of the analogue (Widholm, 1974) or preferential incorporation of the naturally-occurring amino acid (Negrutiu et al., 1978). Another possibility is that not all of the meristems were dead when the piece of curd on which the variant arose was transferred to hydroxyproline-free medium. Results indicate that after 3 weeks exposure to hydroxyproline, 3 mm pieces of curd show no sign of surviving after 5 weeks on hydroxyproline-free medium, but the number of pieces tested this way was small (40) in comparison with the numbers used in mutagenesis experiments.

Apart from the possibility of escapes – the curd system is attractive because most of the cells in a piece of curd are not killed immediately and may nurse resistant cells and shoot primordia through early stages of development.

The initial level of proline measured in the variant which consistently showed higher levels of proline was 3.6 times the control. After 5–6 subcultures, this value was 4.7 times the control. However, after 10–12 subcultures, the mean had dropped to 1.6 times the control. The reason for this reduction may be due to the absence of hydroxyproline in the culture medium. Amino acid over-production may gradually be reduced in the absence of the analogue leading to a reduction in resistance. Widholm (1976) showed that a carrot cell line selected for resistance to ethionine initially required over 1,000 times more ethionine for inhibition of growth than the controls. This resistance was reduced by growth in the absence of ethionine. Van Swaaij et al. (1986) selected hydroxyproline-resistant, proline-accumulating callus of potato, and found that both, shoots regenerated from it, and callus initiated from leaf and stem tissue of these plants, had lower levels of proline than the originally-selected line which had been continuously grown on hydroxyproline (but these levels were still significantly higher than those of the control). The mean proline concentration of the control shoot cultures in the present work also decreased over time, but this was not significant.

A possible explanation for the variation in proline content of shoot cultures which have originated from the same resistant shoot, is that the shoots may be chimeric. The surface of the curd consists of thousands of apical meristems with single cells between them which are capable of dividing to give meristems. Scanning electron microscope studies (Crisp & Walkey, 1974) indicate that each meristem may become vegetative and give rise to a shoot. It is possible that one cell of a pre-formed meristem may have acquired the mutation resulting in over-production of proline, confering resistance on the whole meristem. Continuous subculturing in the absence of selection may lead to segregation to give plants with accumulating and non-accumulating sectors.

Investigations underway support the chimeric nature of leaves of the proline-accumulating, hydroxyproline-resistant variant. Leaf stips on hydroxyproline-containing medium show resistant and sensitive sectors. Regeneration from resistant leaf sectors should give a solid variant in which frost tolerance will be assessed. If fertile plants are regenerated, the heritability of the trait can be studied proving whether or not a stable genetic change has occurred.

In vitro mutagenesis and selection has usually involved callus or cell suspension cultures (Collin & Dix, 1990). While the possibilities offered by regenerable explant cultures have been demonstrated (McCabe et al., 1989) the use of an organised system, such as cauliflower curd, is still rare. We believe the present report shows the approach can be effective and deserves more attention.

Acknowledgements

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