Selection of 3-fluorotyrosine tolerant callus lines in two cultivars of opium poppy (*Papaver somniferum* L.) and regeneration of plants through somatic embryogenesis

Rupali Khanna[1], Ajay Kumar Mathur[1,*] and Nirmal Kishore Mehrotra[2]

[1]Phytocellular Technology Division, Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow 226 015, India
[2]Department of Botany, Lucknow University, Lucknow 226 007, India

Six variant callus lines exhibiting differential tolerance to 3-fluorotyrosine (3FT, a tyrosine analogue) have been isolated in two cultivars of opium poppy ('Sanchita' and 'Shweta'). The selected trait was stably maintained in the absence or under sustained selection pressure of the analogue stress. Growth characteristics of the selected lines showed that lines having threshold tolerance up to sub-lethal level of 3FT (150–200 µM) had a growth index comparable to that of wild type on unstressed medium. One of the lines (Shw L-200) exhibited the capacity to gradually build up its tolerance up to supra-lethal (250 µM) level of 3FT. In the presence of analogue stress, all the selected lines showed a 1.5 to 2.0-fold increase in their free amino acid pool. The selected line Shw L-250 showed a 4.5-fold increase in free tyrosine in the presence of 3FT stress, which was three-fold more than in the wild type maintained on stress-free medium. Selected and non-selected lines showed high frequency of plant regeneration via somatic embryogenesis when the analogue stress was temporarily withdrawn during early stages of organogenesis. Leaves of the regenerated plants of the selected lines showed the capability to initiate callusing on 3FT stressed medium, indicating thereby the persistence of the selected trait at whole plant level. The relevance of the selected variants in relation to their possible exploitation in metabolic engineering of benzyl-isoquinoline alkaloids is proposed.

THERE is much scope for exploiting the metabolic richness of plants for pharmaceuticals, nutraceuticals, flavours, fragrance, colours and cosmetics. The trends in research are also changing from whole plant-based approaches to pathway-oriented manipulation and modifications[1–3]. Because of their centre-stage position in overall plant metabolism, the supply of precursor molecules like aromatic amino acids (tryptophan, tyrosine and phenylalanine) and terpenoid-building units (isopentenyldiphosphate, geranylgeranyldiphosphate and dimethylallyldiphosphate) is one of the most crucial limiting factors in diverting the flux of a secondary metabolic pathway[2,4,5]. Tissues with better sink capacity and availability of such primary precursors are by choice the most appropriate materials for metabolic manipulation of secondary pathways. The present study has got its genesis in the light of this emerging consideration of metabolic engineering and, aimed to select mutants with higher accumulation of the aromatic amino acids that act as primary precursors in the biogenesis of pharmaceutically important alkaloids. To examine such a possibility, four alkaloid-yielding medicinal plants, namely *Papaver somniferum*, *Catharanthus roseus*, *Vinca minor* and *Duboisia myoporoides* were selected as test systems. The selected plant species are the major source of three different classes of alkaloids (benzylisoquinolines, terpenoid indoles and tropanes) that are derived via three different pathways that start with three different amino acids, namely tyrosine, tryptophan and phenylalanine, respectively.

P. somniferum (opium poppy) plants are the exclusive source of several pharmaceutically important benzylisoquinoline type of alkaloids like morphine, codeine, thebaine, noscopine, papaverine, cordaline, protopine and sangunarine. The drug action of these alkaloids varies from analgesic, antitussive, vasodilatory, antiinflammatory, and sedative to antimicrobial activities[6]. The entire biogenetic pathway of opium alkaloids involves 21 steps that have been explicitly resolved at enzyme level[2,6]. As a result, poppy plants and cultured cells are now considered as model tools to understand and verify several options of pathway engineering in plants[1,6]. All benzylisoquinoline alkaloids share a common biogenetic origin beginning with the condensation of two aromatic units, L-DOPA and 4-hydroxyphenylacetaldehyde (4-HPAA), both derived from L-tyrosine. This condensation results in the formation of (S)-norcoclaurine that, through a series of enzymetic reactions, forms (S)-reticuline which is the central branch point intermediate in the pathway. Subsequent synthesis can either proceed towards sanguinarines via (S)-scoulerine formation or towards morphinane group of alkaloids via (R)-reticuline synthesis[6,7]. The gene that codes for enzyme tyrosine or DOPA carboxylase (*TyDC*) has been cloned and heterologously expressed in *E. coli*[7]. *Agrobacterium*-mediated transformation protocols for opium poppy have also been developed[8,9]. It would, therefore, be interesting to generate poppy tissues and plants with larger availability of target precursor/intermediates of the pathway to divert their flux in the desired direction through genetic engineering techniques.

In this communication, we report the isolation of 3-fluorotyrosine (3FT; an analogue of tyrosine) resistant callus lines of *P. somniferum* that can produce and accumulate high levels of tyrosine. The rationale behind the experimental approach employed in this study is based on prior knowledge that resistance to amino acid analogue stress in culture cells generally originates through the overproduction of the corresponding amino acid via an altered/relaxed feedback inhibition control of the allosteric enzymes of the shikimate or aspartate pathway[10–12]. This is a report of a biochemical mutant selected *in vitro* in opium poppy that has stably maintained the acquired trait through a plant regeneration cycle.

*For correspondence. (e-mail: akmcath@yahoo.com)

Seeds of three cultivars of *P. somniferum*, i.e. 'Sanchita', 'Shweta' and 'VPCL' were obtained from National Gene Bank of Medicinal and Aromatic Plants (CIMAP, Lucknow). These were surface-sterilized using savlon (1%) and ethanol for 30 s each, followed by 0.1% $HgCl_2$ for 2 min, rinsed with sterile distilled water and placed on filter paper disc overlaid on Murashige and Skoog (MS)[13] basal medium in sterilized petri plates and kept in dark for germination (72 h). Callus was induced using cotyledonary leaf and hypocotyl explants from 15-day-old seedlings and placed on MS basal medium supplemented with 0.46 µM kinetin (Kn), 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 227.1 µM ascorbic acid, sucrose 3% and agar 0.8% (w/v), hereafter designated as PC medium. The induced callus was multiplied and maintained through regular sub-culturing every fourth week on fresh PC medium. The callus beyond the third sub-culture became embryogenic as evident histologically by the appearance of tiny, shining, white globular embryoids on its surface. The pH of the culture media was adjusted to 5.8 ± 0.1 before autoclaving at 121°C, 1.1 kg cm^{-2} for 20 min. Unless otherwise specified, all *in vitro* cultures were incubated at 25 ± 4°C under 16 : 8 h light : dark photoperiod maintained through cool white fluorescent tube light of 32 µmol $m^{-2} s^{-1}$ intensity.

To determine LD_{50} dose, the wild type calluses of the three cultivars were plated on PC medium fortified with a graded series (0, 100, 125, 150, 200, 250 and 300 µM) of 3FT stress. For this, 3FT was dissolved in 0.1 N NaOH, volume made up with distilled water (pH 7.0–7.5), filter-sterilized (Millipore, Millex GV-25 USA) and dispensed in pre-autoclaved molten medium to obtain the desired level of analogue stress. Following dose-response studies, a modified 'Direct Selection Scheme'[11] was devised to isolate putative 3FT-tolerant variants. Accordingly, 15–20 small callus pieces (200–250 mg) were plated in each petri plate comprising PC medium (25 ml) fortified with sub- to supra-lethal levels (100–300 µM) of 3FT stress. Unstressed medium served as control. Surviving sectors of the analogue-challenged calluses were retrieved and sub-cultured onto the same level of 3FT stress for 3–5 subsequent passages to eliminate escapees. The best proliferating sectors growing at different levels of selection pressure were then divided into five parts and transferred accordingly: part (1) stress-free control medium, part (2) same level of stress, part (3) one level lower than the original level of stress and parts (4) and (5) two levels higher than the original level of stress. The selected 3FT-tolerant callus clones of differential tolerance levels were maintained for 20 cell-doubling cycles on stressed as well as non-stressed media before subjecting to detailed characterization and regeneration studies. The biomass increment in terms of growth index (GI = per cent increase in fresh weight over initial inoculum) of the selected lines was compared with that of non-selected wild type over a 30-day culture cycle on medium fortified with graded series (0–300 µM) of 3FT stress. The variants were also checked for persistence of the acquired trait away from the selection pressure for eight culture cycles.

Free amino acid pool and tyrosine content in the selected and wild-type lines were measured. Briefly, the protocols involved the following steps: freshly harvested callus (100–200 mg) was homogenized in 80% (v/v) cold ethanol (5 ml), centrifuged at 15,000 rpm for 20 min (x3) and the pooled supernatant used as test solution for estimation of total free amino acids[14]. For estimating free tyrosine content, the fresh callus tissue (1.0–1.5 g) was homogenized in 10 ml of 0.2 M potassium phosphate buffer (pH 7.8) having 10 mM glutathione and centrifuged (10,000 rpm) for 10 min; the supernatant was loaded onto a DOWEX 50 X-2 column (1.0×15.0 cm; equilibrated at pH 5.0 with 0.1 N glacial acetic acid) and allowed to stand on the column for 30 min before fractionating out @ 1.0 ml min^{-1}. The column was washed with distilled water (25 ml) and tyrosine was eluted from the column using 0.3 N NH_4OH ($3 \times$ 20 ml). Pooled ammonia fractions were evaporated to dryness on a water bath (80°C) and the residue dissolved in 1.0 ml distilled water. To this, 0.6 N TCA (1.0 ml) was added and left on an ice bath (10 min) followed by centrifugation at 15,000 rpm (Microspin 12: Sorval Instruments, Dupont) and the supernatant used as test solution for estimation of free tyrosine content[15].

For testing the stability of acquired 3FT-tolerant trait in the selected lines through a regeneration cycle, the plantlets were recovered in the presence (75, 100, 125, 150 and 200 µM) and absence of 3FT stress. For plantlet regeneration, the embryogenic callus was transferred to MS basal medium supplemented with 2.46 µM 2-isopentenyladenine (2-iP) and 0.57 µM indole-3-acetic acid (IAA), sucrose 3% and agar 0.8% (w/v), hereafter designated as PR medium. For rooting and plantlet development, the shoots were transferred on half strength MS basal medium (devoid of vitamins) comprising 5.7 µM IAA, sucrose 3% and agar 1% (w/v)[16], hereafter designated as PM medium. The leaf explants were then excised from the regenerants and checked for their callusing potential at 0–250 µM levels of 3FT stress.

The wild type calluses when challenged against a graded series of 3FT stress (0, 100, 125, 150, 200, 250 and 300 µM), exhibited a strong genotypic influence on their sensitivity towards analogue stress (Figure 1). Such genotypic influence has been frequently reported in selection for amino acid analogue tolerance in many plant species[11,12]. The degree of inherent tolerance towards 3FT in terms of callus survival and growth index was in the order of 'Sanchita' > 'Shweta' > 'VPCL'. While 'Sanchita' calluses registered an LD_{50} level around 150 µM 3FT, the corresponding value for 'Shweta' and 'VPCL' calluses was between 100 and 125 µM level of 3FT. None of the wild-type calluses could tolerate analogue stress beyond 250 µM level. To ascertain whether 3FT actually behaved as a true analogue of tyrosine in callus cultures of *P. somniferum*, tyrosine was co-fed at 276.2 and 552.4 µM levels to measure its

counteracting effect on growth inhibition caused by 3FT stress in wild-line callus of cv. 'Sanchita' (Figure 2). Addition of tyrosine in stress-free medium was by itself beneficial for the growth of wild-line calluses registering a 1.5-fold increment in the biomass at the end of the culture cycle. Tyrosine at 276.2 μM level was sufficient to reverse the growth inhibition caused by 3FT up to 150 μM level, whereas its feeding at 552.4 μM level counteracted analogue inhibition up to 300 μM level, suggesting thereby that 3FT behaved as a true analogue of tyrosine. In the presence of excess amount of the target amino acid, the analogue is usually not allowed entry into the cells or is competed out via a dilution effect.

The selection scheme devised in this study led to the isolation of six stable variant lines (San L-150, San L-200, San L-250 of cv. 'Sanchita'; Shw L-150, Shw L-200, Shw L-250 of cv. 'Shweta') over a period of eleven subculture cycles of four weeks each. The wild-type calluses of genotype 'VPCL' failed to yield any stable variant culture. Growth characteristics of some stable 3FT-tolerant callus lines of cvs. 'Sanchita' and 'Shweta' in the absence and presence of 3FT stress are given in Table 1. Amongst the four most stable 3FT-tolerant lines, i.e. San L-150, San L-200, Shw L-150 and Shw L-200, line San L-200 registered the highest biomass gain up to 200 μM 3FT level, recording a growth index of 482.60, which was almost equal to that on unstressed medium (GI = 497.54). This line also showed limited growth at supra-lethal levels of stress, i.e. 250 μM (GI = 97.04) and 300 μM 3FT (GI = 78.84), but appeared to have a threshold tolerance of 200 μM level on which it was originally selected. In comparison, Shw L-200 not only maintained its superiority over other 3FT-tolerant lines as well as wild type up to 200 μM level of stress, but also showed further capacity to gradually build-up its tolerance up to 250 μM level of stress (GI = 278.81), which was comparable to its growth at 200 μM 3FT concentration (GI = 299.42). However, its growth declined drastically when cultured on 300 μM level of stress (GI = 23.72). All the six selected lines showed persistence of the acquired trait when cultured for eight continuous passages in the absence of 3FT, followed by re-transfer to the analogue medium after each cycle on 3FT-free medium.

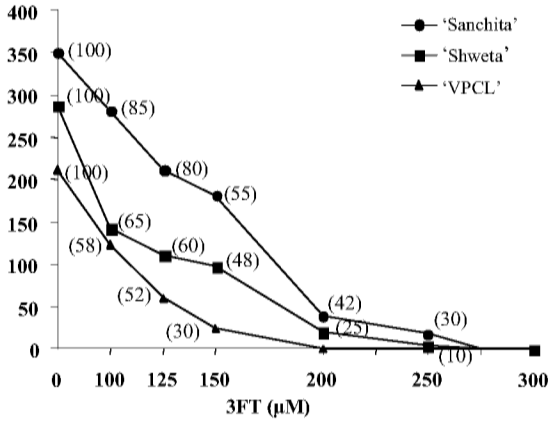

Figure 1. Dose-response of wild-type callus cultures of three cultivars of opium poppy against 3FT stress (values in parenthesis indicate per cent explant survival).

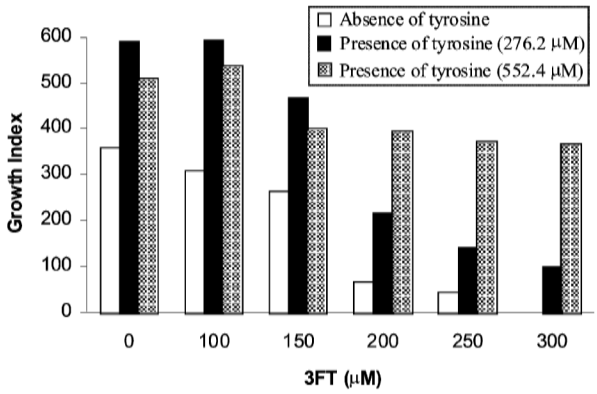

Figure 2. Complementation of growth inhibition caused by 3FT stress by tyrosine supplementation in wild-type callus cultures of opium poppy (cv. 'Sanchita').

The free amino acid pool size of wild type and selected lines of cv. 'Shweta' is given in Table 2. Shw L-150 registered a sudden rise in free amino acid content (6.97 mg g fr wt^{-1}) after 15 days of growth in the presence of 150 μM 3FT, which declined to nearly 50% (3.37 mg g fr wt^{-1}) on the 30th day of growth probably due to higher growth index with age. Shw L-200 and Shw L-250, on the other hand, showed more steady increase in the amino acid pool throughout the culture period and had amino acid content of 6.32 and 7.89 mg g fr wt^{-1} respectively, on the 30th day. Interestingly, none of the lines showed higher amino acid content on 3FT-free medium than the wild-type cultures on 3FT-free medium at any stage of their growth. These results clearly suggest that the enlargement of free amino acid pool was an inductive effect of the analogue stress. The selected and non-selected lines were also prospected for increment of tyrosine content in the presence and absence of 3FT (Figure 3). On unstressed medium the wild type of cv. 'Sanchita' recorded a tyrosine content of 204.98 μg g fr wt^{-1}, which was almost twofold more than the wild type of cv. 'Shweta'. Among all the selected lines, Shw L-250 showed the highest increase in tyrosine content (422.9 μg g fr wt^{-1}) in the presence of the respective analogue stress (250 μM). This was 4.5-fold more than in the absence of 3FT and nearly three fold high than in the wild-type cultures on 3FT-free medium. The other two variant lines, i.e. Shw L-150 and Shw L-200 showed tyrosine content similar to that of the wild type. None of the selected lines of cv. 'Sanchita' showed any significant change in tyrosine level both in the presence and absence of analogue stress. This is probably because of the inherently higher tyrosine content in the wild-type calluses, which was suffi-

Table 1. Growth characteristics of some stable 3FT-tolerant callus lines of *P. somniferum* in comparison to their respective non-selected wild types

	Growth index*					
	'Sanchita'			'Shweta'		
3FT concentration (μM)	Wild type	San L-150	San L-200	Wild type	Shw L-150	Shw L-200
0	355.38 (± 19.88)	477.20 (± 14.39)	497.54 (± 19.19)	252.75 (± 10.64)	307.00 (± 11.59)	354.49 (± 13.42)
100	266.92 (± 13.38)	516.08 (± 19.27)	586.47 (± 22.20)	146.34 (± 24.28)	286.34 (± 16.21)	420.85 (± 14.74)
150	183.41 (± 14.14)	469.60 (± 6.18)	464.70 (± 2.71)	88.45 (± 3.07)	259.00 (± 3.35)	338.90 (± 1.48)
200	38.13 (± 5.21)	239.18 (± 13.62)	482.60 (± 21.13)	20.79 (± 5.80)	239.62 (± 16.82)	299.42 (± 17.48)
250	17.14 (± 3.30)	91.5 (± 5.95)	97.04 (± 2.03)	3.39 (± 0.76)	94.93 (± 1.49)	278.81 (± 15.34)
300	–	–	78.04 (± 3.56)	–	–	23.72 (± 3.37)

*Per cent fresh biomass increment over initial inoculum (200 mg); Data recorded after 30 days of growth; Values in parentheses indicate SE (n = 3); Blank space indicates no survival.

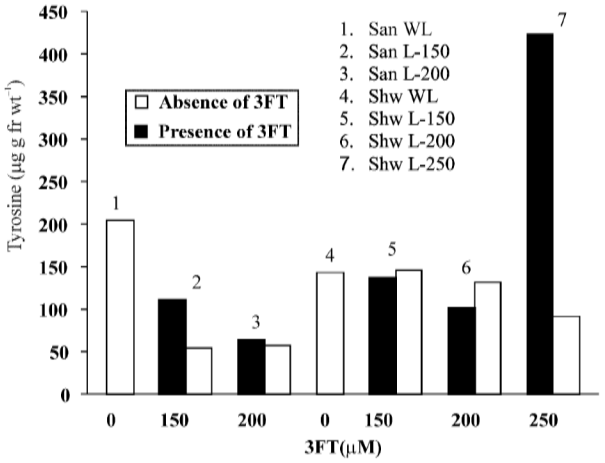

Figure 3. Tyrosine content in some 3FT-tolerant callus lines of opium poppy grown for 30 days in presence and absence of analogue stress.

cient to counterbalance the toxic effect of the analogue as has been reported for S-(2-aminoethyl)-L-cysteine resistant lines of carrot, potato, wheat and maize[17–20].

To test whether the analogue-tolerant trait selected at cellular level is retained through a regeneration cycle and expressed at whole plant level, plant regeneration was attempted from selected and non-selected lines in the presence and absence of 3FT stress imposed at various stages of organogenesis. As mentioned earlier, poppy calluses showed a high tendency to turn embryogenic and developed globular embryos all over their surface on PC medium (Figure 4 *a–c*). Further maturation of these somatic embryos did not occur on PC medium containing 0.46 μM Kn and 4.5 μM 2,4-D and they re-callused upon subculturing on fresh PC medium. For the complete embryo-to-plantlet conversion from selected and non-selected wild types, the calluses were transferred on PR medium containing 2.46 μM 2-iP and 0.57 μM IAA, but the regeneration response was poor. Continuous maintenance of these lines (more than 2 years) in the presence of the analogue (3FT) and/or 2,4-D present in the PC medium, was probably responsible for the poor regeneration response. Toxic antimetabolites (3FT in the present case) are known to adversely influence organogenesis by disrupting the hormonal balance and accumulation of toxic by-products (phenolics) in the cells[12]. Alternately, prolonged exposure to 2,4-D can also reduce the regeneration potential[10,21]. To dilute the effect of 3FT/2,4-D, the callus was transferred to MS basal medium for 5, 10, 15 and 20 days before periodically shifting to regeneration medium containing 2.46 μM 2-iP and 0.57 μM IAA. A minimum of 10 days of such intervening dilution pre-treatment dramatically improved the embryo conversion frequency from <5 to 63% and effectively released the regenerative tissues from the state of developmental block. Green organized meristematic sectors became visible within 10–12 and 15–18 days of transfer to PR medium in 'Sanchita' and 'Shweta' lines respectively (Figure 4 *d–f*). These meristemoids later developed into leafy shoots (6–8 shoots per callus piece) within the next 6–10 days (Figure 4 *g–i*). Individual shoots upon transfer to fresh PR medium, attained a mean height of 8.45 cm within four to five weeks. San L-200 showed the best shoot regeneration frequency of 80%. In comparison to cv. 'Sanchita', the 3FT-tolerant lines of cv. 'Shweta' were less regenerative; Shw L-250 being the poorest, showing regeneration in just 12–15% of the plated calluses. The degree of shoot regeneration response in the selected lines of both the cultivars in the presence of 75 to 125 μM 3FT corroborated with

Table 2. Concentration of total free amino acids as a function of culture age and 3FT stress in selected lines of cv. 'Shweta' of *P. somniferum*

		3FT concentration (μM)							
		0		150		200		250	
Culture age (days)	Line no.	GI	FAA	GI	FAA	GI	FAA	GI	FAA
15	Shw (W)	237.5 (± 19.09)	5.60 (± 0.14)	88.3 (± 1.53)	5.83 (± 0.19)	28.5 (± 3.05)	4.11 (± 0.39)	17.1 (± 3.05)	4.34 (± 0.16)
	Shw L-150	256.8 (± 15.63)	4.88 (± 0.0)	200.1 (± 7.28)	6.97 (± 0.35)	–	–	–	–
	Shw L-200	314.5 (± 22.17)	4.73 (± 0.45)	–	–	223.8 (± 11.58)	5.41 (± 0.0)	–	–
	Shw L-250	250.3 (± 15.91)	4.21 (± 0.45)	–	–	–	–	167.3 (± 1.82)	6.09 (± 0.26)
30	Shw (W)	640.8 (± 7.68)	5.97 (± 0.0)	228.1 (± 11.58)	4.24 (± 0.02)	67.5 (± 3.68)	3.18 (± 0.0)	49.9 (± 2.93)	3.37 (± 0.38)
	Shw L-150	511.1 (± 9.12)	4.16 (± 0.0)	633.8 (± 16.83)	3.37 (± 0.40)	–	–	–	–
	Shw L-200	653.3 (± 19.18)	4.60 (± 0.0)	–	–	714.4 (± 16.42)	6.32 (± 0.48)	–	–
	Shw L-250	460.2 (± 5.43)	4.52 (± 0.0)	–	–	–	–	496.3 (± 7.40)	7.89 (± 0.60)

W, Wild type; GI, Growth index {per cent fresh biomass increment over initial inoculum (200 mg)}; FAA, Free amino acids (mg g fr wt^{-1}); Values in parentheses indicate SE (n = 3); Blank space indicates transfers were not made.

the threshold analogue resistance of the selected lines. The embryogenic calluses of both the wild types did not show embryo maturation or shoot formation at any of the 3FT concentrations tested. Interestingly, it was observed that when 3FT was incorporated in the PR medium, the regeneration process in the resistant lines was less affected, but when 3FT was imposed during exposure to MS basal medium no organogenesis was observed in any of the lines and the globular-stage embryoids started showing signs of degeneration within 6–8 days of transfer. These results support previous reports that early events of organogenesis from globular to heart-shaped embryos are more sensitive to regulatory blocks than the later stages of regeneration[8,21]. Therefore, this simple strategy of removing the antimetabolite stress during early stages of organogenesis may prove useful in obtaining plant regeneration from stress-tolerant biochemical mutants in general. The regenerated shoots of the six selected lines developed normal roots (Figure 4 *j*) upon transfer to PM medium containing half strength MS salts (devoid of vitamin supplements), 1.0% agar and 0.57 μM IAA. The mean number of roots per shoot and their average length varied from 6 to 12 and 2.0 to 4.5 cm respectively. As has been reported earlier by Mathur *et al.*[16], incubation of the cultures at 10–12°C for 2–4 h daily during the initial ten days on the rooting medium enhanced the rhizogenic response to more than 85% besides improving the thickness of the regenerated roots.

The stability of the analogue resistance trait in the selected lines at the whole plant level was tested by plating leaf explants excised from regenerated plantlets of selected and wild-type lines on PC medium supplemented with 3FT at 0, 50, 75, 100, 125, 150, 200 and 250 μM concentration. Callus initiation from the cut ends of the leaf explants became evident in all selected lines within 8–10 days of plating. The frequency of response varied from 65 to 80% in different lines. The leaf explants of the wild type plantlets could not induce callus on medium fortified with 50 μM or more of 3FT. The persistence of the trait was best reflected in the lines San L-150 and San L-200 up to 150 μM level of stress and by Shw L-200 and Shw L-250 at 200 μM level of 3FT. The induced calluses of all the selected lines did not show any mortality or lag in growth and had growth index values from the third cycle onwards, comparable to parent cultures maintained throughout under 3FT stress of corresponding level.

The results obtained in this study indicate that *P. somniferum* is amenable for selection of amino acid analogue resistance at callus level. The availability of 3FT-resistant lines showing differential tolerance to analogue stress and higher tyrosine accumulation and availability in the free amino acid pool (particularly in case of line Shw L-250), provides an appropriate physiological background for genetic transformation using tyrosine/DOPA decarboxylase (*TyDC*) gene construct[19,20,22] for its preferential flux towards tyramine and then to (s)-reticuline synthesis. Though the 3FT-tolerant plantlets still await field transplantation for alkaloid profiling and progeny testing for stability, the stable expression of acquired trait under continued presence of the stress, away from the selection pressure and at whole plant, does suggest a mutant nature of these selected variants. The regenerated plant progeny upon field cultivation will also be utilized in poppy breeding programme involving a

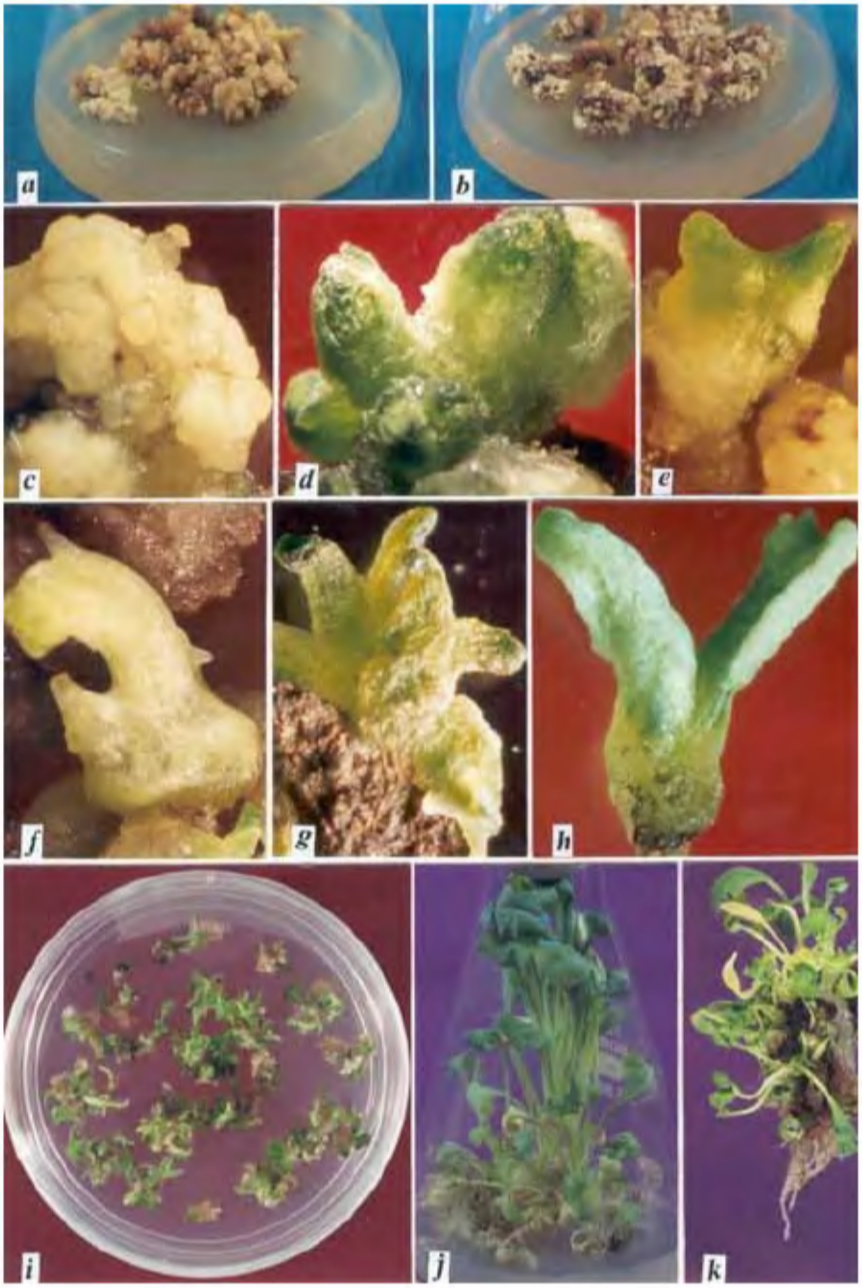

Figure 4. Plantlet regeneration via somatic embryogenesis in *P. somniferum*. Embryogenic callus of lines Shw L-200 (***a***) and San L-200 (***b***), and globular stage embryos after four weeks of growth on PC medium (***c***). ***d–i***, Somatic embryos at sequentially advancing stages of growth and maturation on PR medium. ***j***, Fully grown shoots of line San L-200 after six weeks of culture in the presence of analogue. ***k***, Rooted plantlets obtained on PM medium.

latex- and morphine-less variety 'Sujata' that has been recently developed at CIMAP[23] for EST analysis.

1. Verpoorte, R. and Memelink, J., Engineering secondary metabolite production in plants. *Curr. Opin. Biotechnol.*, 2002, **13**, 181–187.
2. Verpoorte, R. and Alfermann, A. W., In *Metabolic Engineering of Plant Secondary Metabolism*, Kluwer, Dordrecht, 2000.
3. Capell, T. and Christou, P., Progress in plant metabolic engineering. *Curr. Opin. Biotechnol.*, 2004, **15**, 1–7.
4. Mahmoud, S. S. and Croteau, R. B., Strategies for transgenic manipulation of monoterpene biosynthesis in plants. *Trends Plant Sci.*, 2002, **7**, 366–373.
5. Carrari, F., Urbanczyk-Wochniak, E., Willmitzer, L. and Fernie, A. R., Engineering central metabolism in crop species: Learning the system. *Met. Eng.*, 2003, **5**, 191–200.

6. Facchini, P. J. and Bird, D. A., Developmental regulation of benzylisoquinoline alkaloid biosynthesis in opium poppy plants and tissue cultures. *In Vitro Cell Dev. Biol. – Plant*, **34**, 69–79.
7. Facchini, P. J. and De Luca, V., Differential and tissue-specific expression of a gene family for tyrosine/dopa decarboxylases in opium poppy. *J. Biol. Chem.*, 1994, **269**, 26684–26690.
8. Park, S. and Facchini, P. J., *Agrobacterium*-mediated transformation of opium poppy, *Papaver somniferum*, via shoot organogenesis. *J. Plant Physiol.*, 2000, **157**, 207–214.
9. Park, S. and Facchini, P. J., *Agrobacterium rhizogenes*-mediated transformation of opium poppy, *Papaver somniferum* L., and California poppy, *Eschscholzia californica* Cham., root cultures. *J. Exp. Bot.*, 2000, **51**, 1005–1016.
10. Flick, C. E., Isolation of mutants from cell culture. In *Handbook of Plant Cell Culture, Techniques for Propagation and Breeding* (eds Evans, D. A. *et al.*), Macmillan, New York, 1983, vol. 1, pp. 393–441.
11. Maliga, P., Isolation and characterization of mutants in plant cell culture. *Annu. Rev. Plant Physiol.*, 1984, **35**, 519–542.
12. Negrutiu, I., Jacobs, M. and Caboche, M., Advances in somatic cell genetics of higher plants – The protoplast approach in basic studies on mutagenesis and isolation of biochemical mutants. *Theor. Appl. Genet.*, 1984, **67**, 289–304.
13. Murashige, T. and Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 1962, **15**, 473–497.
14. Rosen, H., A modified ninhydrin colorimetric analysis for amino acids. *Arch. Biochem. Biophys.*, 1957, **67**, 10–15.
15. Robins, E., The measurement of phenylalanine and tyrosine in blood. In *Methods of Biochemical Analysis* (ed. Glick, D.), John Wiley, New York, 1969, vol. 17, pp. 287–309.
16. Mathur, A. K., Patra, N. K. and Chauhan, S., *In vivo* and *in vitro* mutagenesis and search for downy mildew resistance in opium poppy (*Papaver somninferum*). In *Mutation Breeding of Oil Seed Crops* (eds Emano, E. and Ashri, A.), IAEA-TEC DOC-781, IAEA, Vienna, 1993, pp. 131–148.
17. Matthews, B. F., Shye, S. C. H. and Widholm, J. M., Mechanism of resistance of a selected carrot cell suspension culture to S-(2-aminoethyl)-L-cysteine. *Z. Pflanzenphysiol.*, 1980, **96**, 453–463.
18. Jacobsen, E., Isolation, characterization and regeneration of a S-(2-aminoethyl)-L-cysteine resistant cell line of a dihaploid potato. *J. Plant Physiol.*, 1986, **123**, 307–315.
19. Kumpaisal, R., Hashimoto, T. and Yamada, Y., Selection and characterization of S-(2-aminoethyl)-L-cysteine resistant wheat cultures. *J. Plant Physiol.*, 1988, **133**, 608–614.
20. Miao, S., Duncan, D. R. and Widholm, J. M., Selection of regenerable maize callus cultures resistant to 5-methyl-DL-tryptophan, S-(2-aminoethyl)-L-cysteine and high levels of L-lysine plus L-threonine. *Plant Cell Tiss. Org. Cult.*, 1988, **14**, 3–14.
21. Nessler, C. L., Somatic embryogenesis in the opium poppy *Papaver somniferum*. *Physiol. Plant.*, 1982, **55**, 453–458.
22. Facchini, P. J. and Park, S. U., Developmental and inducible accumulation of gene transcripts involved in alkaloid biosynthesis in opium poppy. *Phytochemistry*, 2003, **64**, 177–186.
23. Sharma, J. R., Lal, R. K., Misra, H. O., Naqvi, A. A. and Patra, D. D., Combating opium-linked global abuses and supplementing the production of edible seed and seed oil: A novel non-narcotic var. Sujata of opium poppy (*Papaver somniferum* L.). *Curr. Sci.*, 1999, **77**, 1584–1589.

ACKNOWLEDGEMENTS. Financial support from Council of Scientific and Industrial Research and Department of Biotechnology, New Delhi is acknowledged. R.K. thanks Dr C. K. John for useful suggestions on the manuscript. This is CIMAP publication no. 2002-56J.

Received 14 June 2004; revised accepted 6 October 2004

Genetic analysis of cotyledon derived regenerants of tomato using AFLP markers

Poonam Bhatia[1,*], Nanjappa Ashwath[1], Tissa Senaratna[2,3] and Siegfried L. Krauss[2,4]

[1]Primary Industries Research Centre, School of Biological and Environmental Sciences, Central Queensland University, Rockhampton, QLD 4702, Australia
[2]Kings Park and Botanical Gardens, Perth, WA 6005, Australia
[3]Soil Science and Plant Nutrition and [4]School of Plant Biology, Faculty of Natural and Agricultural Sciences, University of Western Australia, Crawley 6009, Australia

MS medium supplemented with 15 μM zeatin was used for direct shoot regeneration from cotyledonary explants. DNA samples obtained from the regenerated shoots and parent cotyledonary explants were subjected to amplified fragment length polymorphism (AFLP) analysis to examine genetic uniformity of tissue-cultured tomato plants. Eighty-five markers were generated using the primer pair M-CAC/E-ACT and M-CTC/E-ACT. Identical fingerprints were generated for each primer pair for the mother and the regenerated plants. AFLP markers proved to be an ideal tool for routine analysis of genetic fidelity of regenerated tomato plants. This is a report on evaluating the genetic fidelity of tissue culture tomato plants using AFLP.

SUSTAINABILITY of the micropropagation technique is dependent upon maintenance of the genetic fidelity of the regenerates. Changes occurring during propagation of tissue culture are either epigenetic or genetic[1]. Genetic changes are heritable and are called as somaclonal variations[1,2]. Somaclonal variations may result due to modifications in the chromosome number or methylation pattern, chromosome breaks, transposon activations, deletions, genome rearrangements, polyploidy or nucleotide substitutions[1,3,4]. The somaclonal variant frequency cannot be predicted and it depends on several factors, viz. species, explant type, and donor genotype. The other factors influencing genetic stability include composition of the culture medium, especially the concentration and type of plant growth regulators used, physical conditions and duration between successive subcultures[5]. Somaclonal variation can pose a severe threat to the genomic integrity of regenerated plants, which is particularly required during the genetic transformation experiments and to achieve genetic uniformity of the propagules. Somaclonal variation can either bring changes at the DNA level or it may induce changes in chromosome numbers. Early detection of the genetic stability provides the opportunity to reevaluate the propagation protocols, and the desired modification in the protocols could be utilized to

*For correspondence. (e-mail: p.bhatia@cqu.edu.au)