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Somatic hybridization of amino acid analogue-resistant cell lines of potato (*Solanum tuberosum* L.) by electrofusion

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Summary. Intraspecific somatic hybridization between amino acid analogue-resistant cell lines of potato (*Solanum tuberosum* L.) has been carried out following electrofusion of protoplasts. In initial analytical electrofusion experiments (1 mm electrode separation) optimal fusion conditions were determined by changing the fusion medium (addition of Ca and/or spermine) and the electrical parameters. Subsequently, in large scale experiments, cell suspension protoplasts of aec-1, a variant resistant to AEC, were fused with the same type of protoplasts of 5mt-26 or 5mt-27, both variants resistant to 5MT and cross-resistant to 3FT. After an extensive selection procedure only somatic hybrid lines of aec-1 + 5mt-26 were obtained. The resistance traits of aec-1 and 5mt-26 were expressed fully, indicating that the variant characters involved are transmitted dominantly. Quantitative examination of the free amino acid content revealed characteristics of both the parental cell lines in most of the somatic hybrids. However, initially selected double resistant colonies from fusions of aec-1 + 5mt-27 lines appeared not to be somatic hybrids.

Key words: Amino acid analogue-resistance – Electrofusion – Potato – *Solanum tuberosum* L. – Somatic hybridization

Introduction

Electric-field mediated protoplast fusion, a newly developed method, has recently received great attention (Zimmermann and Vienken 1982; Bates et al. 1983; Watts and King 1984; Bates 1985; Tempelaar and Jones 1985 a, b).

Initial experiments with this technique have demonstrated the practicality of electrofusion for large scale experiments and that electrofused heterokaryons are viable. Subsequently, successful regeneration of callus and shoots after electrofusion of nitrate reductase deficient mutants of *Nicotiana tabacum* (Kohn et al. 1985) and *N. plumbaginifolia* (Puite et al. 1985) has been reported. For practical applications to crop plant protoplasts, a large throughput of protoplasts fused under mild conditions and yielding high fusion frequencies are required. This has now been achieved for potato.

Ideally, the aim of this type of experiment is to produce only 1:1 (binary) heterologous fusions. Although it is theoretically possible (Zimmermann and Vienken 1982), this has not been achieved in the present practical systems. However, a favourable situation can be exploited, when protoplasts with different fusion responses such as leaf- and suspension protoplasts are available (Tempelaar and Jones 1985 b). In such cases, the fusion process can be directed towards the generation of 1:1 heterologous fusion products by careful selection of electrofusion system parameters. In a number of cases such as in this work, however, mutants will be available only from cells yielding protoplasts with similar fusogenic responses. Thus it has been necessary to carry out preliminary work with the protoplast partners to optimize electrofusion.

Well defined mutants are an important tool for genetic manipulation experiments, such as somatic hybridization. Work on somatic cell genetics of potato has been hampered so far by a scarcity of available mutants. Some lines, resistant to 5-methyltryptophan (5MT), an amino acid analogue of tryptophan, have been isolated by Carlson and Widholm (1978). These lines were used for studies on amino acid synthetic pathways, which did not involve fusion experiments. Recently, a number of potato variants were characterized, i.e. lines resistant to various amino acid analogues (Jacobsen et al. 1985; Jacobsen 1986). With the establishment of a suitable system for the culture and regeneration of potato cell suspension protoplasts (de Vries and Bokelmann 1986), we can examine whether these particular variants can be used as genetic marker in somatic hybridization experiments. As resistance to amino acid analogues is assumed to be transmitted dominantly or semi-dominantly (Maliga 1984), fusion

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Abbreviations: AEC = S-aminoethylcysteine, 3FT = 3-fluorotyrosine, 5MT = 5-methyltryptophan

of two variants, resistant to different amino acid analogues, should result in double resistant hybrid clones.

In this paper we describe electrofusion of protoplasts of amino acid analogue-resistant cell lines of potato, and the selection and characterization of somatic hybrid lines.

Materials and methods

Materials

In this study the amino acid analogue-resistant cell lines aec-1, 5mt-25, 5mt-26 and 5mt-27 were used. All were isolated in the diploid potato clone HH578, kindly supplied by Prof. G. Wenzel, Grünbach, West Germany. The cell lines 5mt-26 and 5mt-27 are resistant to at least 7.5 µM 5-methyltryptophan (5MT), an analogue of tryptophan. Both variants are cross-resistant to 100 µM 3-fluorotyrosine (3FT), an analogue of tyrosine, and to 250 µM parafluorophenylalanine (PFP), an analogue of phenylalanine (Jacobsen et al. 1985). The variant 5mt-25 is also resistant to 5MT and cross-resistant to 3FT and PFP (unpublished results). Aec-1 is resistant to at least 500 µM L-aminoethylcysteine (AEC) (Jacobsen 1985) and cross-resistant to PFP (unpublished results).

Culture conditions of callus and cell suspensions were as described by de Vries and Bokelmann (1986). The culture medium was MS (Murashige and Skoog 1962), supplemented with 2% sucrose, 5 mg/l NAA and 0.1 mg/l BAP, solidified with 8 g/l agar where relevant.

Growth experiments

Various growth experiments were carried out to select suitable fusion partners, to define criteria for the selection of somatic hybrids and to examine the growth characteristics of somatic hybrid cell lines. For these experiments, small pieces of callus (10–25 mg each, 3 pieces/petri dish Ø 5 cm) from the wild-type and one of the variants or from a variant and a somatic hybrid line were cultured on medium containing AEC, 5MT, 3FT, AEC + 5MT or AEC + 3FT. After four weeks of culture, increase in callus weight was determined and compared with the controls, i.e. callus on analogue free medium. For each treatment and the controls three petri dishes (Ø 5 cm) were used. Each experiment was repeated at least twice.

Preparation of protoplasts and electrofusion

Protoplasts were prepared from cell suspension cultures of aec-1, 5mt-26 and 5mt-27 according to de Vries and Bokelmann (1986). In addition, in some experiments protoplasts were treated with 2.5 mM spermine for 5–15 minutes and then washed in 0.5 M mannitol + 0.15–1.0 mM $CaCl_2$. In other experiments, spermine treatment was omitted. Before fusion the protoplasts of aec-1 and 5mt-26 or aec-1 and 5mt-27 were mixed together in the ratio 1:1. Various protoplast concentrations were used, ranging from $1–5 \cdot 10^6$ protoplasts/ml. For analytical purposes, wire electrodes were used (0.1 mm diameter platinum wires, 1 mm separation). The fusion response was monitored by light microscopy in samples containing about 400 protoplasts. For preparative fusion experiments, brass electrodes with 3 mm electrode separation between the strips were constructed, so that the electrode chamber contained a volume of about 0.1 ml. A fusion generator, delivering an alternating collecting field of upto 70 V AC at 1.5 MHz and fusion pulses upto 1000 V (10 µs – 1 ms) was developed by the Central Electronics Department of the University of Groningen. For details of the equipment see de Vries and Tempelaar (1986). Fusion was carried out in a laminar flow bench with constant microscopic observation.

Protoplast culture and selection of somatic hybrids

Protoplast culture was carried out as described previously (de Vries and Bokelmann 1986), using medium 8, containing 1 mg/l NAA and 1 mg/l 2.4D and as osmoticum 0.5 M mannitol. The protoplasts were plated at a density of $1–2 \cdot 10^5$ protoplasts/ml. Selective medium contained 100 µM AEC + 5 µM 5MT or 100 µM AEC + 100 µM 3FT. Protoplasts were either grown on this medium immediately after fusion or grown for one week on non-selective medium (m8), which subsequently was diluted with an equal volume (2 ml) of liquid m8 or with liquid m8, containing 200 µM AEC + 10 µM 5MT. Several weeks later the cultures were diluted with 'soft' culture medium (de Vries and Bokelmann 1986) in which 0.4% agarose (Sea Plack, FMC Corporation, Rockland, USA) was substituted for 0.4% agar. The cultures grown so far under selective conditions were diluted with soft culture medium to which the same concentrations of analogues had been added. The non-selective cultures were diluted either with non-selective soft culture medium or with selective culture medium.

After 1–2 months of culture, a part of the microcalli (Ø 1–2 mm) which had developed were picked up at random and transferred to non-selective solid callus culture medium (25 microcalli/petri dish Ø 10 cm). After further growth selection was carried out by growing small pieces of callus on culture medium containing 100 µM AEC + 5 µM 5 MT or 100 µM AEC + 100 µM 3FT. The selected double resistant colonies were further propagated on non-selective solid culture medium. After a prolonged period of culture in the absence of the antimetabolites the double resistance of the putative somatic hybrids was tested again by growing small pieces of callus (9 pieces/petri dish Ø 10 cm) on medium containing AEC (100 µM), 5MT (5 µM), 3FT (100 µM), AEC + 5MT (100 + 5 µM) or AEC + 3FT (100 + 100 µM). In a subsequent growth experiment the relative growth of one of the somatic hybrid lines and the parental cell lines were compared on medium containing AEC, 5MT or 3FT.

Amino acid composition

For the investigation of the free amino acid content, extracts of callus were obtained according to Bieleski and Turner (1966). The callus cultures had been grown for at least 5 weeks on non-selective culture medium and had been subcultured onto fresh medium 1–2 weeks before extraction. The amino acid composition was determined quantitatively by an amino acid analyzer (Kontron Liquimmat), as described previously (Jacobsen et al. 1985).

Results

Choice of fusion partners, definition of selection criteria

In amino acid analogue-resistant cell lines, cross-resistance, i.e. resistance to analogues other than the one on which initial selection took place, can occur, giving rise to selection problems when two of such variants are used as fusion partners (Cella et al. 1983).

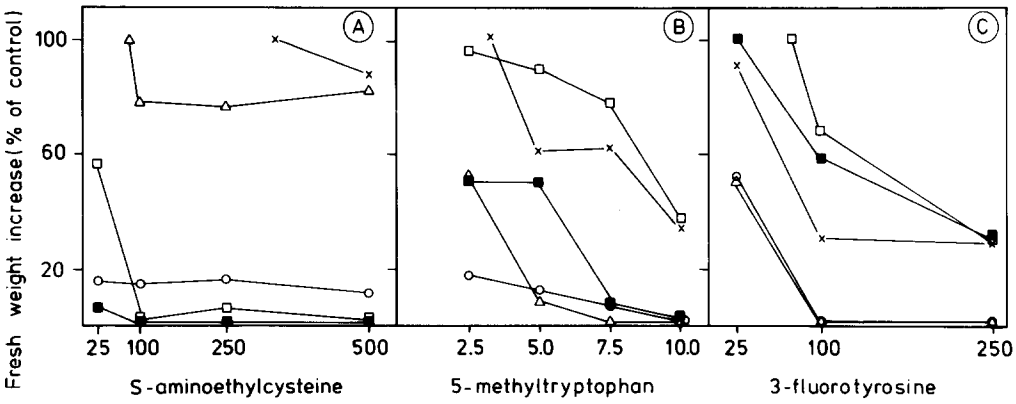

Fig. 1. Effect of S-aminoethylcysteine, 5-methyltrytophan and 3-fluorotyrosine on the relative growth of callus of the wild type HH578 (○) and the variants aec-1 (△), 5mt-25 (×), 5mt-26 (□) and 5mt-27 (■). Values are given as a percentage of the same line grown in the absence of the analogue

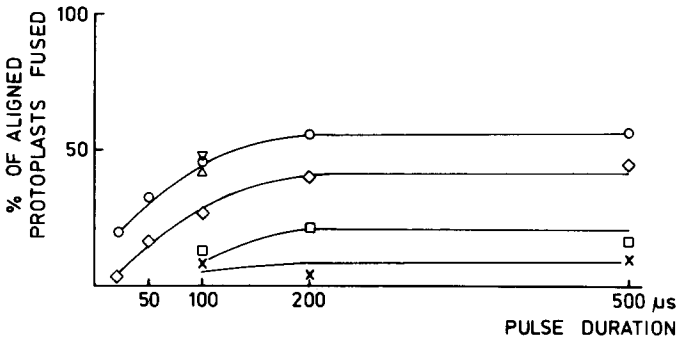

Fig. 2. Induced changes in pulse duration-fusion response curves for aec-1 cell suspension protoplasts, aligned in chains (n = 150–400 protoplasts per measuring point). × : protoplasts in 0.5 M mannitol, 2,000 V/cm; ○ : protoplasts treated with spermine in 0.5 M mannitol +0.15 mM $CaCl_2$, 2,000 V/cm; □ : protoplasts in 0.5 M mannitol +0.15 mM $CaCl_2$, 2,000 V/cm; ✧ : protoplasts treated with spermine in 0.5 M mannitol, 2,000 V/cm; △ : as b, 2,200 V/cm; ▽ : as b, 2,500 V/cm

In order to select suitable fusion partners from the potato variants, the cross-resistance of 5mt-25, 5mt-26 and 5mt-27 to AEC and of aec-1 to 5MT and 3FT was tested in various growth experiments. The variant 5mt-25 appeared to be cross-resistant to relatively high concentrations of AEC (Fig. 1 a). In contrast, callus growth of 5mt-26 and 5mt-27 was inhibited significantly by this analogue (Fig. 1 a). As can be seen, the variant aec-1 is very sensitive to 5MT (Fig. 1 b) and to 3FT (Fig. 1 c).

The clear differences in growth characteristics in the presence of AEC, 5MT and 3FT between aec-1 on one hand and 5mt-26 and 5mt-27 on the other hand, demonstrate that no problems due to cross-resistance will arise when using 5mt-26 and 5mt-27 as fusion partners of aec-1.

In accordance with previous results, callus of 5mt-25 proliferated well on double selective medium, i.e. medium containing AEC + 5MT (25 + 2.5; 100 + 5; 250 + 7.5; 500 + 10 µM, respectively) or AEC + 3FT (100 + 100 µM). The growth of aec-1, 5mt-26 and 5mt-27 was strongly affected when 100 + 5 µM AEC + 5MT or 100 + 100 µM AEC + 3FT supplemented the culture medium. Therefore, these concentrations were chosen for selection of somatic hybrids of aec-1 + 5mt-26 or aec-1 + 5mt-27.

Protoplast fusion and culture

The electrofusion frequency of small suspension culture protoplasts in non conducting media such as 0.5 M mannitol is 10–20% overall fusion of aligned protoplast and thus rather low (see Tempelaar and Jones 1985 a). With the *Solanum tuberosum* variants used, some repulsion of aligned protoplasts was observed when the fusion pulse was applied. Figure 2 indicates the positive effect of increasing the conductivity of the electrofusion medium by addition of Ca^{2+} ions. This increases the fusion frequency, an effect clearly illustrated in pulse duration-fusion response curves. An additional stimulation in fusion frequency was obtained by pretreatment with spermine. This compound was used by Chapel et al. (1984) to agglutinate protoplasts chemically instead of by an AC collecting field, before delivering the fusion pulse. In the present experiments, conditions were chosen so that agglutination did not take place before application of the AC field. After chain formation, there was a

marked increase in the area of contact after spermine treatment and repulsion of protoplasts did not occur.

The modified fusion conditions resulted in an overall fusion response of aligned protoplasts of around 50% at pulse voltages of 2000–2500 V/cm and 100 µs duration (Fig. 2). Agglutination was not permanent and after transfer to culture medium non-fused protoplasts separated.

As the overall fusion percentage of aligned protoplasts does not indicate the number of 1:1 fusions, a number of samples fused under the same conditions as in the preparative experiments were analyzed for frequency and composition of the fusion products. From the pooled results of this analysis it can be seen (Fig. 3) that about 60% of the fusion products are in the 1:1 category. As the tendency towards multifusions increases at high fusion frequencies (Tempelaar and Jones 1985 a), this figure represents the minimum for the proportion of 1:1 heterokaryons, so that at lower overall fusion frequencies a higher percentage of 1:1 fusion products will be present.

Preparative fusion experiments were carried out with several million protoplasts per experiment. In all, 5 experiments were performed with the variants aec-1 and 5mt-26. Protoplasts of 5mt-27 were used in only one experiment. Electrical fusion parameters were chosen using the results from the small scale analytical experiments. As the protoplasts were divided into several samples, slight adjustments could be made in the course of the experiments to get optimum yields in any particular instance.

After fusion, the protoplasts were cultured in liquid medium. Cell suspension protoplasts of aec-1 divided readily. Generally, the first division could be seen 2–3 days after isolation. Protoplasts of the 5MT resistant variants started dividing somewhat later (after 3–5 days).

The process of electrofusion did not seem to influence cell wall regeneration and cell division. No differences between the electrically treated protoplasts and the non-treated controls (heterologous mixtures and cultures of parental cell lines) were observed. After one week a plating efficiency up to 10% was obtained, which is as high as in the wild type cultures (de Vries and Bokelmann 1986).

Selection of somatic hybrids

On culturing protoplasts after fusion, an unexpected phenomenon was found. The addition of AEC+5MT (100+5 µM) or AEC+3FT (100+100 µM) to the liquid or soft culture medium did not influence the development of the protoplast cultures. No differences were observed between the cultures of fused protoplasts grown in selective medium and grown in non-selective medium. Even for the cultures of the heterologous mixtures and the parental cell lines, the amino acid analogues at the concentrations used did not inhibit growth. This was probably due to the relatively high density in which the protoplasts were plated, causing mutual interaction between the cells and subsequent growth.

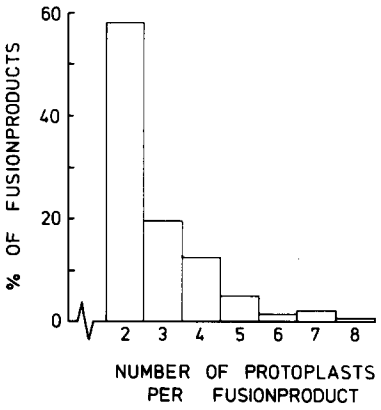

Fig. 3. Yield of fusion products according to their composition. Protoplasts treated with spermine, in 0.5 M mannitol + 0.15 mM $CaCl_2$. Fusion pulse 100 µs, 3 pooled samples with 2,000, 2,200, 2,500 V/cm, as in Fig. 2

After further culture on solid medium, the growth of hundreds of protoplast-derived calli from the fusion experiments and the controls were tested on medium containing AEC+5MT or AEC+3FT (Table 1). At this time selection was successful. From 423 regenerated calli after fusion of aec-1 and 5mt-26 protoplasts, 18 proliferated well on both double selective media. They could be distinguished clearly by their growth and their colour (Fig. 4). From 124 protoplast-derived calli of aec-1 + 5mt-27, 5 proliferating lines were detected on the double selective media. However, their growth rate was lower than for the other combination. No growth was observed in protoplast-derived calli of the parental cell lines and the heterologous mixtures (Table 1).

Further growth experiments

In 8 of the 18 selected colonies of aec-1 + 5mt-26 the double resistance to AEC + 5MT and AEC + 3FT appeared to be stable after prolonged culture (1–3 months) on non-selective medium. After this period, these 8 lines also proliferated well on medium containing the single amino acid analogues. From the 10 remaining lines 6 grew poorly on control medium, so that no clear conclusions could be made. The other 4 lines showed growth characteristics similar to aec-1 or 5mt-26 by growing well on medium containing AEC and being sensitive to 5MT and 3FT or vice versa. The

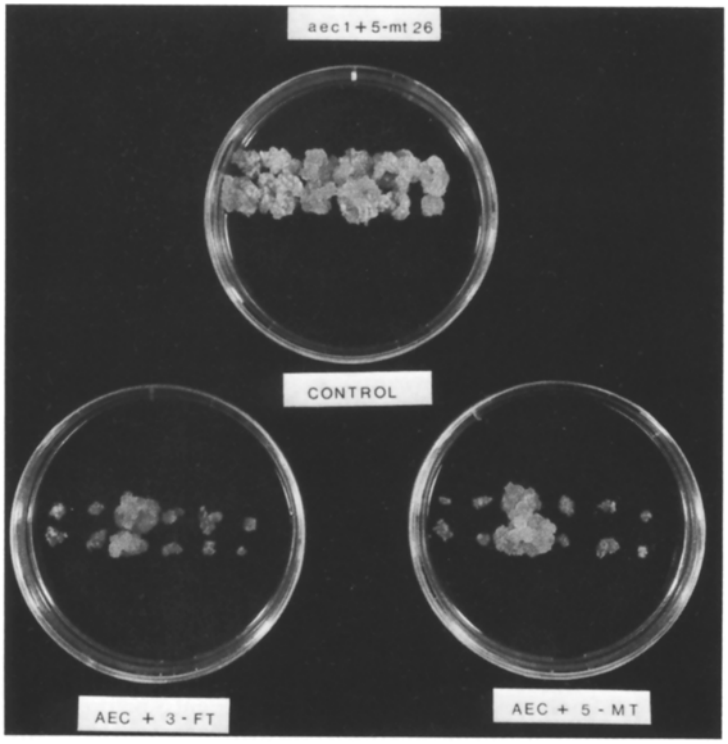

Fig. 4. Selection of somatic hybrids of aec-1 + 5mt-26. One hybrid colony can be distinguished from 5 non-hybrid colonies by its growth on medium containing 100 μM AEC + 100 μm 3 FT (*left*) or 100 μm AEC + 5 μM 5MT (*right*). On control medium (*upper row*) all the colonies proliferated well

Table 1. Fusion and control experiments with aec-1, 5mt-26 and 5mt-27 protoplast-derived calli

Cell type	No. of colonies tested on double selective medium	No. of colonies selected on double selective medium
aec-1	97	0
5mt-26	250	0
5mt-27	14	0
aec-1 + 5mt-26 electrically treated	423	18
aec-1 + 5mt-26 mixtures	202	0
aec-1 + 5mt-27 electrically treated	124	5
aec-1 + 5mt-27 mixtures	59	0

5 selected protoplast-derived colonies of aec-1 + 5mt-27 had the resistance traits of aec-1.

The growth characteristics of one of the somatic hybrid lines of aec-1 and 5mt-26, line 33, are shown in Fig. 5a, b and c. It can be seen that its growth is comparable to that of aec-1 on medium supplemented with AEC (Fig. 5a) and to 5mt-26 on medium, containing 5MT (Fig. 5b) or 3FT (Fig. 5c), indicating that each resistance is fully expressed.

Free amino acid content

In Fig. 6 levels are presented of amino acids related to the analogues to which aec-1 and 5mt-26 are resistant and/or in which differences between the variants were observed.

The variant aec-1 is characterized by a relatively high level of phenylalanine. In addition, the content of tryptophan and of tyrosine is increased (Fig. 6). In agreement with earlier results (Jacobsen et al. 1985), in 5mt-26 the amount of free tyrosine is high when compared to the wild type. The amino acids phenylalanine, tyrosine and tryptophan share the shikimate biosynthetic pathway. Lysine is synthesized via the aspartate pathway.

In the somatic hybrid lines the composition of the free amino acid content appeared to be highly variable. An elevated level of tyrosine as well as an increased content of phenylalanine was found in most of the lines. When elevated, the content of phenylalanine was never higher than the content of tyrosine, as is the case in aec-1. The level of tryptophan also increased. However, in one or more extracts of all these lines, values similar to the wild type were measured as well. In the remaining somatic hybrid lines the content of the amino acids studied was never found to be increased. Although the total free amino acid content was relatively high when compared to the wildtype (Fig. 6), no specific amino acids had accumulated.

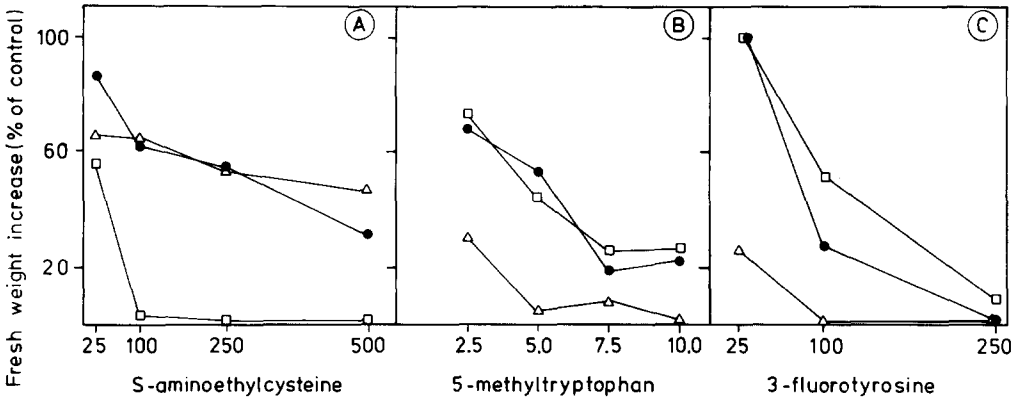

Fig. 5. Effect of S-aminoethylcysteine, 5-methyltryptophan and 3-fluorotyrosine on the relative growth of callus of aec-1 (△), 5mt-26 (□) and somatic hybrid 33 (●). Values are given as a percentage of the same line grown in the absence of the analogue

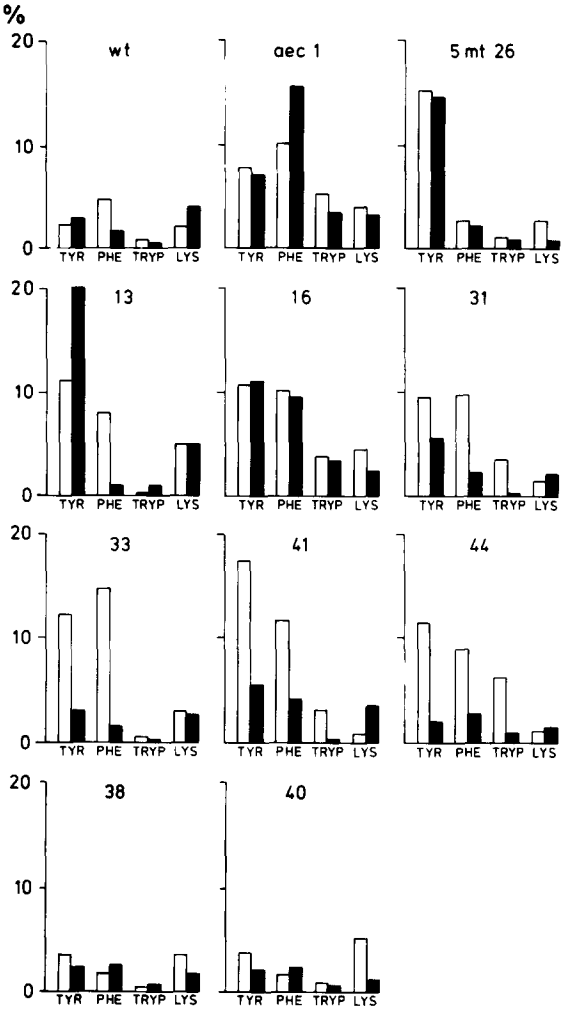

Fig. 6. Free phenylalanine (phe), tyrosine (tyr), tryptophan (tryp), and lysine (lys) content (% of total amino acid content) in extracts of callus of wild type HH578, aec-1, 5mt-26 and 8 somatic hybrid lines of aec-1 + 5mt-26. Values of two independent extracts are given (□ ■). The total free amino acid content (nMol/g callus) was: for wt 2,216 and 2,634, for aec-1 6,800 and 8,312, for 5mt-26 3,581 and 5,351, for 13 4,058 and 7,165, for 16 5,736 and 4,149, for 31 3,984 and 14,972, for 33 1,731 and 3,299, for 41 6,299 and 3,430, for 44 3,101 and 5,248, for 38 8,517 and 6,860 and for 40 4,621 and 9,418

Discussion

Aec-1 as a fusion partner for 5mt-26 and 5mt-27

Our results (Fig. 1 a, b and c) emphasize the importance of examining the growth characteristics of amino acid analogue-resistant variants before using them in somatic hybridization experiments. The potato variants 5mt-26 and 5mt-27 could be used as fusion partner for aec-1. Due to its cross-resistance to AEC, 5mt-25 could not be used.

Electrofusion can be applied for large scale somatic hybridization of potato cell suspension protoplasts

Using electrofusion rather than chemical fusion has some interesting advantages.

First, changes in the fusion medium and electrical parameters can be tested with a good measure of accuracy to rapidly develop optimal yields of the desired fusion products. As shown in Figs. 2 and 3, with fusion pulses of 100 μs duration at 2000–2500 V/cm, about 60% of the fusion products will be binary fusions, at 40–50% overall fusion frequency of aligned protoplasts. Depending on the proportion of aligned protoplasts, which can be as high as 80–90%, it is estimated that at best 25% of the total number of protoplasts treated will take part in 1:1 fusion events. The proportion of protoplasts involved in heterologous fusion would be one half of this percentage if aggregation is at random. This would result in about 6.7% hybrid cells. Improvement of these frequencies may be achieved by reductions of multifusions. This could be done by keeping the chain length down to only a few protoplasts (Tempelaar and Jones 1985a), but would be rather awkward for large scale experiments, since it would require short alignment times that would leave many protoplasts unaligned. Alternatively, sensitization of one of the partners could facilitate more directed 1:1 fusions, as described previously for experiments on leaf-suspension protoplasts (Tempelaar and Jones 1985b). Supposing that the 423 regenerated calli of aec-1 + 5mt-26 are representative for the population of protoplasts which have been electrically treated, the fusion percentage is 1.9 (8 somatic hybrids out of 423 regenerated calli). This is substantially lower than the theoretical maximum percentage of 6.7. However, heterokaryon formation does not always result in nuclear fusion, which might explain the discrepancy.

Second, in preparative electrofusion experiments samples can be subdivided and monitored consecutively before fusing the

next one. This allows adjustments to fusion to be made during the course of an experiment.

Third, although certain chemical additions have been made to the 0.5 M mannitol electrofusion medium, it has not proved necessary to remove these compounds, so that after fusion the contents of the fusion chamber can be diluted directly with the culture medium.

Resistance to AEC, 5MT and 3FT is transmitted dominantly

In the present experiments, somatic hybrids were selected a relatively long time after fusion when compared to other similar experiments (White and Vasil 1979; Harms et al. 1981; Cella et al. 1983). After pre-growing the protoplast-derived calli on non-selective solid culture medium, the screening of hundreds of calli on double selective medium resulted in the selection of double resistant cell lines. The possibility of spontaneously emerging double resistant colonies from one of the parental cell lines is rendered very unprobable by the control experiments (Table 1).

No hybrid calli of aec-1 + 5mt-27 were obtained. Discrimination between double and single resistant calli was much more difficult than in the other combination (aec-1 + 5mt-26). Besides, protoplasts of 5mt-27 appeared to have less regenerating capacity than the protoplasts of the other variants. From the fact that the 5 initially selected seemingly double resistant colonies obtained after fusion of aec-1 and 5mt-27 protoplasts all showed in subsequent experiments the resistance traits of aec-1 only, it might be concluded that in this type of experiment preferentially protoplasts of aec-1 (or homologous fusions of aec-1) regenerated into microcalli.

Our results indicate that in the somatic hybrids of aec-1 + 5mt-26 the amino acid analogue-resistances of both parental cell lines are fully expressed, suggesting dominance of the characters involved. This is in agreement with previous results of Harms et al. (1981, 1982).

It is not clear why the double resistance was maintained in only 8 of the 18 selected cell lines of aec-1 + 5mt-26. Possibly, some of the initially selected lines originated from more than one cell, thus consisting of mosaics of cells of both the parental cell lines, which, through mutual interaction, resulted in double resistant colonies. However, none of the protoplast-derived calli of the heterologous mixtures ever showed similar resistances on the double selective media.

It is also well known that cytological changes can occur in cultured cells. Therefore, the question could be raised whether in the somatic hybrid lines, chromosomes are eliminated, including by chance a chromosome responsible for one of the particular resistances. Examination of the chromosome numbers could not answer this question, as numbers were highly variable and values over 96 were observed in the parental cell lines as well as in the double resistant colonies (M. Ferwerda, personal communication). Chromosome elimination of one of the fusion partners may occur more extensively after somatic hybridization of distantly related species (Gleba and Sytnik 1984). However, elimination of chromosomes has been reported in intraspecific somatic hybrids of *Daucus carota,* but this was not accompanied by loss of one of the parental traits (Harms et al. 1981; Cella et al. 1983).

The present experiments have shown that aec-1 and 5mt-26 are useful genetic markers. In future these variants will be used for interspecific somatic hybridization and studies of partial genome transfer.

Somatic hybrids cannot be distinguished by their free amino acid composition

Until now it has not been clear what mechanisms are responsible for the accumulation of amino acids in aec-1 and 5mt-26, as the biochemical background of the mutations involved are so far not understood (Jacobsen et al. 1985; Jacobsen 1986). Overproduction of amino acids not related to the analogue(s) for which selection has been accomplished, as is the case in aec-1, has been reported before for carrot variants (Cella and Iadarola 1983).

In the somatic hybrid lines the variability in the total amount, as well as the composition, of free amino acids might result from variation of the physiological state of the callus cultures. On the other hand, enzymatic interactions due to the presence of both the parental genomes might also play a role. The combined occurrence of characteristics of both the parental cell lines is in accordance with the double resistance of the hybrid colonies.

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References

Bates GW, Gaynor JJ, Shekhawat NS (1983) Fusion of plant protoplasts by electric fields. Plant Physiol 72:1110–1113

Bates GW (1985) Electrical fusion for optimal formation of protoplast heterokaryons in *Nicotiana.* Planta 165:217–224

Bieleski RL, Turner NA (1966) Separation and estimation of amino acids in crude extracts by thin layer electrophoresis and chromatography. Anal Biochem 17:278–293

Carlson JE, Widholm JM (1978) Seperation of two forms of anthranilate synthetase from 5-methyltryptophan susceptible and -resistant cultured *Solanum tuberosum* cells. Physiol Plant 44:251–255

Cella R, Iadarola P (1983) Characterization of carrot cell lines resistant to 5-methyltryptophan obtained by irradiating suspension cultures with UV-light. Plant Sci Lett 29:327–337

Cella R, Carbonera D, Iadarola P (1983) Characterization of intraspecific somatic hybrids of carrot obtained by fusion of iodoacetate-inactivated A2CA-resistant and sensitive protoplasts. J Plant Physiol 112:449–457

Chapel M, Teissie J, Alibert G (1984) Electrofusion of spermine-treated plant protoplasts. FEBS Lett. 173:331–336

Gleba YY, Sytnik KM (1984) Protoplast fusion. Genetic engineering in higher plants. Springer, Berlin Heidelberg New York

Harms CT, Potrykus I, Widholm JM (1981) Complementation and dominant expression of amino acid analogue resistance markers in somatic hybrid clones from *Daucus carota.* Z Pflanzenphysiol 101:377–390

Harms CT, Oertli JJ, Widholm JM (1982) Characterization of amino acid analogue resistant somatic hybrid cell lines of *Daucus carota* L. Z Pflanzenphysiol 106:239–249

Jacobsen E (1986) Isolation characterization and regeneration of a S-(2-aminoethyl)cysteine resistant cell line of dihaploid potato. J Plant Physiol 123:307–315

Jacobsen E, Visser RGF, Wijbrandi J (1985) Phenylalanine and tyrosine accumulating cell lines of a dihaploid potato selected by resistance to 5-methyltryptophan. Plant Cell Rep 4:151–154

Kohn H, Schieder R, Schieder O (1985) Somatic hybrids in tobacco mediated by electrofusion. Plant Sci Lett 38:121–128

Maliga P (1984) Isolation and characterization of mutants in plant cell culture. Ann Rev Plant Physiol 35:519–542

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

Puite KJ, Wikselaar P van, Verhoeven H (1985) Electrofusion a simple and reproducible technique in somatic hybridization of *Nicotiana plumbaginifolia* mutants. Plant Cell Rep 4:274–276

Tempelaar MJ, Jones MGK (1985a) Fusion characteristics of plant protoplasts in electric fields. Planta 165:205–216

Tempelaar MJ, Jones MGK (1985b) Directed electrofusion between protoplasts with different responses in a mass fusion system. Plant Cell Rep 4:92–95

Vries SE de, Bokelmann GS (1986) Regeneration of callus and plants from cell suspension protoplasts of dihaploid potato (*Solanum tuberosum* L.). J Plant Physiol 122:199–203

Vries SE de, Tempelaar MJ Electrofusion and analysis of somatic hybrids. In: Bajaj YPS (ed) Biotechnology of plant improvement, Vol III, Potato. Springer, Berlin Heidelberg New York (in press)

Watts JW, King JM (1984) A simple method for large scale electrofusion and culture in plant protoplasts. Biosci Rep 4:335–342

White DWR, Vasil IK (1979) Use of amino acid analogue resistant cell lines for selection of *Nicotiana sylvestris* somatic cell hybrids. Theor Appl Genet 55:107–112

Zimmermann U, Vienken J (1982) Electric field-induced cell-to-cell fusion. J Membr Biol 67:165–182