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ALUMINIUM-RESISTANT MUTANTS SELECTED FROM PLANT CELL CULTURES

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ABSTRACT

Mutants resistant to aluminium were isolated from cultured cells of *Nicotiana plumbaginifolia* Viv. using carefully designed selection strategies. To simulate the mineral environment of aluminium toxic soils in cell culture, phosphate, calcium, and pH were lowered to 10 μM, 0.1 mM, and 4.0 respectively, and iron was supplied in unchelated form. All the medium modifications were essential to maximise the expression of aluminium toxicity. In a one-step, direct selection method, cells were plated onto medium containing 600 μM aluminium. An alternative two-step rescue selection method involved culturing suspensions in medium containing 600 μM aluminium for 10 days, then plating onto standard medium for recovery of survivors. Cell density and aggregate size was controlled to inhibit background growth of wild-type cells completely. A total of 246 aluminium-resistant variants were isolated from non-mutagenised homozygous diploid cell cultures, 29 using the direct method and 217 using the rescue method. Resistance was retained in 119 of these variants after each was individually cloned and reselected from single cells. After six to nine months growth in the absence of aluminium, resistance was retained in callus of 67 of the reselected variants. Complete plants were regenerated from 40 of these and all transmit aluminium resistance to their seedling progeny (selfed and backcrossed) in segregation ratios expected for a single dominant mutation.

KEYWORDS

Aluminium toxicity, cell selection strategies, inheritance of aluminium resistance, *Nicotiana plumbaginifolia*, variant characterisation.

INTRODUCTION

One of the most serious environmental stresses limiting the growth of crop plants is aluminium toxicity, a problem widespread in acid soils throughout the world (National Academy of Sciences, USA, 1977). Although the ability to select and breed plants with aluminium resistance is well established (Foy, 1984) the lack of sufficiently resistant germplasm in most crops is a major limitation. As an alternative to screening large germplasm collections in the hope of finding aluminium-resistant accessions, we have developed cell culture methods for the selection of aluminium-resistant mutants.

The success of this cell selection approach depended on the development of stringent aluminium selection strategies before selection experiments began. Important features included the design of a polyurethane culture system, the modification of the inorganic composition of the culture medium, and aluminium dose-response experiments. We summarise here the development of these selection strategies, as well as the selection and characterisation of aluminium-resistant variants from cell culture.

EXPERIMENTAL APPROACH

Polyurethane support system

Aluminium toxicity studies need an acidic pH at which the gelling properties of agar are inhibited. Since plating cells onto a selection medium is desirable for the isolation of variants (Meredith, 1984), it was important to design an alternative to agar for supporting cultured plant cells.

Cells were therefore cultured on Whatman No. 2 filter paper supported by polyurethane foam saturated with liquid medium (Conner and Meredith, 1984). This culture system allowed plated plant cells to grow at pH 4.0, and permitted higher growth rates than conventional agar based medium (Conner and Meredith, 1984). Furthermore, it enabled spent medium to be easily squeezed from the polyurethane so that pH could be estimated after a culture period. The ability to monitor pH was important because pH control is critical in aluminium toxicity experiments, and cultured cells rapidly change the pH of their medium (Conner and Meredith, 1984).

Simulating aluminium soils

Aluminium selection strategies in plant cell culture should closely mimic conditions existing in the soil environment to be of agricultural use (Meredith, 1984). We therefore modified the inorganic composition of the culture medium to simulate conditions existing in aluminium toxic soils (Conner and Meredith, 1985a). The phosphate concentration was reduced from 1250 μM to 10 μM, and pH from 5.8 to 4.0. Without these changes, aluminium precipitated in the culture medium and was not toxic to plated cells of *Nicotiana plumbaginifolia* Viv. A reduction in the calcium concentration from 3.0 mM to 0.1 mM, and the use of unchelated iron enhanced the expression of toxicity. Similar medium modifications are also necessary for the expression of aluminium toxicity to whole plants in solution culture (Foy, 1976).

Of the four medium changes only the reduced phosphate concentration limited the growth of plated *N. plumbaginifolia* cells (Conner and Meredith, 1985a). This was partly overcome by preloading the cells with phosphate before each experiment and via replenishing the low phosphate concentration by transferring the filter paper with adhering cells to fresh medium every second day. This also re-established pH 4.0 which prevented a continual upward drift in medium pH and maintained aluminium in a soluble form.

Aluminium dose-response experiments

Before aluminium-resistant variants were selected and characterised from cell culture, aluminium dose-response experiments were performed to optimise conditions that would distinguish between aluminium-sensitive wild-type cells and aluminium-resistant variants. Strict control of inoculum mass was important for uniform aluminium toxicity to be expressed in *N. plumbaginifolia* cell cultures (Conner and Meredith, 1985b). Higher cell densities or the presence of cell aggregates in plated cells reduced aluminium toxicity. Larger callus inocula resulted in similar responses. These effects were minimised by plating 10^5 cells (from which cell aggregates were sieved out) evenly over each filter paper, or by smearing callus over the filter paper. Once these precautions were taken plated cells, callus cultures, *in vitro* grown shoots, and seedlings of *N. plumbaginifolia* all showed a similar response to aluminium — total inhibition of growth at or above 600 μM. This consistent response in developmentally different plant systems reflects the fundamental cellular basis of aluminium toxicity (Haug, 1984).

Selection strategies

Aluminium-resistant variants from *N. plumbaginifolia* cell cultures were selected using two strategies:
- A one-step direct method in which cells were plated onto the modified medium supplemented with 600 μM aluminium.
- A two-step rescue method in which cell suspensions were cultured for 10 days in the modified medium supplemented with 600 μM aluminium, and then

plated onto standard medium (no aluminium) for the recovery of survivors.

Strict control of cell density and aggregate size was critical to inhibit the background growth of wild-type cells totally and therefore improve the stringency of selection.

RESULTS AND DISCUSSION

Selection strategies

The rescue selection approach proved to be vastly superior to the direct method for selecting aluminium-resistant variants (Conner and Meredith, 1985b). The direct selection strategy was laborious and slow. The filter paper with adhering cells under selection had to be repeatedly transferred to fresh medium to restore the initial pH of 4.0 and replenish the low phosphate supply. Furthermore the resistant variants remained very small after three months selection. In contrast, the rescue selection strategy required medium transfer for only 10 days incubation in suspension culture, and all variants were apparent within three weeks after plating the cells. The rescue approach was also more stringent with complete inhibition in the background growth of wild-type cells against which isolated colonies of aluminium-resistant variants could be easily identified.

Selection and characterisation of variants

Two hundred and forty six aluminium-resistant variants were isolated from non-mutagenised homozygous diploid cell cultures of *N. plumbaginifolia*; 29 via the direct method and 217 via the rescue method (Conner and Meredith, 1985b). Each of these variants was individually cloned and reselected from single cells to eliminate the possibility that they may have been chimeras of aluminium-resistant and wild-type cells. This failed for 127 (52%) variants, indicating they were either escapes through the initiation selection, or the result of a short term acclimatisation to aluminium stress that was lost during a brief culture period in the absence of aluminium. All the 119 variants cloned and reselected from single cells were maintained as callus in the absence of aluminium for six to nine months, then rechallenged with Al (Conner and Meredith, 1985c). Resistance was no longer expressed in 48 of these variants, but was retained in the remaining 67 (four variants were lost through contamination or senescence). The unstable nature of aluminium resistance in the 48 variants was attributed to a transient physiological adaptation to aluminium stress. This was lost only after extended culture in the absence of selection pressure (Conner and Meredith, 1985c). There was no association between the degree of aluminium resistance and callus growth in the absence of aluminium (Conner and Meredith, 1985c). This suggests that resistance is not detrimental in the absence of aluminium stress and not a consequence of increased overall vigour.

Expression and inheritance in seedlings

Fertile plants were regenerated from only 25 of the 48 variants with unstable aluminium resistance. As expected,

they all failed (with one exception) to transmit aluminium resistance to their seedling progeny. Resistance in the one variant was attributed to a somaclonal genetic event that occurred during the plant regeneration phase, independent of the initial cell selection (Conner and Meredith, 1985c). Fertile plants were regenerated from only 40 of the 67 stable variants, all of which transmitted aluminium resistance to their sexual progeny. In all instances the selfed and backcrossed seedling progeny segregated in the ratios expected for a single dominant mutation (Conner, 1985).

Many of the plants regenerated from aluminium-resistant variants showed greatly reduced fertility. Complete male sterility was evident in four mutants, with viable seeds being obtained only after backcrossing to the wild-type as a female parent (Conner, 1985).

A large proportion (31 out of 40) of the aluminium-resistant mutants also segregated for seedling lethality, even in the absence of aluminium (Conner, 1985). This was attributed to the segregation of recessive lethal mutations (somaclonal events), independent of aluminium resistance, that arose during the cell culture phase (Conner and Meredith, 1985c). In seven of the mutants the seedling lethality cosegregated with aluminium resistance, suggesting linkage between these two traits.

Poor regeneration, infertility, and segregation of lethality problems were more severe when the direct selection strategy (i.e. long term selection) was used, or when selection was applied to older cell cultures (Conner, 1985; Conner and Meredith, 1985c). This illustrates a further advantage of the short term rescue selection strategy.

CONCLUSION

Mutants with increased resistance to aluminium can be selected from cell cultures of *N. plumbaginifolia*. This species was carefully chosen since it is one of the most attractive model systems for plant cell genetics. The species allowed techniques to be developed and large scale cell selection and characterisation to be performed in minimum time. Although *N. plumbaginifolia* is not a crop plant, the methodology developed is directly applicable to any crop in which the necessary cell culture manipulations can be performed.

This study also clearly demonstrated the need to select a large number of variants in cell selection experiments. For the successful integration of cell selection into plant breeding programmes, it is important that a genotype not only gains the single selected trait, but that it retains all (or the majority of) its former agricultural attributes. The problems associated with unstable resistance, poor regeneration, infertility, and segregation of recessive lethals in selected variants emphasises the importance of isolating a large number of variants in short term selection experiments (such as the rescue selection strategy), from recently initiated cultures. A large collection of independently selected mutants from cell culture also permits comparisons to be made between potentially

different mutant genes for different levels of resistance and/or undesirable pleiotropic effects.

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SYMPOSIUM DISCUSSION

Dr E.J. Walsh, University College, Dublin
  Have these plants been evaluated in soil with high levels of aluminium?
  How did you know that the plants which you have here are not just suffering from deficiency levels of P.N.K.?
Conner
  Yes, I have tried to grow some of these in the soil that is common in the South Island high country about 100 miles inland from here. Unfortunately, no matter whether I germinated seedlings directly in the soil, or transplanted seedlings, I have seen no difference

between my mutants and the wild type. While that is disappointing, this soil does have a very high level of manganese as well as aluminium and quite possibly the plants are dying of manganese toxicity so we are trying to find a soil without manganese toxicity and with several levels of aluminium.

The second question: although there was reduced growth due to the low level of phosphate, there was still quite sufficient growth to overcome the problems that might arise from phosphate deficiency, and not aluminium toxicity. But there again, it is quite possible that some of these variants may have both aluminium resistance and phosphate deficiency.

Dr H.A. Eagles, Plant Physiology Division, DSIR

Does aluminium resistance occur naturally within the *Nicotiana?*

Conner

I'm not aware of any examples in *Nicotiana* but it is known in quite a few other species.

Dr M.J. Carson, Forest Research Institute

You seem to have developed a system that could be used very effectively for screening. The screening of known resistance could verify your technique, in relation to the problem with other nutrient deficiencies.

Conner

You could not simply just grow seedlings on the polyurethane support, and look for resistant individuals. If you start growing seeds as big as a carrot even, as the root emerges the shoot starts getting away from the medium, and you can lose contact with the medium. You have to use a fairly small seeded species for this system to work — *Nicotiana* is very small seeded.