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INVITED REVIEW

The selection and uses of plant tissue cultures resistant to toxic compounds

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Abstract Beginning in 1970, *in vitro* selection for resistance to various toxic compounds has produced many plant cell lines useful for studying biochemical pathways, altering overall cell metabolism and growth, and regenerating plants with desirable traits. Some toxin-resistant lines have been used to elucidate the genetic and biochemical mechanisms plant cells use to adapt, including gene amplification. Other resistance traits have been used for the selection of protoplast fusion hybrids or to develop selectable markers for nuclear or plastid transformation through cloning the corresponding genes and promoters.

Keywords Allyl alcohol resistance · Amino acid analog resistance · Chlorate resistance · Herbicide resistance · Streptomycin resistance

Introduction

Plant cell and tissue cultures have many uses, including selection for cell lines resistant to various inhibitory substances. The system can be efficient and effective since millions of cells can be subjected to a toxic substance, which enables the selection of resistant cells that arise at very low frequencies, as one would expect for mutations. Such selected lines can then be used for many purposes, including biochemical genetic studies to unravel pathways and controls, regeneration into whole plants that are resistant to herbicides or contain increased levels of amino acids, and cloning of genes that cause resistance, which can then be used as selectable markers for selecting transformants and protoplast fusion hybrids.

These studies have documented a number of resistance mechanisms, including the following: (1) generation of an insensitive target site, (2) degradation of the toxic compound, (3) increase in the target site due to gene amplification or higher transcript levels through higher transcription or messenger RNA (mRNA) stability, or (4) decreased uptake of the toxic compound. In this review article, each of these mechanisms will be examined by highlighting one or more useful examples.

Threonine and Streptomycin Resistance

The first reports of the selection for resistance with plant tissue cultures used the amino acid threonine and tobacco (*Nicotiana tabacum*) cells (Heimer and Filner 1970) and the antibiotic streptomycin and petunia (*Petunia hybrida*) cells (Binding *et al.* 1970). Threonine inhibits cell growth by inhibiting nitrate uptake, while streptomycin inhibits protein biosynthesis by prokaryotic ribosomes in either plastids or mitochondria. Maliga *et al.* (1973) selected streptomycin-resistant tobacco callus and regenerated plants that showed maternally inherited resistance, indicating that the resistance was carried by the genomes of either plastids or mitochondria.

Subsequent work showed that the streptomycin resistance was due to a mutation in the plastid 16S mRNA-encoding gene. This mutated gene also carried another mutation that caused resistance to another antibiotic, spectinomycin, and was used as a selectable marker gene for plastid transformation of tobacco (Svab *et al.* 1990). This breakthrough technique of plastid transformation via homologous recombination into the plastid genome with a selectable marker has been

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used in a number of species for insertion of multiple genes into the plastid, which allows very high expression levels and prevents gene silencing (Daniell *et al.* 2016). Transgenes carried in plastids are also not transmissible through pollen in most species, so they are contained and not spread to other plants.

Amino Acid Analog Resistance

Widholm (1972a, b) selected tobacco and carrot (*Daucus carota*) suspension cultures resistant to the tryptophan (Trp) analog 5-methyltryptophan (5MT), which is a false-feedback inhibitor of the Trp biosynthesis control enzyme anthranilate synthase (AS). The resistant cells contained feedback-resistant AS and increased levels of free Trp. Plants have been regenerated from 5MT-resistant cultures of *Datura innoxia,* corn (*Zea mays*), and rice (*Oryza sativa*) that contained much higher-than-normal levels of free Trp (Ranch *et al.* 1983; Hibberd *et al.* 1986; Wakasa and Widholm 1987), showing that selection in culture can lead to nutritionally important increases in a partially deficient, nutritionally essential amino acid in regenerated plants. In contrast, tobacco plants regenerated from 5MT-resistant, high-Trp cultures did not contain high Trp or the feedback-resistant form of AS, but cultures reinitiated from leaves of these plants did contain high Trp and the feedback-resistant form of AS (Brotherton *et al.* 1986). This indicates that the AS promoter is tissue-culture specific, as shown in studies with soybean (*Glycine max*) where the tobacco AS promoter was used to drive a reporter or selectable marker gene (Inaba *et al.* 2007; Zernova *et al.* 2008).

The feedback-resistant tobacco AS gene was cloned (Song *et al.* 1998) and used as a selectable marker in transformation (Cho *et al.* 2004) including plastid transformation (Barone *et al.* 2009). The rice mutant, feedback-resistant AS was also cloned and used as a selectable marker (Yamada *et al.* 2004).

The active AS enzyme consists of two subunits, alpha and beta, with the alpha being used in the studies described above since it contains the Trp binding site. When the tobacco beta subunit was used in combination with the tobacco alpha subunit to transform tobacco, much greater increases in free Trp were found when compared with using only the alpha subunit (Zhang *et al.* 2015). The reports showing that the rice and tobacco AS alpha subunits alone can increase Trp biosynthesis in other species indicate that these subunits can bind with the beta subunits of other species to form an active enzyme (Yamada *et al.* 2004; Barone *et al.* 2009).

The combination of the amino acids lysine and threonine was shown to inhibit plant cell growth by inhibiting the synthesis of methionine. Hibberd and Green (1982) selected a maize culture resistant to lysine plus threonine and regenerated plants that had increased free threonine in the kernels.

A number of amino acid analogs have been used to select resistant cultures, including the methionine analog ethionine. Ethionine-selected tobacco cells had 100-fold increases in free methionine, due to an increase in the level of the lysine-sensitive aspartokinase enzyme (summarized in Gonzales and Widholm 1985). Hydroxyproline-resistant carrot cells accumulated 15 to 30 times the normal levels of free proline. Tobacco cells selected as resistant to phenylalanine (Phe) analog, p-fluorophenyalanine, had increased phenylalanine ammonia-lyase (PAL) activity that led to a sixfold increase in phenolics. The resistance in this case was due to the detoxification of p-fluorophenyalanine to p-fluorocinnamic acid by the PAL enzyme.

Many of the studies described above involved essential amino acids, the amino acids that animals cannot synthesize. All essential amino acids are synthesized in plastids in plants. Most animals require essential amino acids in their diet, except the ruminant animals, which have bacteria in their stomachs that make these amino acids.

The next section shows that certain herbicides inhibit enzymes involved in essential amino acid biosynthesis, such as the imadazolinones, which inhibit acetolactate synthase involved in the synthesis of isoleucine, valine, and leucine, and glyphosate that inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the Phe and Trp biosynthetic pathways.

Herbicide Resistance

Tobacco suspension cultured cells were selected resistant to the herbicide 4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram) by Chaleff and Parsons (1978). The regenerated plants showed resistance that was controlled in most cases by a single dominant gene. Newhouse *et al.* (1991) reported the selection and regeneration of plants from three corn cell lines selected as resistant to imidazolinone herbicides. The resistance was due to an alteration of the target enzyme acetolactate synthase, an enzyme involved in the synthesis of the branched chain amino acids valine, leucine, and isoleucine, that made it less sensitive to the herbicide. The resistance trait was backcrossed into commercial inbreds and then used to produce herbicide-resistant hybrids. One of these hybrids, called Clearfield® Corn, was first sold commercially in 1992 and is still being sold today (Siyuan Tan, personal communication).

A number of selections for resistance to triazine herbicides were carried out with photoautotrophic cultures that use carbon and energy produced solely by photosynthesis with no sugar added to the culture medium (summarized in Widholm 1992). In all selected triazine-resistant cases, the base and amino acid changes in the photosynthetic reaction center psbA target enzyme were different from those found in five different triazine-resistant weed species: where a base change at position 791 changed codon at position 264 from the amino

acid serine to glycine. The selected triazine-resistant *Nicotiana plumbaginifolia*, *N. tabacum*, *Solanum tuberosum* (potato), and *Chenopodium rubrum* cultures had codon 264 changes of serine to asparagine or changes in other codons in the target *psbA* gene.

The herbicide *N*-(phosphonomethyl) glycine (glyphosate), that acts by inhibiting the shikimate pathway enzyme EPSPS, a nuclear-encoded, plastid-localized enzyme (Steinrucken and Amrhein 1980), was used to select a number of resistant plant cell suspension cultures using gradual selection with increasing concentrations. Nafziger *et al.* (1984) selected a carrot line shown to have a 12-fold increase in EPSPS activity and a tenfold increase in *EPSPS* gene copies (Hauptmann *et al.* 1988). Amplification of *EPSPS* genes was found in glyphosate-selected petunia (Shah *et al.* 1986), tobacco (Goldsbrough *et al.* 1990), *D. innoxia* (Papanikou *et al.* 2004) and *Medicago sativa* (alfalfa), and soybean (Widholm *et al.* 2001). During the glyphosate selection of carrot cells, the EPSPS enzyme activity, mRNA amounts, and gene copy numbers increased gradually, to more than 20-fold, and these increases were relatively stable with a half-life of about 2 yr when grown away from glyphosate (Murata *et al.* 1998).

There are other reports of gene amplification in plant tissue cultures selected with herbicides. Donn *et al.* (1984) used (*RS*)-2-Amino-4-(hydroxy (methyl)phosphonoyl) butanoic acid (phosphinothricin) to select resistant alfalfa cells with amplified glutamine synthetase genes. Sulfonylurea herbicides were used to select resistant lines of tobacco (Harms *et al.* 1992) and carrot (Caretto *et al.* 1994) with amplified acetolactate synthase genes.

Papanikou *et al.* (2004) reported that newly initiated *D. innoxia*, tobacco, and carrot cultures often produce glyphosate-resistant cultures through mechanisms other than *EPSPS* gene amplification, although older cultures usually are resistant due to *EPSPS* gene amplification. The resistance mechanism in cells where gene amplification did not occur was either increased EPSPS levels due to increased mRNA or some other unknown mechanism, because in the latter cases, the EPSPS levels were not increased and the enzyme activity did not exhibit resistance to glyphosate inhibition. Hollander-Czytko *et al.* (1988) also selected glyphosate-resistant *Corydalis sempervirens* cells that contained higher EPSPS activity due to increased mRNA.

The finding by Papanikou *et al.* (2004), that younger cultures often did not show gene amplification while older ones did, might indicate that the control of gene amplification in plant cells may be similar to that in animal cells. Normal animal cells have controls that do not allow the DNA breaks needed for gene amplification, as this occurrence causes the cells to not divide or to die. Tumor cells, in contrast, do not have these controls, so gene amplification can occur. There are examples of the development of resistance to chemotherapy agents due to the amplification of the target enzyme gene (Stark 1993).

Plant cell cultures are known to be genetically unstable, as shown by retrotransposon activation (Hirochika *et al.* 1996), chromosomal breaks and rearrangements (Lee and Phillips 1988), and somaclonal variation that can be described as tissue culture-induced mutations (Larkin and Scowcroft 1981). Somaclonal variations and retrotransposon activation could be caused by epigenetic mechanisms. Usually, the selected resistant cells occurred at frequencies compatible with possible mutations. In some cases, the frequency of resistance was increased by the use of mutagens, which shows that, in most cases, the usual mutation mechanisms are probably involved.

The apparent inability to select glyphosate-resistant cultures with alterations in EPSPS that make the enzyme resistant to glyphosate inhibition may be due to the relative ease of selection for increased EPSPS activity. Shyr *et al.* (1993) found that at least 0.8% of the cultured carrot cells grown in the presence of growth-inhibiting levels of glyphosate were able to grow after a period of time due to *EPSPS* gene amplification that occurred stepwise upon further selection. It is possible that if higher levels of glyphosate are used for selection, rare cells with an altered *EPSPS* gene could be selected.

Several attempts to select glyphosate resistance at the whole plant level, aimed to test if gene amplification could be selected for, were unsuccessful (summarized in Brotherton *et al.* 2007). However, the weed *Amaranthus palmeri* (Palmer amaranth), which became resistant to glyphosate under field conditions, has been shown to contain *EPSPS* genes amplified up to over 160-fold (Gaines *et al.* 2010), showing that gene amplification can be selected for at the whole plant level within certain species. Weeds can also become resistant to glyphosate due to mutations in EPSPS that make it less sensitive to glyphosate inhibition and vacuolar sequestration that reduces translocation.

Selection for Enzyme Deficiency

Allyl alcohol, a compound that can be converted to the toxic compound acrolein by the enzyme alcohol dehydrogenase, was used to treat corn pollen before pollination to produce alcohol dehydrogenase null mutants (Schwartz and Osterman 1976). A suspension culture line of *N. plumbaginifolia* was selected as resistant to toxic levels of allyl alcohol (Widholm and Kishinami 1988). The cells contained one half the normal cell amount of alcohol dehydrogenase activity due to the loss of two of the three isozyme bands on starch gels.

Haploid tobacco cells were selected resistant to chlorate, a compound that is converted by nitrate reductase to the toxic compound chlorite (Muller and Grafe 1978). The selected cells lacked nitrate reductase and thus would not grow in a medium with nitrate as the sole nitrogen source. Chlorate has

also been used with whole plants to select for nitrate reductase deficiency (Nelson *et al.* 1983).

Nitrate reductase auxotrophs were used to make so-called universal hybridizer lines by selecting for resistance to a compound such as the amino acid analog azetidine-2-carboxylate (A2C) that produces a dominant resistance. A2C is toxic when it is incorporated into protein in place of proline. Resistance, in this case, is caused by an accumulation of increased levels of proline in the cells. Protoplasts of the universal hybridizer cell line can then be fused with protoplasts of any wild type cell line that has nitrate reductase to complement the deficiency and is sensitive to A2C (Ye *et al.* 1987).

Protoplast fusion hybrids can also be selected using two cell lines that carry different dominant resistance markers, such as resistance to 5MT and glyphosate (Kothari *et al.* 1986).

Protoplast fusion should be useful since many species can be fused, and the selected hybrids could have a unique combination of genes. Fusion of different species has shown that the mitochondria apparently fuse to allow recombination of their genomes to form new combinations (Kothari *et al.* 1986). Conventional plant breeding does not allow the mixing of different cytoplasmic genomes (plastid and mitochondrial) or the changing of plastid versus mitochondrial combinations; situations that might result in plant improvement. In most plant species, both plastids and mitochondria are maternally inherited during pollination.

Conclusions and Future Prospects

Many things have been learned and commercially important products produced as a result of tissue culture selection for resistance to toxic compounds. There are still possibilities, but the rise of genomics, genetic transformation, and genome editing has shifted the emphasis to these more directed methodologies. However, due to public perception issues and concerns about gene introduction and genome editing approaches, tissue culture selection systems continue to offer promise for the generation of novel and valuable products through the selection of useful variants.

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