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Review

Biotechnology approaches to developing herbicide tolerance/selectivity in crops

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The use of herbicides has revolutionized weed control in many crop production systems. However, with the increasing development of weed resistances to many popular selective herbicides, the need has arisen to rethink the application of chemical weed control. Approaches to maintain the efficiency of chemical weed control include the discovery of new herbicide target sites in plants and the discovery/ synthesis of new, more potent herbicidal molecules. However, these approaches are expensive to execute, take considerably long times to succeed and may lead to increased chemical loads in the environment. Since many existing broad-spectrum herbicides are still effective, an alternative strategy adopted to ensure the safe use of broad-spectrum herbicides in a wide range of crops is the application of biotechnological techniques to engineer crop selectivity to these herbicides. In this review we summarize efforts being made to develop crop herbicide selectivity using biotechnology.

Key words: Biotechnology, herbicide resistance, cell culture, mutagenesis, genetic transformation.

INTRODUCTION

Effective weed control in any crop production system is a prerequisite if high yields and good quality are to be achieved. Over the millennia that humans have practiced crop agriculture, weed control has been the most significant part of cropping operations in ensuring good quality harvests. Initially, hand weeding dominated most weeding practices but it was gradually replaced with mechanical control in the developed world. Mechanical weed control practices are now viewed to be unsatisfactory due to the high-energy requirements and other associated costs, plus the perceived facilitation of soil erosion and compaction. Mechanical weed control has now been largely replaced by chemical weed control using herbicides that can eliminate weeds from crop plantings with minimal soil disturbance.

Herbicide treatment in crop plantings has allowed economically viable weed control and increased productivity. The most preferred herbicides today are those that combine weed killing potency with low- or no-environmental persistence. However, the very effective broad spectrum herbicides available also lack selectivity, thus limiting their use in some cropping operations. On the other hand, the continuous use of the few available selective herbicides is speeding up the development of herbicide resistance in weeds; hence making it difficult to achieve effective control in some crops.

Herbicides generally function by disrupting unique and essential processes in plants e.g. photosynthesis, mitosis, pigment biosynthesis or essential amino acid biosynthesis. Both crops and weeds share these processes. Consequently, at present, selectivity is mostly based on differential herbicide uptake between weeds and crops, controlled timing and site of application or rapid detoxification of the herbicide by the crop plants. Reliance on these natural selective processes limits the effective use of potent herbicides; hence mechanisms to impart better herbicide selectivity in crops need to be investigated.

Two approaches can be pursued to achieve this goal. The first is the design of specific chemicals with broad selectivity for crops. This approach, however, is expensive and the products thereof may be uneconomi-

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cal for use by growers, not to mention that it is also a way to increase the already growing chemical load to the environment. According to Gressel (2002), it has become increasingly difficult to discover new herbicides and even harder to come up with one that has a novel mode of action. In the 1940s only about 500 compounds needed to be screened to select a potential herbicide (Gressel, 2002). By 1989, it was estimated that the discovery of one selective herbicide involved the screening of more than 30 000 compounds and after identification these compounds had to be further modified to improve their toxicity to target weeds and their rapid metabolism in crops (Parry, 1989). In addition "chemical handles" have to be designed to aid the rapid delivery of new chemicals into the target weed plant systems (Owen and DeBoer, 1995). Today the discovery of a potential herbicide requires the screening of nearly 500 000 compounds (Tan et al., 2005).

The second and more popular approach to crop herbicide selectivity is the development of crop cultivars with tolerance to the already existing effective broad-spectrum herbicides so as to expand the crop options in which they can be used. Two methods can be used to develop crops with resistance to herbicides. Conventional plant breeding utilizing lines that are known to be tolerant to specific herbicides is one approach that could confer resistance to susceptible crops from closely related species. However, this approach has limitations in that naturally herbicide resistant plants are found more among weed species than in crops. Also, conventional plant breeding takes a long time to produce a single useful line. A faster approach is the use of biotechnology techniques such as *in vitro* cell culture, mutagenesis and selection in physiologically inhibitory concentrations of herbicides (also referred to as brute force selection) or genetic transformation of already existing crop cultivars with genes than confer resistance to herbicides.

The purpose of this communication is to summarize the results of studies towards the development of crop herbicide selectivity using biotechnology techniques and highlight some of the crop products that have been developed using these techniques.

CELL CULTURE AND SELECTION

Plant tissue culture represents the simplest of the biotechnologies available to plant scientists today. The realization that certain *in vitro* conditions could induce heritable changes, called somaclonal variations, in the genomes of plant cells opened an avenue for the selection of various desirable traits from *in vitro* cultures, including herbicide resistance (Maliga, 1984). Using cell culture procedures, BASF Inc. produced a corn hybrid (DK404SR) that is resistant to the sulfonylurea herbicide, sethoxidim. In their method, a mutant cell line (named S2) was identified following continuous culture of corn embryo tissues under high sethoxidim selective pressure. Plants regenerated from this somaclonal mutant line were found to contain a form of the enzyme, acetolactate synthase (ALS, target of sulfonylureas), which was insensitive to the herbicide. This resistance was subsequently transferred to the commercial hybrid (DK404SR) by backcrossing the S2 line with both of its parental lines. Further investigations showed that the sethoxidim tolerance was inherited as a single partially dominant allele.

Zambrano et al. (2003) selected a glyphosate-tolerant sugar cane cell line in liquid medium containing 0.8 mM glyphosate and regenerated plants that could tolerate up to 5- fold the concentration of glyphosate that killed plants from unselected cells. Analysis of these plants by random amplified polymorphic DNA (RAPD) markers revealed a 564-bp band that was unique to the plants derived from the selected cell line, indicating a possible selection of a pre-existing variability among the cells. A similar method was used to select glyphosate-resistant cells of chicory that subsequently produced glyphosate-resistant plants (Sellin et al., 1992)

Cell culture under lethal concentrations of certain herbicides also results in gene amplification in surviving cells that leads to resistance through the overproduction of enzymes targeted by herbicides. A petunia cell line with resistance to glyphosate was selected in this manner and plants regenerated from it survived lethal levels of glyphosate (Steinrucken et al., 1986). This resistance was found to be due to amplification of the gene encoding 5- enolpyruvylshikimate-3-phosphate (EPSP) synthase that caused its overproduction in the cells (Steinrucken et al., 1986). Similarly Caretto et al. (1994) selected carrot cells and subsequently regenerated plants that were resistant to the sulfonylurea herbicide, chlorsulfuron. Resistance in these plants was due to amplification of the acetolactate synthase gene. *In vitro* development of phosphinothricin (PPT) resistant rice has also been reported by inducing plantlet regeneration in explants collected from 7-day old seedlings on medium supplemented with sublethal doses of PPT (Toldi et al., 2000).

Other *in vitro* cell selection studies have developed resistance to paraquat in tomato cells (Thomas and Pratt, 1982), resistance to glyphosate in carrot and groundnut cells (Murata et al., 1998; Jain et al., 1999) and resistance to a Protoporphyrinogen oxidase (PPO) inhibitor in soybean cells (Warabi et al., 2001); however, no viable plant regeneration was reported in these studies.

MUTAGENESIS

Chemical or physical mutagenesis of seed, microspores or pollen followed by selection under herbicide selective

pressure has also been utilized to develop crop resistance to herbicides. The most common mutagen employed is ethyl methanesulfonate (EMS), which is efficient in producing chloroplast mutants (McCabe et al., 1990). In this method, seeds or pollen are treated with EMS then grown either *in vitro* or *in vivo* in the presence of an herbicide. Surviving plants are selected and grown to maturity to provide seed that is used for further screening with herbicides. Utilizing this method, Sandhu et al. (2002) developed 21 Brazilian rice lines that were resistant to glyphosate. Ashfaq-Farooqui et al. (1997) produced atrazine resistant *Solanum melongena* plants by mutagenizing seeds followed by germination and *in vitro* regeneration of plants from the resultant seedling cotyledons.

Ultra-violet (UV) or EMS treated microspores or pollen can be grown *in vitro* into haploid plantlets whose chromosome number can be doubled to create instant inbred lines bearing a specific herbicide tolerance trait. Ahmad et al. (1991) used microspore UV mutagenesis and haploid culture to develop canola plants that were resistant to chlorsulfuron. Syngenta Seeds Inc. produced the EXP19101T line of imazethapyr-resistant maize using pollen mutagenesis. In this work, EMS-mutagenized pollen was used to fertilize the parent line, UE95, and progeny plants were screened for tolerance to lethal doses of imazethapyr and resistant ones selected. Tolerance in these plants was found to be the result of a single nucleotide substitution within the acetolactate synthase (ALS) encoding gene, which gave a single amino acid change (Ser_{621} to Asn_{621}) in the sequence of the enzyme. This change prevents the binding of the herbicide to the enzyme active site, thus maintaining normal enzyme function. Recently, Venkataiah et al. (2005) reported the production of atrazine-resistant pepper (*Capsicum annuum*) plants regenerated from three-week old seedling cotyledons obtained from EMS-treated seeds. They also observed maternal inheritance of the atrazine resistance trait.

GENETIC TRANSFORMATION

Plant genetic transformation is the science of direct gene transfer and integration, from one plant to another or from a microorganism to a plant, to create plants with altered genetic make-ups to achieve specific crop production goals. The altered plants are generally termed transgenic (or genetically modified, GM). A number of GM crops expressing various traits have been commercialized and several are at various stages of development (TRANSGEN 2005). Herbicide tolerance is the most common trait in commercial transgenic crops, being part of 82% of all transgenic crops in the year 2003 (James, 2003). Transgenesis for herbicide selectivity involves the identification of an herbicide resistance gene from a plant or microorganism, its isolation and manipulation for efficient plant expression (if it is of microbial origin) and (James, 2003) its subsequent delivery, stable integration and expression in the cells of the target crop plant. For the most part, genes coding for useful herbicide resistance in crops are isolated from herbicide degrading soil microorganisms.

Several techniques are now available for the transfer of genes (genetic engineering) into crop plants, including *Agrobacterium*-mediated gene transfer, micro-projectile (or particle) bombardment, polyethylene glycol-mediated DNA transfer and cell (protoplast) electroporation. The most commonly employed techniques in developing herbicide resistant crops are the *Agrobacterium* and the particle bombardment methods, respectively (Tsaftaris, 1996). Herbicide tolerance via genetic transformation can be conferred by one or a combination of these four mechanisms:

1. introduction of a gene(s) coding for an herbicide detoxifying enzyme(s);
2. introduction of gene(s) coding for a herbicide insensitive form of a normal functioning enzyme or over expression of the genes coding for a herbicide target enzyme such that the normal metabolic functioning is still achieved in the plant even though some of the enzyme is inhibited;
3. modification of the herbicide target enzyme in such a way that the herbicide molecule does not bind to it and;
4. the more recently described engineering of active herbicide efflux from plant cells.

These mechanisms have variously been explored in the production of crops that are resistant/ tolerant to various herbicide classes as discussed bellow.

Engineering resistance to photosystem II (PSII) inhibitors

Herbicides targeting the PSII electron transport cascade function by binding to the Q_b site of the D1 protein thereby are blocking the binding of plastoquinone, an electron acceptor at this stage of the photosynthetic electron transport chain. The herbicide bromoxynil is used selectively to control broadleaf weeds in most monocot crops. Apparently, these crops have a metabolic pathway that inactivates the herbicide. The plant genes encoding the enzyme(s) involved in the inactivation process are yet to be identified. To extend the use of this herbicide in dicot crops a gene (*bxn*) coding for an active nitrilase enzyme was cloned from a strain of the soil bacterium, *Klebsiella ozaenae* (Stalker and Mcbride, 1987). This organism was found thriving in

bromoxynil contaminated soils and was able to grow on media with bromoxynil as the sole nitrogen source. Introduction of the *bxn* gene into cotton via *Agrobacterium* transformation produced plants that were able to hydrolyze bromoxynil and ioxynil into non-toxic compounds, thus conferring resistance (Stalker et al., 1996). There are also reports of bromoxynil resistant canola developed using the same system by Calgene Inc and Aventis Crop Sciences Inc (AgBios, GM database).

Engineering resistance to glufosinate

Glufosinate-ammonium (GA) is a non-selective pro-herbicide that is converted by plants into the phytotoxin, phosphinothricin (PPT). The herbicide acts by inhibiting the essential ammonia assimilation enzyme, glutamine synthetase (GS). The strategy to develop glufosinate resistant crops was based on knowledge of where the herbicide was naturally produced. A glufosinate containing tripeptide (bialaphos) was discovered as an antibiotic produced by the fungus, *Streptomyces hygroscopicus* (Mase, 1984). As with most antibiotic producing microbes, *S. hygroscopicus* was found to have a pathway that detoxified this toxin, hence protecting itself from its harmful effects. This pathway is mediated by the *bar* (bialaphos resistance) gene, which encodes a phosphinothricin acetyl transferase (PAT) that converts the herbicidal molecule to a non-toxic acetylated form (Thompson et al., 1987). Wohlleben et al. (1988) also isolated the gene from a related species, *S. viridochromogenes*, and used it to produce transgenic phosphinothricin resistant tobacco, potato and tomato plants.

Since its discovery, the *bar/pat* gene system has been used to engineer glufosinate tolerance in many crops including corn (Gordon-Kamm et al., 1990), rice (Christou et al., 1991), wheat (Vasil et al., 1992), sugarbeet (D'Halluin et al., 1992), oilseed rape and alfalfa (Cobb, 1992), cotton (Keller et al., 1997), lettuce (Mohapatra et al., 1999), sugarcane (Falco et al., 2000), cassava (Sarria et al., 2000) and dry beans (Aragao et al., 2002). Glufosinate tolerant maize, cotton, sugar beet, canola and soybeans have been approved for commercial production by the United States food and drug administration (FDA).

Engineering tolerance to growth regulator herbicides

Growth regulator herbicides were the first class of chemical weed control agents to be commercially introduced for agricultural use in the 1940s (Marth and Mitchell, 1944; Mitchell et al., 1944): they remain the most widely used herbicides today (Rasmussen, 2001). These herbicides mimic auxin activity in plants and show selective activity towards dicotyledonous plants. To date their target site, mode of action and metabolism in affected plants is not fully understood. However, the degradation of 2,4-dichlorophenoxyacetic acid (2,4- D, the first growth regulator herbicide to be synthesized) in the soil has been extensively studied and numerous strains of soil bacteria have been described, which contain plasmid- based gene/ protein cascades for its metabolism (Don and Pemberton, 1981).

The best characterized of the 2,4-D degrading bacteria is *Ralstonia eutrophus (*formerly *Alcaligenes eutrophus*, Don et al., 1985). The gene for the first step of the 2,4-D degradation pathway was isolated from the plasmid, pJP4, of the bacterium (Streber et al., 1987). This gene, *tfdA*, was shown to encode a 2,4-D dioxygenase, which degrades 2,4-D into the inactive compounds, glyoxylate and 2,4-dichlorophenol. Transformation of tobacco with a plant expressible form of the *tfdA* gene conferred 10-fold resistance to the herbicide compared to non-transformed plants (Streber and Willmitzer, 1989; Last and Llewellyn, 1999). The *tfdA* gene in cotton, produced plants that were tolerant to three times the field application rate of 2,4-D used in wheat, corn, sorghum and pasture crops (Bayley et al., 1992). Cotton varieties with tolerance to 2,4-D conferred by the *tfdA* gene have been developed and released in Australia (Llewellyn and Last, 1996) . Recently, the *tfd*A gene has been incorporated into the genome of wine grapes (*Vitis* interspecific hybrid 'Chancellor') and found to confer resistance to up to 20-fold the rate of 2,4-D applied in corn (Mulwa, 2005). This work was carried out to minimize 2,4- D vapor-drift damage in grapes, a serious limiting factor to the establishment of viticulture in grain producing regions of the United States.

Studies are also in progress at the University of Nebraska to genetically engineer crop resistance to dicamba, another volatile growth regulator herbicide. Genes coding for the multi-component dicamba-O-demethylase enzyme of *Pseudomonas maltophilia* strain DI-6 were cloned and expressed in *E. coli* for subsequent efficacy tests for the induction of dicamba tolerance in broadleaf crops (Subramanian et al., 1997). Progress reports on this project indicate that the system has been used to confer dicamba resistance in tomato and tobacco (Anonymous, 2003).

Engineering tolerance to glyphosate

Glyphosate (Round- up^{R}) is a very effective broad spectrum herbicide that inhibits the enzyme 5-enolpyruvylshikimate phosphate (EPSP) synthase (Amrhein et al., 1980). This is a branch point enzyme in the biosynthetic pathway of the aromatic amino acids, tryptophan, tyrosine, and phenylalanine, which are essential for protein synthesis and also as precursors for

hormones, lignins, and other protective compounds such as flavanoids and alkaloids. EPSP synthase uses phosphoenol pyruvate (PEP) and shikimate-3-phosphate as substrates to make EPSP but glyphosate competitively interferes with the binding of PEP to the active site of EPSP synthase, hence blocking the pathway (Anderson et al., 1988; Schönbrunn et al., 2001). For two decades, glyphosate has been a very efficient herbicide with no reports of weed resistance, but recently some weeds with appreciable resistance to this herbicide have emerged (Prately et al., 1999; Lee and Ngim, 2000).

Genetic engineering work to develop glyphosate resistant crops focused on three strategies: overproduction of EPSP synthase, introduction of a metabolic detoxification gene and introduction of an altered EPSP synthase enzyme with decreased affinity for glyphosate (Dill, 2005).

Attempts to alter the structure of the EPSP synthase enzyme in such a way that it is functional in the production of EPSP and phosphate as well as insensitive to the herbicide have dominated research in the last two decades. Comai et al. (1985) were the first to report on studies with altered EPSP enzyme genes for glyphosate tolerance development in plants. They produced glyphosate resistant transgenic tobacco with a modified EPSP synthase encoded by the *aroA* gene of *Salmonella typhimurium* in which an amino acid substitution of a proline for serine caused a decreased affinity for glyphosate without affecting the kinetics of the enzyme. Padgette et al. (1991) concentrated on the Gly_{101} to Ala_{101} substitution of petunia EPSP synthase, but resulting plants were not highly glyphosate tolerant and the EPSP synthase in them bound the PEP substrate comparably to the wild type. A naturally occurring EPSP synthase gene (*cp4*) was later identified from *Agrobacterium* sp. strain CP4, whose protein product had favorable glyphosate tolerance kinetic parameters. This gene was cloned and its expression in several crop plants including soybeans provided acceptable glyphosate tolerance in plants (Padgette et al., 1995).

Glyphosate resistance through metabolic detoxification has also been explored. Two glyphosate detoxification pathways involving a glyphosate oxidoreductase gene (*gox*) are known in microbes. The first involves oxidative cleavage of the N-C bond to yield aminomethylphosphonic acid (AMPA), which is further metabolized into inorganic phosphate (Jacob et al., 1988). The second pathway involves the breaking of the C-P bond by a carbon-phosphorus lyase to yield sarcosine, which is further metabolized into non-toxic components (Dick and Quinn, 1995). The glyphosate oxidoreductase (*GOX*) gene was cloned from *Pseudomonas* sp. strain LBr (Franz et al., 1997) and has been used alongside the *cp4* gene to confer glyphosate resistance in a number of commercially available crops including soybeans, corn, canola, and cotton. This system has formed the basis of the so-called Roundup-Ready crops (Dill, 2005).

Engineering tolerance to plant pigment biosynthesis inhibitors

Chlorophylls and carotenoids are two essential pigment classes in plants whose biosynthetic pathways have been targeted for herbicide design. Herbicides in the broad group of pigment inhibitors target one of two enzymes: protoporphyrinogen oxidase (abbreviated protox or PPO) in the chlorophyll/ heme biosynthetic pathway or phytoene desaturase (PDS) in the carotenoid biosynthetic pathway, respectively.

PPO is the target enzyme for the peroxidizing class of herbicides, which includes diphenyl ether (DPE) and cyclic imide herbicides (Matringe et al., 1989; Wakabayashi and Böger, 2002). PPO catalyzes the oxidation of protoporphyrinogen- IX to protoporphyrin-IX. The inhibition of protox by DPE herbicides leads to an abnormal accumulation of the tetrapyrole intermediate, protoporphyrin-IX, a photo-sensitizer that causes the generation of oxygen radicals (singlet oxygen). This light-dependent free radical production provokes lipid peroxidation in cellular membranes leading to plant death by excessive membrane and cellular constituent damage (Lee et al., 1993). To date, all eukaryotic PPOs that have been characterized are inhibited by DPE herbicides, but the PPO from *Bacillus subtilis* is known to be resistant to the herbicides (Corrigall et al., 1998). The mechanism by which *B. subtilis* PPO exhibits resistance to the herbicides is still unknown but initial transformation studies of tobacco plants with the *B. subtilis* PPO gene, *hemY* (Hanson and Hederstedt, 1992), resulted in plants with very high resistance to the DPE herbicide, oxyfluorfen (Choi et al., 1998). Lee et al. (2000) also produced transgenic rice plants with resistance to the herbicide oxyfluorfen and reported that resistance was higher when the gene was targeted to the chloroplasts than when it was targeted to the cytoplasm.

Mutant PPO genes have also been used as selectable markers in crop transformation studies (Li and Nicholl, 2005). Li et al. (2003) used a mutant DPE herbicide resistant PPO as a selectable marker to develop DPE herbicide resistant corn. Their technique enabled the identification of more than ten lines with field- effective levels of resistance to the herbicides. The use of techniques to produce DPE herbicide-resistant plants utilizing mutant PPO genes is referred to as the Acuron® technology (Holmberg, 2000).

Among enzymes of the carotenoid biosynthetic pathway of plants, PDS is the target of several bleaching herbicides including norflurazon and fluridone, which are non-competitive inhibitors of the enzyme (Sandmann et al., 1989). PDS catalyzes the conversion of phytoene to

–carotene (Sandmann, 1994). Consequently, plants treated with these PDS inhibitor herbicides accumulate phytoene and suffer concurrent bleaching. However, Sandmann and Fraser (1993) reported that the PDS of *Erwinia uredovora*, encoded by the *crtI* gene, was resistant to the bleaching herbicides. Transformation of tobacco with this gene yielded transgenic plants with strong multiple resistance to the herbicides norflurazon, fluridon, fluertamone, fluorochloridon and diflufenican which strongly interfere with the wild type plant PDS (Misawa et al., 1994). This trait could provide broad-spectrum resistance if incorporated into more economically viable crops.

Engineering resistance to acetolactate synthase (ALS) inhibitors

Acetolactate synthase (ALS) is a central enzyme in the biosynthesis of the branched chain amino acids leucine, isoleucine and valine in plants. Herbicides targeting this enzyme belong to four structurally distinct classes: sulfonylureas (SUs, LaRossa and Schloss, 1984; Ray, 1984), imidazolinones (IMs, Shaner et al., 1984), pyrimidine sulfonamides (TPs, Subramanian and Gerwick, 1989) and pyrimidinylsalicylates (pyrimidinyl carboxys, PCs, Shimizu et al., 1994). These herbicide classes offer effective selective control of grass weeds. ALS, as an herbicide target site, is the most widely studied enzyme in terms of kinetics and genetics (Shimizu et al., 2002). It is the target that is reported to have the highest incidence of developing resistance to herbicides. A large number of mutations have been characterized; most of them are due to single amino acid sequence changes that do not affect the enzyme function but easily induce herbicide resistance in plants where they occur (Tranel and Wright, 2002). This has resulted in the emergence of a great number of weeds with resistance to the sulfonylurea and imidazolinone herbicides classes (Tranel and Wright, 2002).

Genetic engineering methods used to generate crops with resistance to ALS inhibiting herbicides include transformation with genes coding for modified forms of ALS and, recently, oligonucleotide mediated plant gene manipulation. Gene transfer has been used to incorporate sulfonylurea resistance into several commercially important crops including cotton (Rajasekaran et al., 1996), soybeans (Aragao et al., 2000), canola (Mike et al., 1990), rice (Li et al., 1992), and flux (McHughen, 1989).

Oligonucleotide-mediated gene manipulation is a new method for plant transformation that was used to develop an imidazolinone resistant corn line (Zhu et al., 2000). The method is designed to address the controversies surrounding the inclusion of other foreign genes (e.g. antibiotic or herbicide resistance marker genes) during conventional genetic transformation. This method utilizes a technique that has for long been used in DNA repair and gene therapy studies in mammalian systems (Yoon et al., 1996). The procedure employed in maize involved the designing of an oligonucleotide made up of a combination of DNA and RNA bases, with a 32-base section having nearly exact homology to the target sequence of the endogenous plant gene (in this case ALS), except that there was a single base mismatch at the point of the desired mutation. The chimeric oligonucleotide was then delivered into the target cells using microprojectile bombardment, where it aligned with the endogenous homologous sequence. In certain cases the normal DNA repair mechanism read the chimeric oligonucleotide as the template gene and corrected the mismatched base in the endogenous gene. This resulted in a direct change in the targeted gene. Specifically, the procedure resulted in a change from Ser_{621} (coded by AGT) to Asn_{621} (coded by AAT), which conferred resistance to imazethapyr and imazapyr in the regenerated plants. According to Tranel and Wright (2002), this method of transformation represents a fast and more convenient way of developing herbicide resistance in a wide variety of crops without going through the rigors and expense of making specific genetic constructs.

Engineering tolerance to mitotic disruptor herbicides

Herbicides in the general class of cell division inhibitors (dinitroanilines, DNAs) act by disrupting the process of mitosis in mersitematic tissues of seedlings. Specifically, these herbicide molecules bind to the tubulin protein subunits, and inhibit tubulin polymerization into microtubules (Morejohn et al., 1987; Appleby and Valverde, 1989). Since microtubules are the major constituent of the spindle apparatus, their inhibition interferes with the movement of chromosomes to the metaphase configuration as well as the migration of daughter chromosomes to their respective poles at anaphase. As a result, cell wall formation fails to occur at telophase. With time in the prophase state the chromosomes coalesce in the middle of the cell and a nuclear envelop reforms, resulting in a polyploid nucleus. Because new cells are not being formed, growth eventually stops and death results at an early seedling stage of the plant.

Efforts to engineer crop resistance to mitotic disruptor herbicides started with the over-expression of mutant - and -tubulin proteins in maize calli (Anthony and Hussey, 1998). This resulted in transgenic maize calli with resistance to DNAs and in which virtually all the endogenous tubulin synthesis was replaced with the transgenic protein (Anthony and Hussey, 1998). However, further development into plants was not possi-

ble because the maize callus lines used were not regenerable. Over-expression of the mutant - and - tubulin system in tobacco has resulted in the regeneration of plants with appreciable resistance to DNA herbicides (Anthony et al., 1999) and presents an opportunity for its application in commercial crops in which DNA herbicides are used.

Potential for the development of multi-herbicide tolerance in crops

Most crop herbicide resistance development has been geared toward one herbicidal chemical or a particular class of herbicides. However, recent studies have demonstrated the possibility for the engineering of multi-herbicide resistance in plants. Windsor et al. (2003) reported the development of cross- resistance to different herbicide classes in transgenic *Arabidopsis* plants facilitated by the overexpression of a plant ATP-binding efflux protein (*A. thaliana* P-glycoprotein 1, AtPgp1) and the apyrase protein. The mechanism by which these two proteins conferred resistance is thought to be related to the decreased retention or increased active efflux of the herbicides from plant cells (Windsor et al., 2003).

The potential to engineer multi-herbicide resistance in crops by the expression of mammalian cytochrome P450 monooxygenase genes has also been demonstrated (Inui and Ohkawa, 2005). Cytochrome P450 (P450 or CYP) monooxygenases consist of a number of enzyme species located on the endoplasmic reticulum of eukaryotic cells. Each of these protein groups exhibit broad and overlapping substrate specificities and play a key role in the oxidative reactions of secondary metabolism and the metabolism of xenobiotics in mammalian systems (Badawi et al., 2001; Niwa et al., 2001). With this background in mind, four human P450 species were introduced into the genomes of potatoes and rice with the resultant regeneration of transgenic plants with cross- resistance to several herbicides of different structural backgrounds and modes of action (Inui et al., 2000, 2001). These studies have opened the avenue for the targeted development of crops that would reduce environmental chemical load occasioned by the use of different herbicides in crop rotational programs.

SUMMARY AND CONCLUSION

Following the development of weed resistances to many selective herbicides and the prohibitive expense and difficulty associated with the development of new herbicides, a need has arisen to seek alternatives to address these challenges. Along with the efforts to discover new herbicide target sites in plants, biotechnology is making major contributions in broadening crop selectivity to the already existing and effective herbicides. Further efforts to create more herbicide tolerant crops are needed to ensure more economical crop production and safeguard environmental quality by reducing the demand for and the number of selective weed killing chemicals required for economical chemical crop protection.

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