CYTOCHROME P450 MONOOXYGENASES AS A TOOL FOR METABOLIZING OF HERBICIDES IN PLANTS

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ABSTRACT
Plants like most other organisms evolved a remarkable battery of metabolic reactions to metabolize many different xenobiotics. In recent years the use of biochemical and molecular techniques associated with enzymatic techniques have greatly advanced our understanding of the metabolic reactions (oxidation, reduction, hydrolysis, conjugation, etc.) not only in plants. In addition recombinant DNA techniques allowed the isolation, cloning and expression in plants of a number of foreign genes, including foreign detoxification genes for tolerance to xenobiotic compounds. Cytochrome P450 are one of the major plant enzyme classes which mediate the oxidative degradation of xenobiotic chemicals, including herbicides. The transgenic tobacco and potato plants expressing CYP1A1 and its fused enzyme with yeast reductase showed tolerance to the herbicide chlorotoluron. Some of them are tolerant to the herbicides diuron and atrazine. The tolerance of the obtained transgenic plants seem to be due to the one and the same mechanism. Our results related to the bulgarian tobacco transgenic plants expressing cytochrome P450 monooxygenase genes showed tolerance to the herbicides chlorotoluron and chlorosulfuron. The herbicide chlorotoluron delayed the germination and development of Orobanche ramosa seeds in case of use of transgenic plants tolerant to the herbicide. New data were obtained about the state of photosystem II by measurement of chlorophyll fluorescence. The lower PSII activity in the control and transgenic plants after application of the herbicide chlorotoluron, increase more rapidly in transgenic plants after the ninth day. Its recovery is due to the better metabolization of the herbicide in the transgenic plants. The transgenic CytP450 plants with increased cyt monooxigenase activity tolerate better oxidative stress after herbicide treatment.

Introduction
Plants function in a chemical environment made up of nutrients and xenobiotics. Xenobiotics (foreign chemicals) are natural or synthetic compounds that cannot be utilized by plants for energy yielding metabolism. Chemicals classified commonly as xenobiotics include pesticides and air pollutants. Plants, like most other organisms, evolved metabolic reactions to defend themselves against the potentially toxic effects of xenobiotics.

The biotransformation of xenobiotics in higher plants are grouped in three main phases: conversion, conjugation and compartmentalization. Phase I reactions include oxidations, reductions and hydrolyses. Phase II includes conjugations. In phase III, xenobiotic conjugates are converted to secondary conjugates or insoluble bound residues and are deposited in the vacuole or other compartments (cell wall) of plant cells (1).

Phase I of xenobiotic metabolism often results in the formation of metabolites with reduced or modified phytotoxicity, in-
increased polarity, or susceptibility to further processing (2, 3). The main enzymes which take part in Phase I are cytochrome P450 monooxygenases (Cyt P450s) and esterases. These enzymes catalyze oxidative and hydrolytic reactions of xenobiotics in plants.

Plant P450 monooxygenases are membrane-bound haem proteins which consist of a protoporphyrin IX and an apoprotein that confers the substrate specificity. Binding of the haem is through a cysteine residue situated about 15% in from the carboxy-terminus. The minimal catalytic system is constituted from the cytochrome P450 and a membrane-bound flavoprotein, the NADPH-cyt P450-reductase, which transfers the two reducing equivalents from NADPH to the cytochrome P450. These enzymes were only positively identified in plants some 15 years after their first description in animal tissue (4, 5). They are oxdoreducting enzymes incorporating an oxygen atom from molecular oxygen into a substrate, the second oxygen atom being converted to water. They are thus monooxygenases and such reactions catalysed by P450 are also classified as mixed function oxygenations. For a typical reaction the following scheme is apparent:

\[
\text{RH} + \text{O}_2 + \text{NADPH}, \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+
\]

Plant P450 have been shown to carry out hydroxylations, epoxidations, heteroatom dealkylations and oxygenations.

Cytochrome P450 enzymes are defined by their absorption spectra (6, 7). They play an essential role in electron transfer as part of the respiratory chain. One can distinguish several distinct cytochrome groups classified by a letter (a, b or c) which corresponds to the ranking, in decreasing order from a to c, of the maximal absorption wavelength in the visible range in their reduced state (Soret band). Cyt P450s are of the b type. The letter P (for pigment) recognizes their coloration in the visible range due to a Soret band around 420 nm. The specific nomenclature of P450 is due to a number of additional properties. Thus, carbon monoxide forms with dithionite-reduced cyt P450 a complex which absorbs maximally at 450 nm (10).

The presence of various other b type cytochromes in microsomes has been reported (15, 16). These haem proteins exhibit peculiar changes in the absorbance around 450 nm and can weaken or obscure the peak of the P450 spectrum. Up to now only two subsets of haem proteins, peroxidases and peroxygenases that are responsible for this interference have been described in plant microsomes. Furthermore, possible contamination of microsomes with respiratory cytochromes of mitochondrial origin might also occur and lead to interference.

The range of reactions in secondary metabolism catalysed by cytochrome P450 is likely to be enormous. They were well described (10):

**Lipids**

Fatty acids have been shown to be hydroxylated and/or epoxidated by P450 enzymes in plants. These reactions are essential to the formation of characteristic features of plants such as cutins and suberins, the protective aerial and underground barriers of plants, which are essentially biopolymers of hydroxylated C16 and C18 fatty acids.

**Phenylpropanoid biosynthesis**

Phenylpropanoids form the basic structural precursors for lignin and may be incorporated directly into the wall especially as ferulic acid. Two hydroxylations in phenylpropanoid metabolism are known to be catalysed by cytochrome P450 (11, 12).

**Flavonoid and coumarin biosynthesis**

Hydroxylations of aromatic groups were long ago proposed as being catalysed by P450 enzymes.

**Terpenoid biosynthesis**

Monoterpenes, sesquiterpenes and diterpenes are all substrates for cytochrome P450s.
Sterol and steroid-like compounds in plants are subject to modification by P450s.

**Alkaloid biosyntheses**

The biosynthesis of two major group of alkaloids, the benzyl-isoquinolines and the monterpenoid indoles both contain steps known to be catalysed by cytochrome P450s. They take part in the biosynthesis of indol alkaloids, tropane alkaloids, in pyridine alkaloid metabolism.

**Xenobiotic metabolism**

Detoxification of xenobiotics is a major function of the range of cytochrome P450s in animals. This role is not precluded in plants since they may be exposed to microbial toxins, but mostly the P450s that function in the detoxification of xenobiotics may well have usual functions in secondary metabolism. However these P450s may become just as familiar through this other route and become synonymous with a role in herbicide and pesticide metabolism.

Plant cyt P450s are multifunctional enzymes catalyzing more than 60 reactions. They exhibit tissue and substrate specificity and there are many sites regulating the expression of cyt P450 genes in plants (1). Important issues such as assigning a specific function to the cloned genes and understanding how cyt P450s are induced in plants have not been resolved. Another question that remains to be answered is to find out how many plant cyt P450s participate in xenobiotic detoxification (1).

Two major mechanisms are responsible for plant resistance to herbicides. Enzyme(s) present in some plants are capable of metabolizing the herbicide to a compound that is no longer phytotoxic, and the plant survives the herbicide treatment. In an alternative mechanism the target site enzyme of the herbicide is modified so that the compound is not able to inhibit the enzyme (13).

According the theme of the review it will be emphasize some results concerning cyt P450s in the process of biotransformation of xenobiotics (herbicides) in plants.

N – and O – dealkylation reactions of xenobiotics are well documented. Cytochrome P450-dependent N-demethylation of herbicide chlorotoluron was characterized in Jerusalem artichoke microsomes (14, 15). This herbicide was also shown to be demethylated in wheat (16).

The herbicide metalochlor is de-ethylated by a P450 from grain Sorghum (17). Many other xenobiotics and especially phenylurea herbicides have been shown to be subject to heteroatom-dealkylation in vivo and reports on their metabolism by P450s increase in the years.

Double bond oxygenation, i.e. ring-hydroxylation, is the most frequent, reaction reported for plant P450-dependent metabolism of xenobiotics. Early work characterized the P450-dependent hydroxylation of the herbicide 2,4-D in cucumber (18).

Corn mycrosomes have been shown to ring-hydroxylate bentazon (19). Wheat microsomes also carry out this type of reaction on diclofop (20, 21). Chlorotoluron has been shown to be hydroxylated on its ring methyl by a cyt P450 enzyme in wheat (15) and corn (22).

Plant P450s have a significant role in detoxification which is reminiscent of their role in animal systems. They are therefore of great importance commercially not only from the point of view of herbicide resistance but also in terms of ecotoxicology.

Since there now more than 60 reported reactions in plants catalyzed by cytochrome P450s, there are certainly potentially many multiple forms, it is important to understand the potential range of genetic diversity of this group of gene products. They also have enorous biotechnological significance.

Localization studies have been mainly biochemical and detection of cytochrome P450 in microsomal fractions is routine. Spectra with mitochondrial fractions have usually been attributed to microsomal contamination.

Since cytochrome P450s show a large di-
versity in their site of action within metabolism, each one is expressed in response to developmental and environmental cues. As P450s function at steps in all the major pathways it is expected that the level of these proteins would be co-ordinately regulated with other proteins involved in the same pathway (10).

In many cases, the first step in the metabolism of a xenobiotic molecule is an oxidation reaction. Cytochromes P450 form a family of catalysts outranking any other family of enzymes by the number of substrates, the number of reactions catalyzed and the number of inducers (23).

The mechanisms by which the cytochrome P450 enzymes are induced in response to external signals, like foreign chemicals, is a fundamental question (23). It was shown that chlorotoluron metabolism which could not be detected in microsomes from *Lolium rigidum* plants resistant to this and other herbicides, was induced after exposing the plants to blue light (Powles, personal communication).

Phytoalexins are low molecular weight compounds produced in plants exposed to pathogens or to elicitors (fractions derived from fungi). The construction of P450 enzymes is an important step in the production of different classes of phytoalexins (23).

Induction of P450 in wounded tissues was reported (24, 25).

Induction by chemicals is one of the most striking features of P450 enzymes (26, 27, 28).

All plant P450s depend on NADPH-cytochrome P450 reductase for providing the reducing equivalents needed to activate molecular oxygen (23). To what extent the reductase may actually control the activity of different P450 isoforms remains unknown. It is observed that the reductase is not induced by chemicals.

Herbicide selectivity can be defined at the differential sensitivity of plant species to a given application rate of a herbicide. Herbicide selectivity, in which target weeds are controlled and the crop species is undamaged, has led to the widespread use of these chemicals in crop production. Herbicides are the most used crop protection chemicals (29).

There are three primary mechanisms by which herbicide selectivity is achieved in crops: 1. Less absorption or translocation of the herbicide by the crop compared to the weeds; 2. More rapid metabolic detoxification of the herbicide by the crop than the weeds; 3. The presence of an insensitive herbicide target site in the crop and a sensitive target site in the weeds (30). The second mechanism has been the most important for herbicide selectivity. However, with the introduction of genetically engineered or selected crops for herbicide resistance, the third mechanism is becoming more important (30).

Cytochrome P450 monooxygenases (P450) are among the enzymes responsible for herbicide metabolism. The trivial chemistry of the herbicides has changed. New herbicides (sulfonylureas, imidazolines and sulfonamids) are more susceptible to attack by P450s. They, also, are characterized by very low application rates. This is very important for herbicide P450 detoxification and for a rapid metabolism to avoid phytotoxicity. Many of the enzymes, including P450, involved in herbicide metabolism in plants are inducible. The enzymes can be induced by light, herbicides, heavy metals, wounding, etc.

A typical P450 catalyzed reaction yields a hydroxylated metabolite of the herbicide. The hydroxylated herbicide metabolites are often further metabolized through conjugation with a glucosyl residue (31).

The enzyme system on microsomes consists of many P450 species and a few NADPH-cytochrome P450 oxydoreductase molecules (32). In mammals, a number of microsomal P450 species are involved in xenobiotic metabolism. Ohkawa et al. (1997) (32) produced the fused enzyme
between rat CYP1A1 and rat reductase on microsomes in the recombinant yeast cells, which showed enhanced specific activity as compared with the original enzyme system (33). Then the authors attempted to express rat CYP 1A1 and its fused enzyme with yeast reductase in transgenic plants, which appeared to exhibit monooxygenase activity towards xenobiotics including herbicides. Enhancement in herbicide metabolism in transgenic plants appeared to result in resistance towards herbicides. Ohkawa et al. (1997) (32) successfully worked on metabolism of some herbicides by fused enzyme between rat CYP1A1 and yeast reductase in transgenic tobacco and potato plants.

**Metabolism of the herbicide chlorotoluron in plants**

The phenylurea herbicide chlorotoluron is used for selective weed control in winter wheat and barley. This herbicide is metabolized in plants by a combination of oxidative N-demethylation and hydroxylation of the ring-methyl group. P450 has been suggested to be involved in both N-demethylation and ring-methyl hydroxylation of chlorotoluron (34, 35, 36). So, enhancement of P450 monooxygenase activity related to chlorotoluron metabolism in plants may result in tolerance to the herbicide.

**Transgenic tobacco plants expressing the fused enzyme between rat CYP1A1 and yeast reductase**

Ohkawa et al. (1997) (32) examined the expression of the fused gene between rat CYP1A1 cDNA and yeast reductase gene under the control of CaMV35S promoter and NOS terminator in transgenic tobacco plants. The fused enzyme was mainly located on the microsomes, which showed about 10 times higher monooxygenase activities towards 7-ethoxycoumarin and benzo-pyrene than those of the control plants. The transgenic tobacco expressing the fused enzyme exhibited tolerance to chlorotoluron (37). Based on analysis of chlorotolurom metabolites, it was found that transgenic plants metabolized the herbicide more rapidly as compared with the control plants through both ring-methyl hydroxylation and N-demethylation, although the control plants metabolized the herbicide through N-demethylation (38). Since the N-demethylated chlorotoluron was still phytotoxic, the activity in ring-methyl hydroxylation of the transgenic plants appeared to be attributable to tolerance of the herbicide.

The human P4502B6 and yeast reductase fused enzyme was also expressed in transgenic tobacco plants. Molecular analysis of the transgenic plants revealed that the fused enzyme cDNA seemed to be integrated into the tobacco genome and transcribed into its mRNA. The microsomal fraction of the transgenic plants where the fused enzyme protein was localized, exhibited 4 to 6 times higher monooxygenase activity toward 7-ethoxycoumarin than that of the control plants. These results suggested that the P4502B6/reductase fused enzyme was functionally expressed in the transgenic tobacco plants (39).

The transgenic plants, expressing P4501A1 and P4502B6 were expected to metabolize a number of foreign chemicals. There seems to be apparent differences in the toxicity and the metabolism of xenobiotics between human and experimental animals. The present strategy to express human P450 monooxygenases in transgenic plants may be useful for production of “humanized crops” having the ability to detoxify agrochemicals and environmental contaminants (39).

**Transgenic Potato Plants Expressing CYP1A1 and its fused enzyme with Yeast Reductase (32)**

Both rat CYP1A1 cDNA and its fused gene with yeast reductase gene were each expressed in transgenic potato plants under the control of CaMV35S promoter and NOS terminator. Both enzymes mainly localized on the microsomes, which exhibi-
ited several times higher 7-etoxycoumarin O-deethylase activity than that of the control plants. Both transgenic potato plants expressing CYP1A1 and its fused enzyme showed tolerance to chlorotoluron. Both transgenic plants metabolized 14C-chlorotoluron more rapidly to give N-demethylated and ring-methyl hydroxylated metabolites as compared with the control plants. The tolerance of both transgenic potato plants to chlorotoluron seemed to be due to the same mechanism as found with the transgenic tobacco plants. In addition, both transgenic potato plants showed tolerance to diuron, probably due to enhancement in N-demethylation of the herbicide, since it has no ring-methyl group (10).

Inui (1999) (40) was also attempted to express human CYP1A1 and CYP1A1/yeast reductase fused enzyme in transgenic potato plants. The P450-dependent monooxygenase activity of some of the transgenic plants was from 3.5 to 4.2 times higher than that of the control plants. The clear tolerance toward the herbicides chlorotoluron and atrazine was found in one clone of the transgenic potato plants.

The co-expression of human CYP1A1, CYP2B6 and CYP2C19 in transgenic potato plants was attempted. One of the transgenic clones was tolerant towards all herbicides which were metabolized by these three P450 species (40).

These transgenic potato plants expressing mammalian P450 species have a great impact on phytoremediation of the environment as well as on generation of herbicide resistant crops.

Our studies were related to the tolerance of cv. Xanthi to the herbicide chlorotoluron – T0 progeny, but also herbicide tolerance of the transgenic plants – T1 and T2 progenies in greenhouse and field conditions. Transgenic plant material T0, expressing a genetically engineered fused enzyme between rat CYP1A1 P450 and yeast NADPH cyt P450 reductase was kindly conceded from prof. Ohkawa, Japan. As a result of the herbicide treatments with the field dose (200 g dka-1) chlorotoluron (commercial product Tolurex), all transgenic plants T0, T1 and T2 seed progenies, showed tolerance to the herbicide in greenhouse and field conditions (41).

In our studies it was developed also genetically engineered tobacco plants, expressing rat P4501A1/yeast reductase fused enzyme using five economically important Bulgarian tobacco cultivars: Burley 21, Nevrokop 1146, Krumovgrad 90, Khan Tervel 39 and Kocker 254. It was obtained transgenic tobacco plants for herbicide tolerance toward phenylurea herbicide chlorotoluron (42). These cultivars were transformed with the construct PGFC2 (kindly provided by Prof. Ohkawa, Kobe University, Japan), carrying rat P4501A1 and yeast P450 reductase genes under the control of CAMV35S promoter and Nos terminator, conferring resistance to the herbicide chlorotoluron. The genes were introduced into tobacco plants by leaf disks genetic transformation mediated by Agrobacterium tumefaciens.

The phytotoxicity of the herbicide chlorotoluron was examined in transgenic tobacco plants and in the control plants. The transgenic tobacco plants showed normal growth at 10, 20 and 50 µM chlorotoluron, a little chlorosis and delayed development at 100 µM chlorotoluron 45 days after having been transplanted in greenhouse conditions.

These results indicated that transgenic tobacco plants showed resistance to chlorotoluron at concentrations from 10 to 50 µM while for control plants even 10 and 20 µM were phytotoxic. The obtained results correspond to these, obtained by other author (43). For difference from his studies, we carried out herbicide treatments of the transgenic tobacco plants with a field dose of the herbicide chlorotoluron (Tolurex) (200g dka-1). After these treatments the transgenic tobacco plants were resistant and had very low symptoms of damage in
contrast to nontransformed control plants, which had severe damages and died after 21 days.

The herbicide chlorotoluron is selective mainly systematic herbicide. The normal application of the herbicide in agriculture for weed control varies from 160 to 240 g dka−1. Our results showed that the fused rat P4501A1/yeast reductase gene was expressed in plants at level enough for resistance to high dose of the herbicide chlorotoluron – 200 g dka−1.

It was established that when a rabbit liver P450cDNA was integrated into tobacco plants, the transformants showed marked phenotypic changes, with a tendency to rapid senescence caused by accumulation of degradative metabolite of nicotine alkaloids (44). However such phenotypes were not observed in our studied transgenic tobacco plants, with exception in cv. Nevrokop 1146 (42). Transgenic plants from this cultivar were characterized with short internodes, branched stems and reduction of seed production. Tobacco plants from resistant lines (T0) were self-pollinated. The seeds were collected and tested for herbicide resistance in the T1 progeny. For all eight tested lines the segregation gave soundness to suggest that genes were introduced in a single locus in the genome of tobacco plants.

Over 3500 species of parasitic plants occur in plant kingdom. They represent serious agricultural problem in many parts in the world. Most dangerous parasitic weeds are species from the genera Orobanche, distributed mainly in Eastern Europe, Asia and Mediterranean region.

Tobacco is one of the most economically important crops in Bulgaria that is seriously affected by a strong root parasite Orobanche ramosa. This parasite can cause a reduction of the yield of tobacco up to 40% (45). There are not natural sources of resistance to broomrape identified in tobacco (46). The absence of naturally occurring tobacco cultivars resistant to Orobanche species is a barrier to obtain tobacco plants resistant to broomrape by the methods of classical selection. The use of genetically engineered herbicide resistant plants is one of the most effective ways for broomrape control.

In this relation it was performed also transformation of commercially important Bulgarian tobacco cultivars with the same gene CYP1A1, made in the laboratory of Prof. Ohkawa. The transformants To were tested to the action of the herbicide chlorotoluron in greenhouse conditions at a field dose 200g dka−1. The aim of the study was to test whether the chlorotoluron treatment of the transgenic plants could lead to inhibition of the germination of broomrape (Orobanche ramosa) (41). The results showed inhibition of the broomrape germination at the field dose of the herbicide chlorotoluron. It were not found any data on such kind of studies for the Orobanche ramosa germination.

The resulting transgenic plants with higher herbicide tolerance to chlorotoluron helped to study effects of the expression of rat Cytochrome P4501A1 on plant – parasite interactions (like Orobanche ramosa). That is why in our studies we attempted to generate transgenic tobacco plants resistant to the herbicide chlorotoluron and chlorosulfuron by expression of two mammalian cytochrome P450 monooxygenase genes and to investigate the effects of the expression of the used P450 genes on tobacco – parasite Orobanche ramosa interactions (47). The obtained transgenic tobacco plants, carrying the fused rat cytochrome P4501A1/yeast reductase gene from T2 progeny were used to investigate the efficiency of chlorotoluron for broomrape control in pots.

That kind of investigations was carried out for the first time. There are not any literature data on such kind of studies.

Herbicides were applied on the tobacco plants at the vegetatively stage of 4–5 leaves. Tobacco plants, expressing the
fused rat cytochrome P4501A1/yeast reductase gene were treated with chlorotoluron (Tolurex) in greenhouse conditions at a dose 200 g dka⁻¹. It was observed that transgenic tobacco plants were resistant and had no symptoms of damage in contrast to control plants that had severe damage and died after 20 days.

Tobacco plants from resistant lines (T₀) were self-pollinated. The seeds were collected and tested for resistance in the T₁ and T₂ progenies.

Three stable transgenic lines in T₂ progeny – two for Khan Tervel 39 and one for Burley 21 were obtained. The effect of the herbicide chlorotoluron on germination of Orobanche ramosa seeds was studied in vitro and in greenhouse conditions.

It was established that the herbicide chlorotoluron at concentrations 0.1–0.5 mM decreased the percent of germination of Orobanche ramosa seeds and the full inhibition was observed at concentration 12.5 mM.

The basic idea for broomrape control is to apply herbicides in the field with herbicide resistant tobacco (48).

According to our experiments chlorotoluron resistant tobacco plants in T₂ progeny were grown in soil infested with broomrape (47). It was observed that the broomrapes on transgenic tobacco plants, expressing the fused rat P4501A1/yeast reductase gene treated with 0.5 mM chlorotoluron, developed 3–4 weeks later than did nontreated control and transgenic plants in greenhouse conditions. These results indicated that Orobanche ramosa was affected when herbicide chlorotoluron was applied before appearance of the parasite above the soil. However this herbicide had delayed the germination and development of Orobanche ramosa seeds with 3–4 weeks in comparison with nontreated tobacco plants but did not kill the parasite.

In our studies we obtained also transgenic tobacco lines, expressing human cytochrome P4502C9 gene. Transgenic tobacco plants demonstrated to have chlorosulfuron resistant phenotype by treatment with Glean at a dose 1 g dka⁻¹ (47). Ten days after treatment all control plants died while transgenic ones were resistant. Seeds from resistant plants were collected and planted to investigate the segregation in T₁ progeny. These resulting transgenic plants with higher herbicide tolerance to chlorotoluron and chlorosulfuron are useful for further investigations of the effect of the expression of used P450s on tobacco parasite Orobanche ramosa interactions.

It was investigated the effect of the herbicide chlorosulfuron on the changes of the proline content and on the activity of catalase, peroxidase and superoxide dismutase in the control nontransformed and transgenic tobacco plants after treatment with the herbicide Glean 0.5 g dka⁻¹ (49).

It was established that chlorosulfuron induced higher proline content in control and transgenic plants on the first day after herbicide treatment. The proline content in the control plants is higher in comparison to that in the transgenic plants. It is indicated that nontransformed plants are higher stressed.

Catalase and guaicol – peroxidase activity in transgenic plants are not affected by the herbicide chlorosulfuron treatment during whole tested period.

In the transgenic plants the activities of antioxidant enzymes (catalase and peroxidase) do not affect by chlorosulfuron treatment in comparison with nontransformed plants.

Superoxide dismutase activity in the transgenic tobacco plants is higher that it in the control plants on the first day after the herbicide treatment, but after that the decrease of the superoxide dismutase activity is higher in the transgenic plants. These results are due of the fact, that the transgenic tobacco plants, expressing human cytochrome P4502C9 gene metabolize faster the herbicide chlorosulfuron in comparison to the control plants. This faster
metabolization of the chlorosulfuron has less negative influence on the transgenic plants including the oxidative stress. The lower oxidative stress in transgenic plants could be explain with the increased monooxygenase activity, leading to faster obtaining of nonphytotoxicity metabolites as a result of chlorosulfuron metabolism (50).

Detoxification of chlorotoluron and chlorosulfuron in plants is mediated by cytochrome P450s (51).

**Coupling of P450 Monooxygenase to Photosynthetic Electron Transfer Systems in Chloroplasts (32)**

Higher plants evolve molecular oxygen and produce NADPH and ATP during the process of photosynthesis in chloroplasts. In contrast, P450 monooxygenases on microsomes utilize both molecular oxygen and NADPH for monooxygenase reaction. P450 monooxygenase may utilize electrons from photosynthetic electron transfer systems to exhibit an enhanced monooxygenase activity under light irradiation. The enhanced activities were reduced to the level of the control plants under dark conditions, as well as under light conditions in the presence of the herbicide diuron, which is an inhibitor of photosynthetic electron transfer. Immunogold electron microscopic observations revealed that the P450 enzymes largely located on thylakoid membranes. Thus, it was suggested that microsomal P450 monooxygenases utilized electrons through coupling with photosynthetic electron transfer systems in tobacco chloroplasts to exhibit an enhanced monooxygenase activity under light irradiation. The experiments of the authors (32) also showed that these transgenic tobacco plants were tolerant to chlorotoluron.

The study of the light - induced in vivo chlorophyl (Chl) fluorescence of green plant tissue provides basic information on the function of the photosynthetic apparatus and on the capacity and performance of photosynthesis (52).

In our investigations we analysed the functional activity of photosynthetic apparatus in control nontransformed and transgenic tobacco plants, *Nicotiana tabacum* L. a bulgarian cv. Khan Tervel, carried the fused rat cytochrome P4501A1 and yeast reductase gene (PGF-6 construct), treated by chlorotoluron.

Chlorophyll fluorescence measurements were used to detect, follow and define the influence of this herbicide on photosynthetic activity up to 21st day after plant treatment (53).

The transgenic plants, expressing the fused rat cytochrome P4501A1/yeast reductase gene, were much more chlorotoluron resistant than the control plants. In nontransformed tobacco plants the electron transport flow to PQ pool was strongly inhibited one day after treatment with herbicide, whereas it was still existing in transgenic plants although a little reduction was observed.

The quantum yield of photosystem II (ΦPS2) which is related to the quantum yield of whole-chain electron transfer was much more inhibited by chlorotoluron than the primary PS II photochemistry, measured by the ratio Fv/Fm. Lower PS II activity was found for herbicide treated nontransformed plants up to the 9th day. Then it started to increase in both control and transgenic plants, but more rapidly in transgenic ones, and its values were near to the control level at the 21st day after chlorotoluron treatment.

The finding that the PS II activity started to recover after 9th day from the herbicide application is in agreement with that of (54), who observed recovery from damaging treatment with 10^{-5} M diuron of nontransgenic *Hypogymnia physodes* within weeks.

It was not found any other data in the literature on such kind of studies for the effect of the herbicide chlorotoluron or other herbicides on the state of photosystem II by the measurement of chlorophyll fluorescence.
cence of the tobacco resistant lines, expressing cytochrome P450 genes.

The state of photosystem II given by chlorophyll fluorescence is of importance to the physiology of a plant. Damage to PSII will often be the first manifestation of stress in a leaf.

Conclusions

Introduction of detoxification genes into crops provides the opportunity to enhance the selectivity of herbicides. Because of the practical importance of herbicide resistance of weeds advances in this area and commercial exploitation of herbicide-resistant transgenic crops are expected to increase in the near future.

Ohkawa et al. (1997) (32) concluded that mammalian microsomal CYP1A1 species shows a broad substrate specificity to xenobiotics. Expression of this P450 species in transgenic plants was found to exhibit cross tolerance to the structurally related phenylurea herbicides. This technology seems to be useful for genetic engineering to herbicide resistant crops as well as for phytoremediation. In addition, since many of plant P450 species are involved in secondary metabolism, expression of such a P450 species in plants appears to be useful for green chemistry. Particularly, coupling of a P450 monooxygenase with photosynthetic electron transfer systems in chloroplasts has a great potential for utilization of photoenergy in both oxidative degradation and synthesis of various chemicals as well as for reduction of oxygen stress in plants.

Chlorophyll fluorescence can give insights into the ability of a plant to tolerate environmental stresses and into the extent to which those stresses have damaged the photosynthetic apparatus. Chlorophyll fluorescence analysis can be applied successfully in ecophysiological studies.

The transgenic plants expressing mammalian P450 species have a great impact for generation of a model system for human metabolism of pesticides and for safety evaluation of agrochemicals and environmental contaminants.

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