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Enhanced herbicide cross-tolerance in transgenic rice plants co-expressing human CYP1A1, CYP2B6, and CYP2C19

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Abstract

We introduced an expression plasmid, pIKBACH, co-expressing the human cytochrome P450 genes *CYP1A1*, *CYP2B6*, and *CYP2C19*, into rice plants (*Oryza sativa* cv. 'Nipponbare'). The transgenic rice plants (pIKBACH rice) were screened by a combination of hygromycin resistance, PCR, and Western blot analysis. The pIKBACH rice plants expressed all three P450 species and exhibited tolerance towards various herbicides with different chemical structures and different modes of action, including a photosynthesis inhibitor (chlortoluron), very long-chain fatty acids (VLCFAs) inhibitors (acetochlor, metolachlor, and thenylchlor), a carotenoid biosynthesis inhibitor (norflurazon), and a root-elongation inhibitor (pyributicarb). In addition, the pIKBACH rice plants showed high tolerance to two mixtures of three herbicides [quizalofop-ethyl (0.15 μM), metolachlor (2 μM), and norflurazon (0.4 μM); and mefenacet (2.5 μM), thenylchlor (2 μM), and pyributicarb (1.5 μM)]. Thin-layer chromatography analysis revealed that the pIKBACH transgenic rice plants exhibited high metabolic activity towards chlortoluron, metolachlor, and norflurazon. The metabolism of herbicides by the pIKBACH rice plants was enhanced additively by the introduced P450 species. Assuming that public and commercial acceptance is forthcoming, pIKBACH rice plants may become useful tools for the breeding of herbicide-tolerant crops and for phytoremediation of environmental pollution by organic chemicals.

Keywords: Cytochrome P450; Phytoremediation; Detoxification; Chlortoluron; Metolachlor; Norflurazon

1. Introduction

Herbicides are widely used for the purpose of crop cultivation and are usually removed from the environment by natural degradation and degradation by bacteria and plants. Metabolism of herbicide in higher plants comprises three main stages: conversion, conjugation, and compartmentalization [1]. In the conversion step, cytochrome P450 monooxygenases insert one atom of oxygen to herbicides and the hydrophobic herbicides are converted to more hydrophilic metabolites through the oxidation, peroxidation or reduction. The herbicides usually are converted to less phytotoxic metabolites and some pro-herbicides are activated to herbicides in this process [2,3]. In the conjugation step, herbicides are directly conjugated with glutathione or the metabolites produced in the conversion step are conjugated with sugars or amino acids. These conjugated metabolites become highly water-soluble. Finally, the conjugated metabolites are converted to secondary conjugates or insoluble bound residues to be deposited in vacuoles or into the cell walls of plants [1].

Weeds resistant to various herbicides have been reported all over the world, covering the majority of known herbicide site of action and their chemical classes [4,5]. The resistance could result from modification of the target site, enhanced

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detoxification, and alterations in uptake, translocation, and compartmentalization of the herbicide [2]. Three enzyme systems including P450, glutathione *S*-transferase and arylacylamidase are known to be involved in resistance due to increased metabolic herbicide detoxification [6]. In the case of an agricultural crop, tolerance to herbicides is due to the ability of the crop to metabolize the herbicide. Two enzyme families that play major roles in conferring tolerance to herbicides are P450 monooxygenases and glutathione *S*-transferases [2].

Genome sequencing has revealed that P450 species constitute the largest family of enzymatic proteins in higher plants [7]. The *Arabidopsis* genome houses 273 P450 genes [8,9]. The current *Oryza sativa* genome-sequencing project has suggested that rice houses 452 named P450 genes [10]. Among those plant P450 species, relatively few involved in xenobiotics have been identified in plants [11–14].

In contrast, the enzymatic functions of mammalian P450 species have been well studied. Human P450 species that metabolize xenobiotic chemicals are almost exclusively in the CYP1, CYP2, CYP3 and to a lesser degree, CYP4 families [15]. Among them, 11 P450 species related to xenobiotic chemical metabolism are involved in over 90% of P450-dependent drug metabolism in the human liver [16]. Mice lacking expression of CYP1A1, CYP1B1, and CYP2E1 seem to be outwardly normal, indicating that these P450 species have no critical roles in development and physiological homeostasis [17,18]. These P450 species involved in xenobiotic metabolism exhibit broad and overlapping substrate specificity towards xenobiotics.

Inui et al. [19] reported that 11 human P450s metabolize various chemicals including 27 herbicides and four insecticides using recombinant yeast microsome expressing human P450 species. Transgenic plants harboring such mammalian P450 genes involved in xenobiotic metabolism were expected to be tolerant to the herbicides and be able to clean up the residual agrochemicals [2]. Phytoremediation—the use of plants to clean up polluted soils, sediments, and waters could prove to be an inexpensive and sustainable technology for bioremediation [20]. However, field trials have suggested that the rate of contaminant removal using conventional plants is insufficient [21]. Genetic manipulation of plants is considered to be able to increase the remedial capacity of plants significantly [22,23]. Application of the mammalian P450 enzyme system for genetic engineering of plants may prove useful for phytoremediation.

Transgenic tobacco plants have been engineered by introducing rat CYP1A1 to detoxify herbicide chemicals [24]. To monocots, human CYP1A1 has been introduced into rice plants (*Oryza sativa*), and the transgenic rice plants showed tolerance towards herbicides, including chlortoluron and quizalofop-ethyl [25]. Transgenic rice plants expressing human CYP2C9 and CYP2C19 showed tolerance towards the herbicides chlorsulfuron and norflurazon, respectively [26]. These transgenic rice plants expressing single P450 genes had a certain range of herbicide tolerance, but if P450 genes can be accumulated, the plants would show a much broader spectrum of herbicide tolerance.

We attempted to co-express three human P450 species—CYP1A1, CYP2B6, and CYP2C19—in rice plants. These three P450 species are supposed to metabolize many kinds of herbicides with different chemical structures and modes of action in plants because they can metabolize these herbicides in vitro [19]. The plants' herbicide tolerance and metabolic activities towards herbicides were evaluated with a view to assessing their usefulness in phytoremediation.

2. Materials and methods

2.1. Chemicals

^{14}C-ring-labeled chlortoluron—*N*-(3-chloro-4-methylphenyl)-*N*, *N*-dimethyl urea (specific activity, 2.99 MBq mmol^{-1}; radiochemical purity, >98%)—was provided by Novartis Crop Protection Inc. (Basel, Switzerland). ^{14}C-ring-labeled metolachlor—2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide (specific activity, 2.33 GBq mmol^{-1}; radiochemical purity, >98.9%)—and ^{14}C-ring-labeled norflurazon—4-chloro-5-(methylamino)-2-[3-(trifluoromethyl)phenyl]-3(2H)-pyridazinone (specific activity, 2.22 GBq mmol^{-1}; radiochemical purity, >99%)—were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, England).

2.2. Plasmid construction and plant transformation

Expression plasmid pIKBAC harboring human *CYP1A1*, *CYP2B6*, and *CYP2C19* cDNA in tandem was described previously [27]. A 2-kbp DNA fragment harboring hygromycin B phosphotransferase (HPT) was inserted into the *Cla*I site of pIKBAC, giving rise to pIKBACH (Fig. 1). The constructed expression plasmid was introduced into *Agrobacterium tumefasciens* strain EHA101, which was subsequently used for transformation of *Oryza sativa japonica* cv. 'Nipponbare' [28]. Regenerated plants (R_0) on culture medium containing 50 mg L^{-1} hygromycin were screened by PCR using three sets of human P450 specific primers (described below). The transgenic plants containing all three P450 genes were grown in a greenhouse and their seeds (R_1) were harvested. R_1 seeds were screened three times by germination tests in a 9-cm Petri dish with 40 mL of MS solid medium [29] containing 0.5 μM norflurazon, 100 μM chlortoluron, or 2.5 μM of thenylchlor. The screened R_1 progeny were used for further experiments.

Transgenic plants expressing single human P450 species (CYP1A1, CYP2B6, or CYP2C19) were also generated in the same manner [28] by using the expression plasmids shown in Fig. 1. R_1 seeds were screened with 40 mL of MS