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BRIEF COMMUNICATION

Influence of the herbicide chlortoluron on photosynthetic activity in transgenic tobacco plants

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Abstract

Photosynthetic activity of leaf disks from chlortoluron (2 μmol per plant) treated and non-treated non-transgenic and transgenic (PGF-6) tobacco plants was measured from 1 up to 21 d after treatment under greenhouse conditions. PGF-6 plants, expressing the fused rat cytochrome P4501A1/yeast reductase genes were used. PGF-6 plants were much more chlortoluron-resistant than control plants. In non-transgenic tobacco plants the electron transport flow to PQ pool was strongly inhibited 1 d after treatment with herbicide whereas it was still existing in PGF-6 plants although some reduction was observed. The quantum yield of photosystem 2 (Φ_{PS2}) which is related to the quantum yield of whole-chain electron transfer was much more inhibited by chlortoluron than the primary PS2 photochemistry, measured by the ratio F_v/F_m. Lower PS2 activity was found for herbicide-treated non-transgenic plants up to the 9th day. Then it started to increase in both control and PGF-6 plants, but more rapidly in PGF-6 ones, and its values were near to the control level at the 21st d after chlortoluron treatment.

Additional key words: chlorophyll fluorescence; photosystem 2 activity; rat cytochrome P4501A1.

Chlortoluron as a selective phenylurea herbicide negatively affects photosynthetic activity in plants. Similar to triazines and other phenylureas, this herbicide displaces plastoquinone (PQ) at the Q_B binding site on the D1 protein and thereby blocks electron flow from Q_A to Q_B (Ohkawa *et al.* 1998).

Cytochrome P450 monooxygenases are important in oxidative metabolism of many xenobiotics, including herbicides. Detoxification of chlortoluron in plants is probably mediated by cytochrome P450s (Ohkawa *et al.* 1998). Since mammalian P450 species show broad substrate specificity, the expression of each of these species in plants produces transgenic plants resistant to various herbicides (Ohkawa *et al.* 1997).

The study of the light-induced *in vivo* chlorophyll (Chl) fluorescence of green plant tissue provides basic information on the function of the photosynthetic apparatus and on the capacity and performance of photosynthesis (Lichtenthaler and Rinderle 1988, Krause and Weis 1991). At physiological temperatures fluorescence originates mainly from Chl *a* of PS2 (Papageorgiou 1975) and reflects the primary processes of photosynthesis, such as PAR absorption, distribution and transport of the exciting energy, and the photochemical reaction in PS2 (Krause and Weis 1984). However, due to the functional relation of PS2 to the other photosynthetic apparatus components, fluorescence yield is an indirect indicator for the condition of the integral photosynthetic process.

The aim of present investigation was to analyse the functional activity of photosynthetic apparatus in control non-transgenic and transgenic (PGF-6) tobacco plants treated by chlortoluron. Chl fluorescence measurements were used to detect, follow, and define the influence of this herbicide on photosynthetic activity up to the 21st d after plant treatment.

Genetically modified tobacco plants from *Nicotiana tabacum* L. cv. Khan Tervel 39 carry the fused rat cytochrome P4501A1 and yeast reductase genes for resistance to chlortoluron at dose 50 μmol per plant (Yordanova *et al.* 2000). The transgenic To line PGF-6, with the highest resistance to the herbicide, was multiplied *in vitro* and the obtained genetically identical plantlets were grown in

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Abbreviations: Chl – chlorophyll; F_0 – dark, F_m – maximal, F_v – variable, and F_s – steady state Chl fluorescence; F_0' and F_m' – ground and maximal Chl fluorescence measured in light state; PQ – plastoquinone; PS2 – photosystem 2; q_P and q_N – photochemical and non-photochemical fluorescence quenching.

a greenhouse at 25 °C, irradiance of 30 μmol m^{-2} s^{-1}, and photoperiod of 16/8 h light/darkness. After one month, control and PGF-6 tobacco plants were treated with 2 μmol per plant chlortoluron (commercial name Tolurex) at vegetative stage of 5-6 leaves. Leaf disks from treated and non-treated control and PGF-6 plants were taken up to 21 d after treatment.

Chl fluorescence kinetics from the upper leaf surface was measured with a pulse amplitude modulation fluorometer (*PAM 101-103, H. Walz*, Germany) as described by Schreiber *et al.* (1986). The initial fluorescence yield F_0 in weak modulated radiation (0.075 μmol m^{-2} s^{-1} photon flux density, PFD), and maximum total fluorescence yield F_m during a saturating "white light" pulse (1 s, over 3 500 μmol m^{-2} s^{-1} PFD, by *Schott KL 1500* radiation source) were determined. The leaf disc (1-cm diameter) was then irradiated with continuous red radiation (125 μmol m^{-2} s^{-1} PFD). When the measuring radiation was applied alone, a modulation frequency of 1.6 kHz was used, otherwise the modulation frequency was set to 100 kHz. The short pulses (with 20-s interval) on the background of a red radiation were used to obtain the fluorescence intensity F_m' with all PS2 reaction centres closed in any light adapted state. F_v/F_m estimated the photochemical activity of PS2, measured after dark adaptation. Three Chl fluorescence parameters were calculated from the measurement of F_m', F_0', and F_s (maximum, ground, and steady-state fluorescence emission, respectively) for a leaf exposed to actinic radiation: (*1*) F_v'/F_m', which estimates the relative photochemical efficiency of open (oxidised) PS2 traps, (*2*) Φ_{PS2} calculated as $(F_m' - F_s)/F_m'$ that is a relative quantum yield of PS2 photochemistry, and (*3*) the photochemical fluorescence quenching q_p which estimates the relative change of PS2 photochemical yield depending upon the concentration of open PS2 reaction centres. Induction kinetics were registered and analysed with a program *FIP 4.3* (Tyystjärvi and Karunen 1990). Experimental values were processed statistically by the Student's *t*-test.

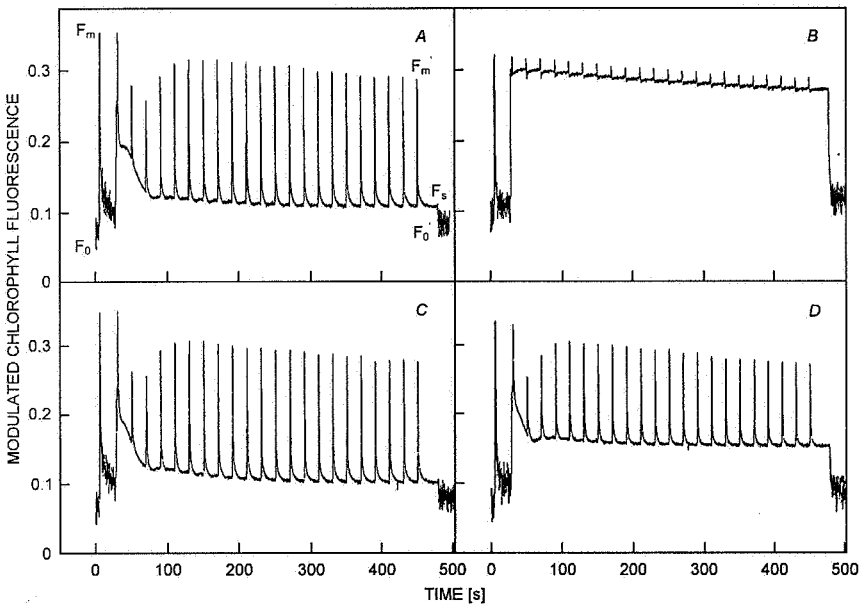

Fig. 1. Time course of modulated chlorophyll fluorescence measured 1 d after chlortoluron treatment. *A* - control (non-transgenic) plants; *B* - chlortoluron treated non-transgenic plants; *C* - transgenic plants; *D* - chlortoluron treated transgenic plants.

The original Chl fluorescence traces measured in control (Fig. 1*A*) and PGF-6 (Fig. 1*C*) tobacco plants prior to and 1 d after their treatment with the herbicide (Fig. 1*B, D*) show that this herbicide strongly inhibited the electron transport flow to PQ pool in control plants and to a much lesser extent in transgenic plants. The photochemical activity of PS2 measured after dark adaptation as F_v/F_m decreased by 20 % 1 d after spraying the control tobacco plants with chlortoluron and was significantly lower than control up to the 9th d (Fig. 2*A*). Than it began to increase and reached the control value at the 21st d. The reduction of F_v/F_m was mainly due to increased F_0 whereas F_m did not change considerably. Chlortoluron induced a 36 % increase in F_0 1 d after the treatment of control plants and *ca.* 150 % increase after 9 d (values not shown). Chlortoluron reduced slightly the F_v/F_m ratio in PGF-6 plants during the first days after their treatment, but later F_v/F_m reached the control value (Fig. 2*A*).

F_v'/F_m' sharply decreased 1 d after chlortoluron treatment of control tobacco plants (by 55 %), then started to increase but it was significantly lower than control 12 d after herbicide treatment (Fig. 2*B*). In PGF-6 plants this ratio was more influenced by chlortoluron treatment than the primary PS2 photochemistry (F_v/F_m) measured after dark adaptation (Fig. 2*A*). F_v'/F_m' declined by 27 % 1 d after herbicide treatment of these plants and then it started

to increase.

The values of Φ_{PS2} measured in non-treated control and PGF-6 plants were similar (Fig. 2*C*). However, chlortoluron reduced Φ_{PS2} by 90 % in control plants and by 30 % in PGF-6 plants 1 d after their treatment. Φ_{PS2} was strongly reduced up to the 9th d after chlortoluron treatment especially in control plants, then it started to recover but was still under the control level up to the 21st d.

Chlortoluron induced similar changes in the photochemical fluorescence quenching q_P in all investigated groups of plants (Fig. 2*D*), but q_N was higher than the control by 91 and 46 % in control and transgenic plants, respectively, only 1 d after the herbicide treatment (values not shown).

Thus transgenic tobacco plants were much more resistant to the herbicide than control plants. Also trans-

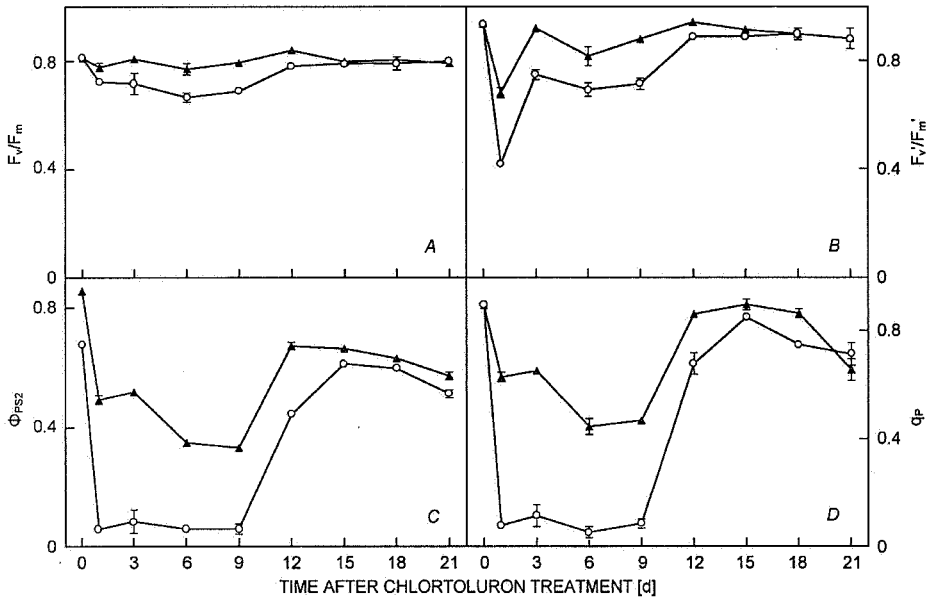

Fig. 2. Changes in PS2 activity of transgenic and non-transgenic tobacco plants after chlortoluron treatment estimated by: the photochemical activity of PS2 measured after dark adaptation (F_v/F_m, *A*) and in light adapted state (F_v'/F_m', *B*), and in quantum yield of PS2 photochemistry (Φ_{PS2}, *C*) and photochemical fluorescence quenching (q_P, *D*). o - chlortoluron treated non-transgenic plants; ▲ - chlortoluron treated transgenic plants.

genic tobacco plants *Nicotiana tabacum* L. cv. Xanthi expressing the same rat cytochrome P4501A1/yeast reductase fused enzyme (Shiota *et al.* 1994) showed resistance to chlortoluron and metabolised it more rapidly than did the control plants. Five metabolites in the extracts of both plant types were produced but large amounts of non-phytotoxic metabolites were obtained in transgenic than control plants. Thus herbicide resistance of transgenic plants may be due to enhanced monooxygenase activities in the metabolism of chlortoluron yielding these non-phytotoxic metabolites (Shiota *et al.* 1996). We found that the electron transport was strongly inhibited after treatment of control plants with chlortoluron whereas it still existed in PGF-6 plants although some reduction was observed (Fig. 1).

The ratio F_v/F_m is considered a measure of PS2 effectiveness in the primary photochemical reactions (Butler 1977). This ratio is remarkably constant among non-stressed dark-adapted plants regardless of the species studied. Stress factors, affecting mainly PS2 function, reduce the F_v/F_m values. Lower F_v/F_m was found for herbicide-treated control plants from the first up to the ninth day. Reduced PS2 activity as a result of herbicide application was often found (Vidal *et al.* 1995, Carter *et al.* 1996, Fernandez *et al.* 1999, Jensen *et al.* 1999).

We found that PS2 activity was inhibited to a greater extent in chlortoluron-treated control plants than in transgenic plants. The reduction of F_v/F_m was mainly due to increased F_0. In a native photosynthetic object F_0 reflects the state of antenna Chl and is a measure for the initial distribution of energy to PS2 and the efficiency of excitation capture in P680. The inactivation of D1-protein of the PS2 reaction centre leads to an increase in F_0 (Rintamäki *et al.* 1994).

In our experiments F_v'/F_m', Φ_{PS2}, and q_P were more inhibited than primary PS2 photochemistry, measured as F_v/F_m. It is not surprising since chlortoluron inhibits electron transport to the PQ pool. These results also confirmed the higher sensitivity to chlortoluron of control plants in comparison to the transgenic ones. A measurement of all these parameters revealed that the PS2 activity in chlortoluron treated transgenic plants was at least 50 % higher than in chlortoluron-treated control plants.

The finding that the PS2 activity started to recover after the 9th d from the herbicide application (Fig. 2*A, B, C*) is in agreement with that of Jensen *et al.* (1999) who

observed recovery from damaging treatment with 10^{-5} M diuron of *Hypogymnia physodes* within weeks. Vidal *et al.* (1995) found that plant tissues detoxified the herbicide methabenzthiazuron, and one month after plant emergence, photosynthesis was fully recovered in herbicide-treated plants; biomass production recovered and even increased at the end of the growth period. Triazine resistance, which is one of the most prevalent types of herbicide resistance found in weeds, is mostly due to a modification at the target site of D1 protein of the PS2 in chloroplasts. Foes *et al.* (1999) showed that one *Kochia scoparia* biotype was resistant to herbicide atrazine. In this case resistance to this herbicide was conferred by amino acid substitution in D1 protein. Another mechanism of resistance that is widely spread in most crops is due to the ability of the crop to metabolise the herbicide and thereby prevent injury. In mammals many cytochrome P450 species are involved in oxidative metabolism of xenobiotics. Detoxification of chlortoluron in transgenic tobacco and potato plants, expressing the fused enzyme between rat cytochrome P4501A1 and yeast reductase is due to enhanced metabolism of the herbicide (Ohkawa *et al.* 1997). Hall *et al.* (1995) examined the effect of chlortoluron using two resistant biotypes *of Alopecurus myosuroides.* They found no significant differences between resistant and susceptible biotypes in rates of herbicide uptake and translocation. Similarly, the herbicide target PS2 was equally sensitive to inhibition by chlortoluron in all biotypes, indicating that changes at the herbicide active site were not responsible for resistance. Both resistant biotypes had a markedly enhanced ability to metabolise chlortoluron. Chlortoluron metabolism was decreased and herbicide phytotoxicity correspondingly increased by treatment in the presence of cytochrome P450 inhibitor 1-aminobenzoiriazole, suggesting that increased activity of cytochrome P450 monooxygenases may be responsible for herbicide degradation and thus for herbicide resistance in two resistant biotypes.

References

Butler, W.L.: Chlorophyll fluorescence: a probe for electron transfer and energy transfer. – In: Trebst, A., Avron, M. (ed.): Photosynthesis I. Pp. 149-167. Springer–Verlag, Berlin – Heidelberg – New York 1977.

Carter, G.A., Cibula, W.G., Miller, R.L.: Narrow-band reflectance imagery compared with thermal imagery for early detection of plant stress. – J. Plant Physiol. **148**: 515-522, 1996.

Fernandez, R., Whitwell, T., Riley, M., Bernard, C.: Evaluating semiaquatic herbaceous perennials for use in herbicide phytoremediation. – J. amer. Soc. hort. Sci. **124**: 539-544, 1999.

Foes, M., Liu, L., Vigue, G., Stoller, E., Wax, L., Tranel, P.: A kochia (*Kochia scoparia*) biotype resistant to triazine and ALS-inhibiting herbicides. – Weed Sci. **47**: 20-27, 1999.

Hall, L., Moss, S., Powles, S.: Mechanisms of resistance to chlorotoluron in two biotypes of the grass weed *Alopecurus myosuroides.* – Pestic. Biochem. Physiol. **53**: 180-192, 1995.

Jensen, M., Linke, K., Dickhauser, A., Feige, G.: The effect of agronomic photosystem II herbicides on lichens. – Lichenologist **31**: 95-103, 1999.

Krause, G.H., Weis, E.: Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals. – Photosynth. Res. **5**: 139-157, 1984.

Krause, G.H., Weis, E.: Chlorophyll fluorescence and photosynthesis: The basics. – Annu. Rev. Plant Physiol. Plant mol. Biol. **42**: 313-349, 1991.

Lichtenthaler, H.K., Rinderle, U.: The role of chlorophyll fluorescence in the detection of stress conditions in plants. – CRC crit. Rev. anal. Chem. **19**: S29-S85, 1988.

Ohkawa, H., Hiromasa, I., Shiota, N., Inui, H., Ohkawa, Y.: Molecular mechanisms of herbicide resistance. – Rev. Toxicol. **2**: 245-252, 1998.

Ohkawa, H., Shiota, N., Inui, H., Sigiura, M., Yabusaki, Y., Ohkawa, Y., Ishige, T.: Herbicide-resistant tobacco and potato plants expressing mammalian P450 monooxygenases. – In: Hatzios, K.K. (ed.): Regulation of Enzymatic System Detoxifying Xenobiotics in Plants. Pp. 307-312. Kluwer Acad. Publ., Dordrecht 1997.

Papageorgiou, G.: Chlorophyll fluorescence: an intrinsic probe of photosynthesis. – In: Govindjee (ed.): Bioenergetics of Photosynthesis. Pp. 319-371. Academic Press, New York – San Francisco – London 1975.

Rintamäki, E., Salo, R., Aro, E.-M.: Rapid turnover of the D1 reaction-center protein of photosystem II as a protection mechanism against photoinhibition in a moss, *Ceratodon purpureus* (Hedw.) Brid. – Planta **193**: 520-529, 1994.

Schreiber, U., Schliwa, U., Bilger, W.: Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. – Photosynth. Res. **10**: 51-62, 1986.

Shiota, N., Inui, I., Ohkawa, H.: Metabolism of the herbicide chlortoluron in transgenic tobacco plants expressing the fused enzyme between rat cytochrome P4501A1 and yeast NADPH-cytochrome P450 oxidoreductase. – Pestic. Biochem. Physiol. **54**: 190-198, 1996.

Shiota, N., Nagasawa, A., Sakaki, T., Yabasaki, Y., Ohkawa, H.: Herbicide-resistant tobacco plants expressing the fused enzyme between rat cytochrome P4501A1 (CYP1A1) and yeast NADPH-cytochrome P450 oxidoreductase. – Plant Physiol. **106**: 17-23, 1994.

Tyystjärvi, E., Karunen, J.: A microcomputer program and fast analogy to digital converter card for the analysis of fluorescence induction transients. – Photosynth. Res. **26**: 127-132, 1990.

Vidal, D., Martinez-Guijarro, J., Simon, E.: Chlorophyll fluorescence and photosynthetic O_2 evolution in *Vicia faba* plants treated with methabenzthiazuron. – Photosynthetica **31**: 9-20, 1995.

Yordanova, E., Gorinova, N., Bachvarova, R., Nedkovska, M., Atanassov, A., Ohkawa, H.: Genetic transformation of tobacco with rat cytochrome P4501A1 monooxygenase gene. – Bulg. J. agr. Sci. **6**: 1-7, 2000.