THE CHANGES IN SOME PHOTOSYNTHETIC CHARACTERISTICS OF TRANSGENIC TOBACCO PLANTS, RESISTANT TO BACTERIA

PSEUDOMONAS SYRINGAE PV. TABACI

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ABSTRACT

The changes in the photosynthetic reactions of tobacco plants, infected by Pseudomonas syringae pv. tabaci were investigated. The protection against pathogen were studied in sensitive to wildfire disease tobacco plants - Nevrokop 1146 (N1146) and pigment deficient “aurea-like mutant”, A1146. The responses were compared with a transgenic resistant line of Nevrokop 1146 - (T1146) developed by Batchvarova et al, (1998) and carrying the ttr (tabtoxin resistance) gene. The changes in thylakoid electron transport, especially on PS2, were evaluated by induction curves of variable and the delayed fluorescence. Correlation with some changes in enzymatic processes related to fast and slow protection against pathogen was also investigated. More expressed effects of infection on photosynthesis in N1146 and A1146 than in T1146 were probably due to the direct tabtoxin action on both chlorophyll a and b. The hypersensitive reaction was expressed during the initial stages of the bacterial infection as evaluated by enhanced peroxidase activity. The increase in reduced sugars and α-amylase activity was related to development of later stages of protection against pathogen. The investigations showed that the changes in the photosynthetic apparatus could not only be correlated to the tabtoxin action and thence limited photorespiration. The resistant T1146 did not develop typical disease symptoms because of the limited tabtoxin action but pathogen induced changes in photosynthesis were obtained. The differences in the effects in N1146 and A1146 were probably due to their possible different chloroplast structure.

Introduction

Wildfire is one of the most economically imported diseases in tobacco plants. The pathogen is the bacteria Pseudomonas syringae pv. tabaci that produces tabtoxin (21) induces the chlorotic haloes on the tobacco leaves and destroys photosynthetic machinery (15). A technique for introducing the gene for resistance in different

Abbreviations: A1146 – Aurea tobacco mutant; chl a and b – chlorophyll a and b, respectively; car – carotenoid; HR – hypersensitive reaction; DF – delayed fluorescence; F – fluorescence; FW – fresh weight; GDHP – guaiacol dehydrogenated product; N1146 – Nevrokop tobacco cultivar; PFD – photon flux density; PS1, PS2 – photosystem 1 and 2, respectively; Qₐ and Qₘ – the first and second electron acceptor of PS2; Rfd – vitality index
tobacco cultivars has been developed (3). The plants possess different protection mechanisms against pathogens. Common and fast response of plants to infection by bacteria and other incompatible pathogens is the development of hypersensitive reaction (HR). The HR is localized necrosis, which is a result of the development of programmed cell death and correlates with the active defense of the plants (14, 11). HR includes activation of reactive oxygen species. For metabolic and protective processes is necessary to support the balance between synthesis and cleavage of reactive oxygen species, especially during the stress conditions. Among many enzymes, important place in these reactions have peroxidase enzymes as ascorbate peroxidase and guayacol peroxidase (Asada, 1992). After HR slower reactions could be included in protective mechanisms against pathogen infection. The increased activity of α-amylase in barley and tobacco was observed (Heitz et al., 1991). Starch hydrolysis provides metabolic substrates for stressed plant cells.

The photosynthesis is the most sensitive process during abiotic and biotic stresses (2). The biotic stress has particular effects on photosynthesis (Osmond et al., 1990, 19, 7) and especially on photosystem 2 (18, 12, 20). The tabtoxin destroys chloroplasts and inhibits the many key enzymes in the photorespiratory nitrogen cycle. The influence of the bacterial infection on the electron-transport reactions in the thylakoid membranes, especially on PS2 level and on distribution of photoassimilates remains obscure. The damages of the photosynthetic apparatus, which are possibly result of the bacterial infection, were mainly in the acceptor side of photosystem 2 and were related to the electron transfer between QA and QB (15). Some changes in infected tobacco leaves were probably a result of the modification in the mechanisms of pH-gradient accumulation, but not of the ATP-ase damaging as it was evaluated by photoacoustic measurements. This suggestion is in agreement with data from millisecond delayed fluorescence, DF (15).

In the present study, some changes in the photosynthetic reactions of tobacco plants, infected by Pseudomonas syringae pv. tabaci were investigated. The thylakoid electron transport reactions, especially changes in the PS2 electron transport, evaluated by chlorophyll a fluorescence, F, and induction curves of the delayed fluorescence, DF (8) were used. Slow phase in F and DF induction curves are dependent from changes in the oxygen metabolism (Schreiber et al. 1996) and changes in dark reactions. Also, the correlation with changes in some enzymatic processes connected with fast and slow developed protection against pathogen were studied in sensitive to wildfire disease tobacco plants - Nevrokop 1146 (N1146) and in a transgenic resistant line of Nevrokop 1146 - (T1146), carrying the trr (tabtoxin resistance) gene. A pigment deficient tobacco mutant similar to Aurea (6) was used for a better characterization of the processes in photosystem 2 - line A1146 (1).

Materials and Methods

Plant material and inoculation

Eight-week-old tobacco plants (Nicotiana tabacum L.) were used in the experiments. All plants were grown as soil cultures in greenhouse conditions with 120 µmol. m⁻².s⁻¹ PFD, 16h photoperiod, 25/20 °C day/night temperature and 60-80% relative humidity. More details were presented in Milanov et al. 1997. The transgenic tobacco plants resistant to wildfire (T1146), carrying trr-gene, were obtained by genetic transformation according to Horsch et al. (13), on the basis of N1146 (3). Aurea mutant tobacco plants (A1146), obtained from Nevrokop 1146 (1) were also infected for investigation.

The investigations were carried out with healthy and infected plants - 1, 5 and 10 days after bacterial inoculation. Eight-
week-old tobacco plants with 6 to 8 fully expanded leaves were infected with inoculum prepared from bacteria *Pseudomonas syringae* pv. *tabaci*. Bacteria were grown as described by Bachvarova et al. (3). Inoculum concentration was adjusted spectrophotometrically at 10^8 cells.ml⁻¹. Fifth and sixth fully expanded leaves were pricked with a needle, sprayed with the bacterial suspension and grown at high humidity (95%) in a greenhouse at 25 °C.

**Fluorescence and delayed fluorescence measurements**

F and DF induction curves were measured simultaneously at room temperature using a fluorometer Fl-2006 (Test, Russia). The registration of induction transients (Kautsky effect) was described in details elsewhere (21). Signal recording (Fig. 1) and data acquisition were performed on a personal computer using Fl2006 data software.

**Pigment analysis**

Pigment analysis was carried out by absorption spectrophotometer (Shimadzu UV 200, Japan) according to a standard procedure, described by Lichtenthaler (16). The pigments were extracted by 80% (v/v) acetone.

**Enzyme analyses**

**Reduced sugars.** Reduced sugars were determined spectrophotometrically with DSA (3,5 - dinitro salicylic acid) according to Plummer (17). The results were expressed as mg glucose.g⁻¹ FW.

**The activity of α-amylase (EC 3.2.1.1.)** was estimated by modified method of Plummer (17). The activity was expressed as mg maltose.mg⁻¹ protein.
**Protein content** was estimated by the Bradford method (5).

**Peroxides activity** was evaluated by Hart et al. (10). The activity was expressed as $\mu$mol guaiacol dehydrogenated product, GDHP.mg$^{-1}$.protein.min$^{-1}$, using extinction coefficient of GDHP ($\varepsilon=26.6$ mM$^{-1}$.cm$^{-1}$).

**Statistics.** The data were presented on the figures as average values from two independent experiments. For verification of the results the Student’s t-criteria at $p<0.05$ was used.

**Results and Discussion**

There are different responses to bacterial infection with *Pseudomonas syringae* pv. *tabaci* in tobacco leaves. N1146 developed typical symptoms of the wildfire disease. On the beginning of the infection chlorotic haloes were appeared. Further they were converted into necrotic spots, caused by the tabtoxin action. In A1146 mutant plants the infected leaves became yellow and necrotic lesions was not observed. Despite the lack of necrosis, the decrease in the studied photosynthetic parameters was the same and even greater extent than in N1146.

**Changes in the pigment composition**

Non-infected tobacco leaves had higher pigment concentrations in T1146 plants (Fig 2A-C). Plants of A1146 are characterized with decreased chl $b$, which is typical for aurea type tobacco leaves. The infection development caused a sharp decrease in chlorophyll $a$ and chlorophyll $b$ concentrations in N1146 and A1146 (Fig. 2A and B), which was related to tabtoxin action. In both N1146 and A1146 the carotenoid concentration increased up to the fifth day after infection, and then returned to the control values (Fig. 2C). The decrease in ratio chl $a$ (Fig. 3) supposes some destruction in thylakoid membrane system, while changes in chl $a+b$ to carotenoid ratio (Fig. 4) are a result of the protection against photooxidation processes. The pigment changes in T1146 were poorly expressed (Fig. 2A-C, 3 and 4). It is probably as a result of the development of resistance to the tabtoxin action.
The changes in peroxidase and α-amylase activity and reduced sugars.

The HR was expressed during the initial stages of the bacterial infection as evaluated by enhanced peroxidase activity (Fig. 5). Except of N1146, the activity of peroxidase (Fig. 5) possessed fast response first day after infection in comparison to other studied enzymes (see Fig. 6 and 7). There are no changes in peroxidase activity in all

![Graph showing changes in chl a/b ratio in tobacco plants infected with Pseudomonas syringae pv. tabaci](image1)

**Fig. 3.** Changes in chl \(a/b\) ratio in tobacco plants infected with *Pseudomonas syringae pv. tabaci* (1d, 5d and 10d) wild type, N1146 (circles, solid line), transgenic, T1146 (down triangles, dashed line) and aurea, A1146 (up triangles, dotted line) tobacco leaves. Presented data are from 4 independent experiments and data are average from 6 to 9 replicas.

![Graph showing changes in chl a+b/car ratio in tobacco plants infected with Pseudomonas syringae pv. tabaci](image2)

**Fig. 4.** Changes in chl \(a+b/car\) ratio in tobacco plants infected with *Pseudomonas syringae pv. tabaci* (1d, 5d and 10d) wild type, N1146 (circles, solid line), transgenic, T1146 (down triangles, dashed line) and aurea, A1146 (up triangles, dotted line) tobacco leaves. Presented data are from 4 independent experiments and data are average from 6 to 9 replicas.
Days after infection

Fig. 5. Changes in peroxidase activity in tobacco leaves infected with *Pseudomonas syringae pv. tabaci* (1d, 5d and 10d) wild type, N1146 (circles, solid line), transgenic, T1146 (down triangles, dashed line) and aurea, A1146 (up triangles, dotted line) tobacco leaves. Presented data are from 2 independent experiments and data are average from 4 to 6 replicas.

Fig. 6. Changes in α-amylase activity in tobacco leaves infected with *Pseudomonas syringae pv. tabaci* (1d, 5d and 10d) wild type, N1146 (circles, solid line), transgenic, T1146 (down triangles, dashed line) and aurea, A1146 (up triangles, dotted line) tobacco leaves. Presented data are from 2 independent experiments and data are average from 4 to 6 replicas.

non-infected leaves (Fig. 5). The first day after infection A1146 showed more expressed changes, only. N1146 and T1146 had similar changes 5 days after infection, while peroxidase activity in A1146 increased significantly. Peroxidase activity in 5 and 10 days infected T1146 began to decrease, while activity in A1146 remained to increase. It was observed that peroxidase activity in A1146 was almost 4 fold higher than in non-infected leaves. Limited HR causes increased pathogen induced damages in N1146. An additional difference between infected and uninfected plants
Fig. 7. Changes in reduced sugars content in tobacco leaves infected with *Pseudomonas syringae* pv. *tabaci* (1d, 5d and 10d) wild type, N1146 (circles, solid line), transgenic, T1146 (down triangles, dashed line) and aurea, A1146 (up triangles, dotted line) tobacco leaves. Presented data are from 2 independent experiments and data are average from 4 to 6 replicas.

During development of HR was the greater amount of total carotenoids, reaching maximal concentration in N1146 and A1146 five days after the infection (Fig. 2C).

The changes in reduced sugars and α-amylase activity are related to development of later stages of protection against pathogen. The activity in α-amylase tended to the increase but slower than peroxidase activity in infected leaves (Fig. 6). Again, A1146 had highest activities 10d after infection. Increased enzyme activities possibly correlated with enhanced metabolic activity in infected plants, expressing accelerated carbohydrate breakdown especially starch.

The highest concentration of reduced sugars in non-infected A1146 was detected 1.320 mg glucose•mg⁻¹ FW (Fig. 7). It was not observed the great differences between N1146 and T1146. The infection increased in A1146 and there was less increase in N1146 and especially in T1146 10 days after infection. The increase in reduced sugars and α-amylase activity is related to development of later stages of protection against pathogen, when HR is finished. On the other hand, T1146 are resistant to tabtoxin action. In this case, T1146 have no need to develop the above-mentioned mechanisms for protection against pathogen invasion.

**Changes in parameters of the induction curves of F and DF**

Typical traces of F and DF induction curves registered simultaneously by fluorometer FL2006 are shown on Figure 1. The reasons for the altered photosynthetic characteristics were different in N1146 and A1146. The investigations showed that the changes in the photosynthetic apparatus could not only be correlated to the tabtoxin action and thence limited photorespiration. T1146 did not develop typical disease symptoms because of the limited tabtoxin action. On the other hand, changes in F and DF induction curves were observed (Fig. 10B). In this case, pathogen induced changes in photosynthesis in T1146 was independent by photorespiratory activity (15).

The fluorescence ratio (I-O)/(P-O) evaluated by Kautsky effect registered at very low
The changes in \((I-O)/(P-O)\) fluorescence ratio in control and infected with bacteria *Pseudomonas syringae* pv. *tabaci* (1d, 5d and 10d) wild type, N1146 (circles, solid line), transgenic, T1146 (down triangles, dashed line) and aurea, A1146 (up triangles, dotted line) tobacco leaf disc with area 1 cm\(^2\). The measurement is conducted in 5min dark adapted samples. The registration of Kautsky effect was made at AL of 50 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) PFD. The results are average from 3 – 6 replicas.

The fast phase of DF curve, \(I_1D_1I_2D_2\), of induction curves (Fig. 1) reflected increase in recombination of separated charges in PS2 reaction centers that is influenced by accumulated membrane electric gradient, \(\Delta\psi\) (23). The slow phase, \(D_2\text{max}S\) gives information about possibility for increased charge recombination during accumulation of transmembrane proton gradient, \(\Delta\text{pH}\) (8, 22). Figure 10 represents changes in the induction curves of the millisecond DF. In the uninfected leaves fast phase has the highest DF intensity in A1146 (Fig. 10C). And it was the lowest in T1146 (Fig. 10B), which suggested increasing of electron flux through PS2 to PS1 (charge recombination in PS2 were lower). In infected N1146 relative share of \(I_1\) towards \(I_2\) increased during infection development. On the other hand, the relative share of \(I_2\) towards \(I_1\) remained higher in infected T1146 (Fig. 10B).

Minimum \(D_2\) reflects accumulation of closed reaction centers of PS2, i.e. \(Q_A^-\) after photoinduced plastoquinone pool reducing (22). In closed centers possibility for charge recombination that induced millisecond DF was decreased. \(D_2\) disappear and it quickly reached \(I_{\text{max}}\) during 1 day after the infection. During further stages of infection \(D_2\) reached values closer to \(I_1\) and \(I_2\) (Fig. 10A-C).

Intensity of \(I_{\text{max}}\) and whole slow phase of...
DF decreased in plants infected before 5 and 10 days (Fig. 10A-C). The rate of decrease in $I_{\text{maxS}}$ was accelerated in first day infected N1146 and A1146 sensitive to disease tobacco plants.

Except of 1d-infected N1146, the decreased number of $Q_B$-nonreduced PS2 reaction centers (Fig. 8) with infection development was not related to the increase of the photochemical activity, detected by changes in DF (Fig. 10A-C). Possibly, the damages in acceptor side of PS2 between $Q_A$ and $Q_B$ are occurred (15). The increased level of $Q_B$-nonreduced PS2 centers in N1146 could be a result of tabtoxin-induced decreased photorespiratory activity, which would be reversibly limited photochemical activity on PS2 level due to over-reduction of plastoquinone pool. In this case the $Q_B$-nonreduced PS2 centers remains functional. The changes in Rfd express the development of the additional regulatory mechanisms on photochemical activity (Fig. 9). HR results in a faster accumulation of the proton gradient due to increased electron transport rate and faster reaching to $I_{\text{max}}$. During the late stages of the infection, the proton gradient dissipation was probably a result of some tabtoxin induced uncoupling (15) and/or increased thermal dissipation as estimated by increased Rfd (Fig. 9) and faster decreases in $I_{\text{maxS}}$ (Fig. 10). Transgenic plants showed the highest chlorophyll content in the leaves, and the highest photochemical activity, evaluated by F and DF. The $O_2$- evolution measurements with a Clark electrode (data not shown) also confirmed it. The differences in the effects in N1146 and A1146 were due to their possible different chloroplast structure. Such effects were also expressed in transgenic plants but in smaller extent.

In conclusion, the availability of changes in photosynthetic activity in transgenic tobacco plants independently of well-known tabtoxin effects on tobacco leaves supposed different influence of bacteria on
Fig.10. Millisecond DF induction curves in control and infected with bacteria *Pseudomonas syringae pv. tabaci* (control, 1d, 5d and 10d) wild type, N1146 (A), transgenic, T1146 (B) and aurea, A1146 (C) tobacco leaves. The conditions of the measurement are the same as presented on the fig. 1. The curves are average from 3 – 6 replicas.

photosynthesis in T1146.

REFERENCES