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Effects of biotic stress caused by *Potato virus Y* on photosynthesis in *ipt* transgenic and control *Nicotiana tabacum* L.

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Abstract

We studied the effect of biotic stress caused by *Potato virus* Y^{NTN} (PVY) on photosynthesis in transgenic P*ssu-ipt* tobacco overproducing endogenous cytokinins (CK) in comparison with control (non-transformed) plants. Both control and transgenic tobacco were grown as rooted or grafted plants. Content of viral protein increased significantly in control tobacco within ca. 18 days after inoculation, whereas transgenic plants exhibited much lower accumulation. This corresponded also with the presence of visible symptoms of PVY infection; while they were always present in control, rooted tobacco, they never developed in transgenic grafts. Contents of CKs (mostly in the forms of *N*- and/or *O*-glucosides) increased in all infected plants except transgenic grafts, where the highest amount of CKs was found already prior the inoculation. The photosynthetic rate (P_N) was significantly inhibited by PVY infection in control and transgenic rooted plants, while both grafted types were less affected. Reduction of P_N was caused not only by stomata closure, but also by the decrease of ribulose-1,5-bisphosphate carboxylase/oxygenase activity, contents of chlorophylls and xanthophyll cycle pigments, and activity of photosystem II (PSII). The negative effect on PS II was promoted by high irradiance treatment particularly in both rooted types infected by PVY.

Keywords: *Potato virus Y*; Photosynthesis; Transgenic tobacco; *ipt*; Cytokinins

1. Introduction

Plant virus diseases have highly damaging effects on crop productivity [1]. *Potato virus Y* (PVY) belongs to genus *Potyvirus* (family Potyviridae), the largest group of plant viruses [2]. PVY is highly variable due to a wide range of host species. PVY^{NTN} isolates belong to PVY^{N} subgroup according to their reaction with characteristic necrotic symptoms on *Nicotiana tabacum*. The isolates of PVY have a capacity to infect tobacco systemically. In tobacco, PVY^{NTN} causes the veinal necrosis in the leaves, occasionally also a leaf distortion and stem necrosis. *Potyvirus* genom is formed by a single molecule of RNA. It is translated into one protein molecule. Single functional proteins are released from that large polypeptide by three different viral proteases. This results in several proteins with different function including a coat protein (CP). Potyviruses induce *in vivo* formation of cytoplasmic and nuclear inclusions in host cells containing aggregates of viral proteins. For PVY infection the inclusions called ''pinwheels'' and ''bundle-like'' structures are typical [2]. PVY induce also the formation of non-crystalline amorphous inclusions within the cytoplasm of infected cells.

Most research concerning plant viruses has been directed towards understanding the structure, genetics, transport and localization of viruses in plants. However, much less is known about the impact of virus infection on host plant physiology [3].

Many studies reported that viral infections lead to sugar accumulation and alter photosynthetic capacity [4,5]. The symptoms of virus infection seem to correlate with the presence of viral proteins inside the plastids. Deterioration of chloroplast ultrastructure, pigment composition and electron transport can

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be attributed to the damage caused mostly to photosystem II (PSII) during virus infection [6].

Virus infected plants display also a wide range of such symptoms that could be linked to the action, or reduction in action, of plant hormones [7]. As concern cytokinins (CK), Clarke et al. [8] found that 10 days after inoculation with *White clover mosaic potexvirus* total CK content was similar as in control plants. Nevertheless, 3 days following inoculation when virus content began to increase, the contents of CK free bases and ribosides declined. This could indicate that the decline in active CKs is needed prior to virus replication. Further studies proved that supplementing the xylem stream with low concentration of CKs inhibited virus replication at the dsRNA level [9,10], and also prevented virus-induced decline in several enzymes involved in the scavenging of free radicals [11].

Transgenic plants with the bacterial gene for isopentenyltransferase (*ipt*), a key enzyme of CK synthesis pathway, accumulate higher contents of endogenous CKs [12]. This affects plant growth, development, and senescence and also the resistance to abiotic and biotic stresses [13,14].

In present study, we have used P*ssu-ipt* tobacco to study the effects of *Potato virus Y* on photosynthetic and water relation characteristics of transgenic plants with elevated content of endogenous CKs. The main aim of this study was to find out, if high content of CKs could improve the resistance of transgenic plants against the PVY infection or diminish its negative impacts on photosynthesis. We correlated our findings with CK contents in both healthy and virus-infected plants.

2. Material and methods

2.1. Plant material

Control tobacco (*N. tabacum* L. cv. Petit Havana SR1) was grown as rooted plants (C) from seeds or as grafted onto control rootstock (C/C). Transgenic tobacco (P*ssu-ipt*) containing a supplementary *ipt*-gene under a control of the promoter for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) was generated by means of the *Agrobacterium tumefaciens* transformation system and grown *in vitro* as shoots unable to form roots. The transgenic shoots were grafted on C rootstock and grown as grafts (T/C) as described by Beinsberger et al. [15]. P*ssu-ipt* transgenic plants (T), i.e. the autogamic progeny of the transgenic grafts, which are able to form a small root system, were grown from seeds, selected on agar medium with kanamycin (*in vitro*) and then transferred into soil. Although the growth and development of transgenic plants was slower than that of controls, they went throughout all developmental stages as controls.

All plants were grown after *in vitro* precultivation in pots with soil substrate in a greenhouse under temperature of 25 °C day/18 °C night, and relative humidity 60%. Natural photosynthetic photon flux density (PPDF) mean ca. 500 μmol (quanta) m^{-2} s^{-1} was prolonged by the additional illumination (*AgroSon T* and HT9 lamps, ca. 200 μmol (quanta) m^{-2} s^{-1}) to 16 h.

For inoculation, plants at early vegetative stage with total number of 4–5 leaves (C, 7–8 weeks old; T, 10–12 weeks old; C/C shoots 3–4 weeks after grafting; T/C shoots 4–6 weeks after grafting) were used.

2.2. Inoculation of plants with PVY^{NTN} isolate

Mature leaves at the bottom of the plant were mechanically inoculated with virus isolate of PVY^{NTN} (Lebanon, provided by Dr. P. Dědič—Institute of Potato Research, Havlíčkův Brod, Czech Republic) on the adaxial surface. Leaf samples from infected plants were taken ca. 15–18 days after the inoculation from young symptomatic mature leaves. Samples from healthy plants were taken at the same time from the same leaf insertion level.

2.3. DAS-ELISA

Leaf samples were frozen in liquid N_2 and stored at −75 °C. The extent of viral infection was determined by DAS-ELISA [16] in homogenates of the leaves of infected and control plants using polyclonal antibodies raised against the PVY [17].

2.4. Cytokinin extraction and purification

CKs were extracted overnight at −20°C with Bieleski solvent [18] from leaves (1 g), grounded under liquid nitrogen. For MS quantification, deuterium-labelled cytokinins ([2H5]Z, [2H5]ZR, [2H5]Z-7G, [2H5]Z-9G, [2H5]Z-OG, [2H5]ZR-OG, [2H3]DZ, [2H3]DZR, [2H6]iP, [2H6]iPR, [2H6]iP-7G, [2H6]iP-9G; Apex, UK) were added as internal standards. After centrifugation, the extracts were purified using Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA) and evaporated to water phase. After acidifying with HCOOH, CKs were trapped on an Oasis MCX mixed mode, cation exchange, reverse-phase column (150 mg, Waters Corporation, Milford, MA, USA) [19]. After two washes (with 1 M HCOOH and 100% MeOH), CK phosphates (CK nucleotides) were eluted with 0.17 M NH_4OH in water, further CK bases, ribosides, and glucosides were eluted with 0.17 M NH_4OH in 60% (v/v) MeOH. The latter eluate was evaporated to dryness. NH_4OH was evaporated from the eluted fraction with CK nucleotides. About 0.1 M Tris (pH 9.6) was added to samples and after treatment with alkaline phosphatase (30 min at 37 °C) CK nucleotides were analysed as their corresponding ribosides. After neutralization, the solution was passed through a C18 Sep-Pak cartridge. CKs were eluted with 5 ml 80% (w/w) methanol and evaporated to dryness. Samples were stored at −20 °C until further analysis.

2.5. Quantitative analysis of cytokinins

Purified CKs samples were analysed by LC–MS system consisting of HTS PAL autosampler (CTC Analytics, Switzerland), Rheos 2000 quaternary pump (FLUX, Switzerland) with Csi 6200 Series HPLC Oven (Cambridge Scientific Instruments, England) and LCQ Ion Trap mass spectrometer