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Transgenic P*ssu-ipt* tobacco under biotic stress

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Introduction

Phytohormones, particularly CK, together with light, play the most important role in plastid development (Parthier 1979). Exogenously applied CK during early stages of plant development caused only minor changes in grana formation and stacking (*e.g.* Branca et al.1994). Regardless of other effects on plant metabolism and photosynthesis, CK also affect processes caused by viral infection and particularly those associated with the development of symptoms (Dermastia and Ravnikar 1996). Although conflicting results of CK activity in virus-infected plants have been reported, several reports demonstrate that enhanced tolerance to various viral infections may be related to elevated CK content (Petrovic et al. 1997).Virus-infected plants show strong morphological and physiological alterations, with symptoms such as chlorosis and necrosis associated with changes in chloroplast structure and function. Various studies proved that plant viruses reduce photosynthesis of their hosts and increase susceptibility to photoinhibition. Photosystem II (PS II) electron transport is often limited and energy-dissipating mechanisms are affected (Rahoutei et al. 2000).

The main aim of our experiments was to find out how PVY affected photosynthetic apparatus and whether elevated CK content in transgenic tobacco could play any role in plant protection.

Material and methods

As a control, non-transformed tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) was used (SR1). Control grafts were made from SR1 shoots grafted onto SR1 rootstock (SRG). Transgenic tobacco containing a supplementary *ipt*-gene under a control of the promoter for the small subunit of RuBPCO (P*ssu-ipt*) was grown as grafts (G) on SR1 rootstock as described by Synkova et al. (1999). The kanamycin resistant progeny of the transgenic grafts, P*ssu-ipt* plants (SE) were grown as rooted plants. All plants were grown in pots in a greenhouse, where also mechanical inoculation by virus was done. Virus isolates of PVY (Lebanon) were provided by Dr. P. Dědič (IPR, Havlíčkův Brod, Czech Rep.).

Chlorophyll fluorescence kinetics and quenching parameters were measured with PAM Chlorophyll Fluorometer (Walz, Germany) on adaxial side of the young fully developed leaf attached to the plant and calculated according to van Kooten and Snel (1990). Samples for transmission electron microscopy (TEM) were taken from the central part of the same leaf and after overnight fixation in 3 % glutaraldehyde were embedded in Spurr‘s resin. Ultrathin

sections were stained by uranyl acetate and Reynold's lead citrate and examined in JEM 1010 (Jeol, Japan). Analysis of serial sections by xxx program enabled three dimensional (3D) reconstructions of chloroplasts.

Results

Visible effects of PVY infection were observed in all rooted plants (both SR1 and SE), while both types of grafts (SRG and G) showed none or very mild symptoms two weeks after inoculation. Chlorophyll fluorescence kinetic parameters proved more damaging effects of PVY in the rooted plants. Maximal photochemical efficiency of PS II (F_v/Fm), the quantum yield (Φ_{II}), and photochemical quenching decreased in infected SR1 and SE, while both grafted types remained almost unchanged (Table 1). Non-photochemical quenching decreased in infected SR1 contrary to the increase in transgenic SE. Both grafted types were affected only moderately (Table 1).

Table 1. Chlorophyll fluorescence kinetic parameters in healthy (control) and PVY infected plants.

	SR1		SRG		G		SE	
	control	PVY	control	PVY	control	PVY	Control	PVY
F_v/F_m	0.775	0.478	0.770	0.674	0.705	0.659	0.680	0.393
Φ_{II}	0.637	0.350	0.618	0.549	0.558	0.518	0.614	0.215
1- q_P	0.064	0.137	0.098	0.041	0.126	0.126	0.044	0.246
q_N	0.374	0.254	0.343	0.324	0.293	0.293	0.253	0.374

TEM examination of ultrathin sections revealed massive occurence of virus in mesophyll cells of SR1 and SE. However, typical "pinwheels" marking the presence of PVY particles were found in all infected plants and grafts. Contrary to G and SRG, virus formed large bundle-like aggregates adjacent to chloroplasts in both rooted types. PVY particles were observed also inside plastids, usually when the plastid envelope was broken (Fig. 1).

Regardless to PVY, numerous anomalies in chloroplast ultrastructure were found in uninfected transgenic tobacco in comparison to control SR1. Irregular, amoeboid shape of plastids was often accompanied by the occurrence of distinct peripheral reticulum with regular tubular or vesicular structures."Cups" formed by chloroplast frequently contained other organelles, such as mitochondria or even peroxisomes, which was proved by 3D reconstruction (Fig.2).

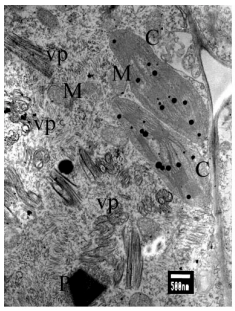

Fig.1. PVY infected SR1cell. C=chloroplast, M=mitochondrion, P=peroxisome, vp=virus particle.

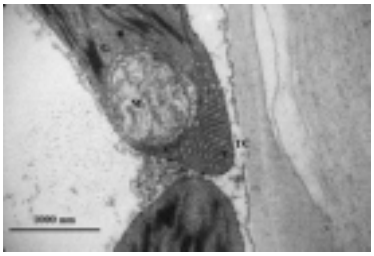

Fig.2. Mitochondrion (M) inside the cup formed by chloroplast and peripheral reticulum (TC) in P*ssu-ipt* tobacco.

Fig.3. Crystalloids (B) inside chloroplasts of transgenic tobacco. G=grana, PG = plasto-globuli, ST=starch, Insertion on right shows more detailed view.

Besides of round-shaped protein bodies inside plastid stroma, bounded by a thylakoid membrane (not shown), large crystalloids with a fine membrane-like structure were the most prominent particles found in chloroplasts (Fig.3).

3D reconstruction showed that crystalloids occupied often considerable part of inner plastid volume and came through the entire chloroplast like large desks.

Discussion

Our results proved that PVY affected differently PS II in rooted and grafted tobacco regardless of genetic transformation and CK content. Energy-dissipating mechanisms were more influenced by plant origin. Both transgenic and control grafts seemed to be less susceptible to PVY, though the reason is not clear. *Potyviridae* are known to multiply in cytoplasm (Shukla et al. 1994). The presence of virus particles and aggregates inside and around chloroplasts was reported for some other members of *Potyviridae* family, though it was not shown for PVY. The plastid envelope was often broken, which could be the result of direct virus action or some stage of plastid destruction in already dying cell.

The occurence of anomalies in chloroplast ultrastructure was restricted to transgenic P*ssu-ipt* tobacco and it was probably the effect of genetic transformation or high content of endogenous CK. The presence of amoeboid plastids have been observed under various conditions and they might be a temporary stage of plastid development (Hudák 1997). Plastids require components synthesised in the cytoplasm and pleiomorphic forms increase the plastid surface area over which such an exchange of metabolites can take place. This might be also the case of P*ssu-ipt* tobacco, where surface area is increased also by peripheral reticulum. Similar structures have been reported in *Nymphoides indica* (van Steveninck et al. 1971). In transgenic tobacco, the systems of 'channels' like vesicles often included other organelles. This might support the idea that organelles need to co-operate more closely under certain conditions (Mckenzie and McIntosh 1999). Large crystalloids, observed in P*ssu-ipt* chloroplasts, had not been reported to our knowledge. There is some resemblance to "macrograna" which were found in various mutants and also during senescence (*e.g.*, Dinkins et al. 1997). Contrary to giant grana stacks, the structures observed in P*ssu-ipt* chloroplasts form rather large protein crystals with a fine lamellar structure with unit size approximately

a=12nm, b=12nm, γ=102°. Although their occurrence in *ipt* transgenic tobacco is quite frequent, their composition and physiological function is completely unknown. Nevertheless, our hypothesis is that those particles are formed by LHC protein, aggregating in a form of 2D crystals, which then constitutes membrane stacks. Šiffel and Vácha (1998) proposed that such aggregates of LHC may develop during degradation of the photosynthetic apparatus. Evident but rather scarce occurrence of similar structures in senescent leaves of SR1 could support this idea.

As some of the above mentioned alterations were observed by other authors under stress conditions or during senescence, they might be the results of plant acclimation to permanent stress caused probably by CK overproduction. However, short-term biotic stress led rather to destruction of photosynthetic apparatus.

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