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Modulation of carbohydrate metabolism and chloroplast structure in sugarcane leaves which were infected by Sugarcane Yellow Leaf Virus (SCYLV)

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ABSTRACT

Non-symptomatic sugarcane plants infected with Sugarcane Yellow Leaf Virus showed starch in mesophyll and bundle sheath cells. In situ-hybridization of mRNAs of sucrose-phosphate phosphatase and ADP-glucose pyrophosphorylase revealed that infected leaves contained SPPase and AGPase in mesophyll cells, Kranz cells and bundle sheath cells. In contrast virus-free leaves contained SPPase only in Kranz cells and AGPase only in bundle sheath cells. Infected leaves exhibited ultrastructural changes in Kranz cell chloroplasts and a shift of the chlorophyll *a*/*b* ratio. No obstruction of plasmodesmata was observed. The results indicate that SCYLV-infected plants, even when visually non-symptomatic, underwent strong metabolic and ultrastructural changes.

1. Introduction

The luteovirus *Sugarcane Yellow Leaf Virus* (SCYLV) was identified as the cause of the sugarcane disease *Yellow Leaf*, formerly called YLS [1,2]. The disease was noticed in the 1990s when plantation fields turned yellow and a substantial yield loss was suffered [3]. Symptomatic leaves had low levels of carbohydrates so that a decline of carbon assimilation because of chlorophyll loss was suspected, especially in the veins where the leaf yellowing started. The disease stayed unnoticed for long time, because the symptoms were not always observed, in fact for most of the time the viral infection stayed latent without obvious symptoms in the plant [4]. Since tests for SCYLV were available [5,6], this virus was found in nearly all sugarcane growing areas of the world. The infected sugarcane in the non-symptomatic stage showed, in contrast to the symptomatic, yellow leaf, elevated levels of carbohydrates compared to virus-free plants [7].

The so far best studied luteoviruses are *Barley Yellow Dwarf Virus* and *Potato Leaf Roll Virus*. Luteoviral infection is confined to the phloem and the severe necrotic symptoms derive primarily from sieve plate obstruction [8,9], which then may eventually lead to anatomical deterioration of mesophyll cells. These viruses, depending on the virus strain, severely damage or even kill the infected plants [10–12]. The luteoviral genome codes, among others, for a movement protein (mp) which allows passage of the virus from the primary infection site, the sieve tube, to the companion cells and possibly other phloem cells. The viral movement proteins were thoroughly studied in the context of protein and RNA trafficking into the phloem and the inter-organ regulation of plant development by phloem-mobile peptides and RNAs [13,14]. The mp-mediated increase of the plasmodesmal size exclusion limit was expected to affect the pressure-driven mass flow of assimilates. Potato plants were transformed to express the *Tobacco Mosaic Virus* mp either constitutively in all cells or specifically in phloem or mesophyll. Expression by a mesophyll-specific promotor decreased the foliar sucrose and starch levels, but increased sucrose efflux from cut petioles [15], whereas expression with a phloem-specific promotor increased foliar carbohydrate levels and decreased sucrose efflux from cut petioles [16]. Expression of *Potato*

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; Bp, base pairs; BSA, bovine serum albumin; cDNA, complementary DNA; cRNA, complementary RNA; DEPC, diethyl pyrocarbonate; Dig-dUTP, digoxigenin-11-2′-deoxy-uridine-5′-triphosphate; DNase I, deoxyribonuclease I; dNTP, deoxyribonucleotide phosphate; EDTA, ethylendiamintetraacetate; LB, Luria Bertani broth; NBT, 4-nitroblue-tetrazoliumchlorid; PCR, polymerase chain reaction; RACE, rapid amplify cDNA ends; RNase, ribonuclease; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; TMV, Tobacco Mosaic Virus; mp, movement protein.

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Leaf Roll Virus mp in tobacco resulted in decreased sugar levels at low expression, interpreted as export stimulation, and elevated sugar levels at high expression, interpreted as export inhibition [17,18]. In addition cytological effects were observed in the mp-transformed plants, for example obstruction of mesophyll plasmodesmata by callose [19]. Because of these unexpected ultrastructural changes and because of the bicollateral vascular bundle type in potato and tobacco, in contrast to most other angiosperm species, it is questionable whether the observations with transformed Solanaceae plants can be generalized to the effects of a luteoviral infection of a "natural" monocotyledonous host such as sugarcane.

The assimilate export route in sugarcane is preceded by the bidirectional symplastic trafficking of the primary photosynthetic products between bundle sheath cells and surrounding Kranz mesophyll cells as a crucial part of the C_4-mode of carbon assimilation [20]. Triosephosphates are photosynthetically produced in the bundle sheath cells, whereas all sucrose were found in the mesophyll and all starch again back in the bundle sheath [21]. Sucrose export from mesophyll to the phloem follows a symplastic path across a suberine lamella, which surrounds the bundle sheath and the Kranz cells. Then it is loaded apoplastically into the sieve tube–companion cell complex, which is symplastically isolated from the other phloem cells. A similar path is assumed for sucrose which derived from starch mobilization in the bundle sheath [22].

It is unknown, whether the infection of sugarcane by SCYLV changes this complex intercellular compartmentalization of carbohydrate metabolism, and whether the SCYLV movement protein exerts similar effects in sugarcane as the movement proteins of TMV or PLRV do in transformed Solanaceae. The distribution of exemplary enzymes of sucrose and starch metabolism in leaves was studied by in situ methods to reveal how the carbohydrate backup observed in non-symptomatic, infected leaves [7] changed the enzyme compartmentalization. In addition some electron micrographs were performed to observe effects of SCYLV-infection on chloroplast ultrastructure and plasmodesmatal cell junctions. Emphasis was posed on the non-symptomatic state of leaves, where neither chlorophyll loss nor growth inhibition is noticed yet.

2. Materials and methods

2.1. Chemicals, enzymes and software

All chemicals were used at highest available grade and purchased either from Merck, Roth, Applichem, Roche Diagnostics GmbH, Sigma–Aldrich, MBI Fermentas (St. Leon-Rot), Biomol and DIFCO, BD, located all in Germany or from Buchs, Switzerland.

General PCRs for screening of clones and incorporation of restriction sites were carried out using sequence-specific primers from MWG (MWG Biotech, Ebersberg, Germany) or Biomers (Biomers.net, Ulm, Germany).

The sources for enzymes and kits were Roche Diagnostics GmbH, Mannheim, Germany, MBI Fermentas, St. Leon-Rot, Germany, Stratagene La Jolla, CA, USA, BD Bioscience, Palo Alto, CA, USA, Qiagen GmbH, Hilden, Germany and Promega, Mannheim, Germany.

Software for gene analysis: Chromas for sequence reading, Blast (http://www.ncbi.nlm.nih.gov/BLAST/), Entrez Pubmed (http://www.ncbi.nlm.nih.gov/Entrez/), GCG for various sequence analysis (Wisconsin), MACAW for multiple alignment (http://www.ncbi.nlm.nih.gov/).

2.2. Plant material

The sugarcane plants for the in situ studies were grown in the greenhouse in Bayreuth. Surface-sterilized seed pieces were germinated in a humid chamber and then set into humous soil mixed with sand in 2:1 ratio in 25 L pots. They were regularly watered and fertilized. Leaf material was taken from the top visible dew lap (TVD) leaf of 5–6 month-old-plants. Virus-free and SCYLV-infected plants were separately grown in different compartments of the greenhouse. The leaves for the chlorophyll determination were collected from field-grown plants as described elsewhere [4].

2.3. Preparation of RNA and RNA-probes

2.3.1. Preparation of RNA and primers

Plant tissue was homogenized and the RNA was purified from the lysate by extraction with phenol–chloroform. Many samples were processed simultaneously. The separation of RNAs on the agarose gel was performed after Lehrach et al. [23].

RNA was extracted from leaves, subjected to cDNA synthesis by hexamer random primers followed by using specific primers to synthesize an approximately 600 bp fragment. The primers were designed to bind to the conserved sequence parts of the RNAs. The primer pairs were following: (1) for a 600 bp fragment of rRNA: 5′-AGGGAGCCTGAGAAACGGCTACCACATC-3′and 5′-TGGTTGAGACTAGG ACGGTATCTGATC-3′. (2) For a 550 bp fragment of SCYLV: 5′-CACA-CATCCGAGCGATAGTGAATGAAT-3′ and 5′-GTCTCCATTCCCTTTGTA-CAGC AACCA-3′. (3) For a 600 bp fragment of ADP-glucose pyrophosphorylase 5′-ATHGCBDSHATGGAAGTNTAYRTH-3′ and 5′-ATHGCBDSHATGGAAGTNTAYRTH-3′. And (4) For a 500 bp fragment of sucrose-phosphate phosphatase 5′-AARSARYTRAGRAARGA AARCC-3′ and 5′-GGWGTHATGGTNRSYAATKCVCAA-3′.

2.3.2. Synthesis of digoxigenin-labelled RNA probe

Plasmid DNA was isolated from small-scale (3 ml) bacterial cultures or from large-scale (500 ml) according to Birnboim and Doly [24] and Ish-Horowicz and Burke [25]. The amplification of DNA fragments using PCR was performed according to Wybranietz and Lauer [26]. Degenerate PCR- and RACE-amplified products were cloned into T-vector using the TA-cloning kit (Promega, Mannheim, Germany). PCR products were checked by agarose gel electrophoresis and extracted from the gel by using activated silica-based gel extraction kit (Roche Diagnostics GmbH, Mannheim, Germany).

2.3.3. Synthesis of riboprobe

The synthesis of digoxigenin labelled probe was performed according to Krieg and Melton [27]. The DNA fragment (1 μg) was subcloned into an appropriate vector and linearized. Complementary RNA (cRNA) transcripts were generated and used as RNA probes for the transcribed gene. The length of RNA probes was reduced to approximately 200 bases by hydrolysis.

2.4. In-situ-hybridization of RNA

The in situ hybridization was performed according to Woo et al. [28]. The leaf tissues were fixed in a formaldehyde fixation solution, the tissue samples (ca. 1 cm^2) were cut into the solution, kept for 50 min and infiltrated with vacuum. Then the samples were washed and dehydrated through a series of ethanol steps and infiltrated with paraffin (paraplast) through a series of ethanol/Histoclear solutions. Tissue blocks were cast in plastic disposable frames and 8–10 μm sections were cut. The slides were placed in Coplin jars, deparaffinized in Histoclear, rehydrated through a graded ethanol series, incubated with Proteinase K, rinsed and dipped in a 4% formalin–PBS solution, then in 100 mM triethanolamine–acetic anhydride. Then slides were washed in PBS, dehydrated by a graded ethanol series and air-dried. Each section was covered with 200 μl of prehybridization solution and incubated in