Cell Wall-Bound Invertase Limits Sucrose Export and Is Involved in Symptom Development and Inhibition of Photosynthesis during Compatible Interaction between Tomato and *Xanthomonas campestris* pv *vesicatoria*[W][OA]

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Cell wall-bound invertase (cw-Inv) plays an important role in carbohydrate partitioning and regulation of sink-source interaction. There is increasing evidence that pathogens interfere with sink-source interaction, and induction of cw-Inv activity has frequently been shown in response to pathogen infection. To investigate the role of cw-Inv, transgenic tomato (*Solanum lycopersicum*) plants silenced for the major leaf cw-Inv isoforms were generated and analyzed during normal growth and during the compatible interaction with *Xanthomonas campestri*s pv *vesicatoria*. Under normal growth conditions, activities of sucrolytic enzymes as well as photosynthesis and respiration were unaltered in the transgenic plants compared with wild-type plants. However, starch levels of source leaves were strongly reduced, which was most likely caused by an enhanced sucrose exudation rate. Following *X. campestri*s pv *vesicatoria* infection, cw-Inv-silenced plants showed an increased sucrose to hexose ratio in the apoplast of leaves. Symptom development, inhibition of photosynthesis, and expression of photosynthetic genes were clearly delayed in transgenic plants compared with wild-type plants. In addition, induction of senescence-associated and pathogenesis-related genes observed in infected wild-type plants was abolished in cw-Inv-silenced tomato lines. These changes were not associated with decreased bacterial growth. In conclusion, cw-Inv restricts carbon export from source leaves and regulates the sucrose to hexose ratio in the apoplast. Furthermore, an increased apoplastic hexose to sucrose ratio can be linked to inhibition of photosynthesis and induction of pathogenesis-related gene expression but does not significantly influence bacterial growth. Indirectly, bacteria may benefit from low invertase activity, since the longevity of host cells is raised and basal defense might be dampened.

The coordinated sequence of assimilate production, allocation, and utilization is essential for normal plant growth and development. Photosynthetic CO_2 fixation occurs in source leaves producing an excess of assimilates that are allocated, mostly in form of Suc, to sink tissues via the phloem. Sink tissues, such as developing leaves, roots, meristems, fruits, and flowers, are unable to produce sufficient amounts of assimilates themselves and therefore require their net import. Carbohydrate partitioning between source and the different competing sink tissues is a highly dynamic process that is influenced by environmental and developmental cues. Plant pathogens like viruses, fungi, oomycetes, and bacteria are known to interfere with the source-sink balance (Biemelt and Sonnewald, 2006; Berger et al., 2007; Seo et al., 2007), and in the case of a successful interaction, pathogens are believed to reprogram a plant's metabolism to their own benefit. This comprises the suppression of plant defense responses and the reallocation of photoassimilates to sufficiently supply the pathogen with nutrients. In accordance with this, the infected leaf is assumed to undergo a source to sink transition or retains its sink character (Horst et al., 2008). Indicative for this are a stimulation of cell wall-bound invertase (cw-Inv) and a decreased rate of photosynthesis, which has been shown in response to different plant pathogens, including fungi (Chou et al., 2000; Swarbrick et al., 2006), viruses (Herbers et al., 2000), and bacteria (Biemelt and Sonnewald, 2006; Bonfig et al., 2006).

Cw-Inv is an extracellular enzyme catalyzing the cleavage of the transport sugar Suc into Glc and Fru. Since hexoses are not transported in the phloem, they remain at the site of formation. Hence, cw-Inv activity increases the local hexose availability and is therefore thought to be a key enzyme for supplying sink tissues with carbohydrates. In fact, it has been shown in a number of studies that cw-Inv is involved in the regulation of developmental processes such as seed and pollen development (Miller and Chourey, 1992, Weber et al., 1996; Goetz et al., 2001) as well as in carbohydrate partitioning (for review, see Roitsch and González, 2004). For instance, overexpression of a yeast invertase in the apoplast of transgenic tobacco (*Nicotiana tabacum*) plants interfered with Suc export and caused a strong accumulation of soluble sugars and starch,

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leading to an inhibition of photosynthesis, stunted growth, and the development of bleached or necrotic leaf areas (von Schaewen et al., 1990).

In plants, cw-Invs are encoded by small gene families. From fully sequenced plant genomes, six cw-Inv isoforms have been annotated in Arabidopsis (*Arabidopsis thaliana*; Sherson et al., 2003) and nine in rice (*Oryza sativa*; Ji et al., 2005). Cw-Inv gene families have also been reported in other plant species (Huang et al., 2007), such as four isoforms in tomato (*Solanum lycopersicum*), referred to as Lin5, -6, -7, and -8, showing different tissue-specific expression (Godt and Roitsch, 1997; Fridman and Zamir, 2003). Expression of the individual isoforms was found to be regulated in response to various stimuli at the transcriptional and translational levels (for review, see Roitsch et al., 2003; Huang et al., 2007). For instance, expression of Lin6 was shown to be up-regulated in response to fungal elicitors or polygalacturonic acid as well as after wounding (Godt and Roitsch, 1997; Sinha et al., 2002). Interestingly, a quantitative trait locus for increased sugar yield in tomato fruits was mapped to Lin5, which is expressed in conductive tissues of developing tomato fruits (Fridman et al., 2004).

Among others, cw-Inv expression is induced upon microbial infection and is most likely associated with an apoplastic hexose accumulation during the infection process. The hexoses formed are thought to aid the pathogen's nutrition (Biemelt and Sonnewald, 2006; Berger et al., 2007; Seo et al., 2007). Fungal pathogens even produce their own invertases to ensure their nutritional supply (Chou et al., 2000; Voegele et al., 2006). Beside being a source of nutrients, soluble sugars have been shown to act as signals leading to the down-regulation of genes involved in photosynthesis and reserve mobilization (Koch, 1996) as well as to an induction of defense gene expression (Herbers et al., 1996b; for a recent review, see Rolland et al., 2006).

In fact, down-regulation of photosynthetic activity and gene expression has been observed along with induction of cw-Inv activity and accumulation of soluble sugars in compatible interactions (Chou et al., 2000; Herbers et al., 2000; Swarbrick et al., 2006) but also during incompatible interactions (Scharte et al., 2005; Swarbrick et al., 2006). A direct comparison of both types of interactions in barley (*Hordeum vulgare*) leaves using susceptible and *Mla12* or *mlo* resistant lines revealed that cw-Inv increased more rapidly and to a much greater extent during the incompatible interaction (Swarbrick et al., 2006). There were also differences in responses to pathogen attack regarding photosynthesis and the induction of *PATHOGENESIS-RELATED* (*PR*) gene expression, which were found to be faster and stronger in incompatible interactions (Swarbrick et al., 2006; for review, see Berger et al., 2007; Seo et al., 2007). Consistently, large-scale expression profiling using the Affymetrix GeneChip demonstrated that responses in compatible and incompatible interactions are qualitatively similar but differ in quantity and kinetics (Tao et al., 2003).

These data indicate that the speed of reprogramming of plant metabolism might determine the outcome of an infection. A rapid accumulation of soluble sugars caused by an early induction of cw-Inv activity is thought to promote their utilization for host defense reactions, supporting the successful establishment of resistance (Scharte et al., 2005; Swarbrick et al., 2006; Essmann et al., 2008). For instance, callose deposition and the production of phenolic compounds (including salicylic acid) are both known outputs of plant defense and require large amounts of metabolizable sugars (Herbers et al., 1996a; Scharte et al., 2005). In addition, hexose signals are known to trigger the induction of *PR* gene expression and to amplify plant defense reactions (Herbers et al., 2000). Thus, an early reprogramming and redirecting of carbon flow seems to support plant defense. This is in accordance with the model of "high sugar resistance" (Horsfall and Dimond, 1957) and with the observation that transgenic tobacco plants overexpressing a yeast invertase in the apoplast were found to be resistant against potato virus Y infection (Herbers et al., 1996a). This phenomenon was accompanied by the accumulation of carbohydrates, the inhibition of photosynthetic capacity and gene expression, an increase of transcripts encoding *PR* genes, and callose deposition (Herbers et al., 1996a, 2000). In contrast, an accumulation of sugars during later stages of infections might serve as nutrients for the invading pathogen, resulting in disease development (Seo et al., 2007).

So far, no functional analysis of the role of cw-Inv in compatible interactions using knockout mutants or RNA interference (RNAi)-silenced plants has been published. Recently, transgenic tobacco plants with RNAi-mediated inhibition of cw-Inv were generated to investigate the significance of cw-Inv for the establishment of plant defense in an incompatible interaction (Essmann et al., 2008). In our study, we exploited the compatible interaction between tomato plants and *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) as a model system to elucidate how reduced cw-Inv affects carbon metabolism and disease development.

Xcv is a gram-negative bacterium causing bacterial spot disease on pepper (*Capsicum annuum*) and tomato plants. Bacteria penetrate the plant through stomata or wounds and colonize the intercellular space. Typical symptoms are the appearance of water-soaked lesions that develop into necrotic spots. Pathogenicity of *Xcv* depends on a type III secretion system (T3SS), which is used to inject a set of proteins collectively referred to as bacterial effectors (Alfano and Collmer, 2004). A functional T3SS is required for pathogenicity, since mutants lacking the T3SS are unable to cause disease in susceptible plants or to elicit the defense-associated hypersensitive response in resistant interactions.

To achieve our aim, we generated transgenic tomato plants with strongly reduced cw-Inv activity in source leaves using a Lin8-RNAi construct. These plants were thoroughly analyzed for carbohydrate metabolism, photosynthesis, and their compatible interaction with

Xcv. The possible role of cw-Inv in the regulation of photosynthesis and the establishment of disease will be discussed.

RESULTS

RNAi-Mediated Silencing of Leaf-Specific Cw-Inv Isoforms in Transgenic Tomato Plants

An increase in cw-Inv activity and gene expression has been observed during various plant-pathogen interactions (for review, see Biemelt and Sonnewald, 2006; Seo et al., 2007). In order to investigate the role of cw-Inv during plant-pathogen interactions in more detail, we aimed at generating transgenic tomato plants with strongly reduced cw-Inv activity in source leaves. Two isoforms of tomato, Lin6 and Lin8, were reported to be expressed in leaves (Fridman and Zamir, 2003). Since both sequences are highly homologous, the intention was to silence both isoforms using a single intron-spliced hairpin RNA (RNAi) construct. To this end, primers were designed from a highly homologous region of both sequences sharing 81.9% identity at the nucleotide level, which is thought to be sufficient to allow silencing of both isoforms using a single construct (Le et al., 2006a, 2006b; Chen et al., 2008). For vector construction, a 400-bp fragment of Lin8 was inserted in the sense and antisense orientations into the plant transformation vector pK7GWIWG2(II) (Karimi et al., 2002), designated Lin8-RNAi (Fig. 1A). This construct was transformed into tomato plants (cv Moneymaker) by means of *Agrobacterium tumefaciens*-mediated gene transfer, and 65 kanamycin-resistant tomato plants were generated. After transfer to the greenhouse, plants were screened for reduced cw-Inv activity in source leaves. Ten lines showed strongly decreased activity, which was about 10% to 15% of that of wild-type leaves (data not shown). The transgenic plants did not display growth alterations under greenhouse conditions (Supplemental Fig. S1), but seed size was reduced in the best RNAi lines (data not shown).

Three transgenic lines (lines 33, 50, and 57) with clearly diminished cw-Inv activity were selected for a more detailed analysis (Table I). To prove whether cw-Inv was affected in leaves only, samples were taken from different organs during plant development and the activity of cw-Inv was determined. As indicated in Figure 1B, the strongest reduction of cw-Inv activity was measured in source leaves. While sink leaves and petioles of the selected transgenic lines still had 40% to 60% of wild-type activity, there were only minor or no alterations found in all other organs and tissues investigated. Thus, expression of the Lin8-RNAi construct caused a predominant decrease of cw-Inv activity in source leaves.

Effects of Reduced of Cw-Inv Activity on Primary Metabolism of Source Leaves

In order to investigate the impact of reduced leaf cw-Inv activity on carbohydrate metabolism, the activities of key enzymes involved in Suc cleavage and metabolism as well as the amounts of soluble sugars and starch were measured in the three selected lines in the middle of the light period (Tables I and II).

Activities of vacuolar and neutral invertases were not affected in the Lin8-RNAi lines. Also, the activity of Suc synthase (SuSy), catalyzing the reversible cleavage of Suc in the cytosol, was not altered in the transgenic lines in comparison with the wild type. Moreover, activities of Glc and Fru, phosphorylating glucokinase and fructokinase, were similar in wild-type and trans-

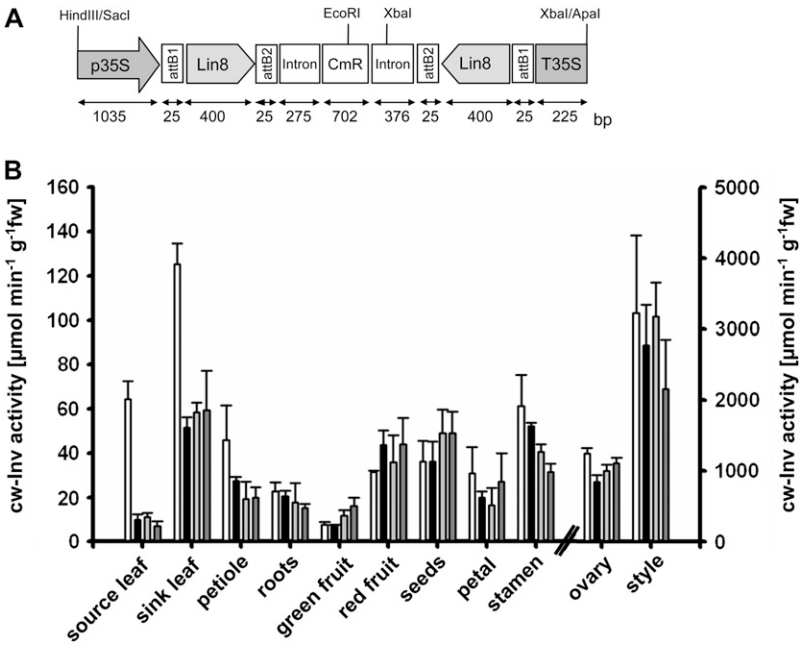

Figure 1. RNAi-mediated silencing of one cw-Inv isoform in tomato. A, Schematic illustration of the Lin8-RNAi construct used to generate transgenic tomato plants. The leaf-specific cw-Inv isoform Lin8 was inserted in the sense and antisense orientations into pK7GWIWG2(II) (Karimi et al., 2002) under the control of the constitutive 35S cauliflower mosaic virus promoter and designated Lin8-RNAi. B, Cw-Inv activity in different tissues of three independent transgenic Lin8-RNAi lines (line 33, black bars; line 50, light gray bars; line 57, dark gray bars) compared with the wild type (white bars). Samples were taken from three different plants and assayed for cw-Inv activity. Each value represents the mean ± SD of five independent samples. fw, Fresh weight.

Table I. *Activities of enzymes involved in carbohydrate metabolism in source leaves of wild-type and Lin8-RNAi plants*

Data represent means ± SD of eight replicates taken from source leaves of four different plants. Asterisks indicate significant differences ($P = 0.005$) from the wild type according to the *t* test.

Plant Line	Cw-Inv	Vacuolar Invertase	Neutral Invertase	SuSy	Glucokinase	Fructokinase	AGPase
	μmol m⁻² min⁻¹			*nmol min⁻¹ mg⁻¹ protein*			
Wild type	40.54 ± 12.86	4.20 ± 1.03	4.80 ± 1.60	49.18 ± 10.74	0.31 ± 0.13	2.75 ± 0.40	5.17 ± 2.50
Lin8-RNAi line 33	3.41 ± 0.67*	3.63 ± 1.38	3.28 ± 0.39	41.06 ± 7.20	0.24 ± 0.09	2.78 ± 0.54	8.41 ± 3.13
Lin8-RNAi line 50	3.58 ± 1.29*	5.55 ± 1.10	2.27 ± 0.97	42.83 ± 12.76	0.20 ± 0.08	3.11 ± 0.50	11.37 ± 4.32
Lin8-RNAi line 57	1.50 ± 0.45*	3.95 ± 1.55	3.65 ± 1.28	38.95 ± 9.04	0.33 ± 0.18	3.32 ± 0.72	9.26 ± 2.60*

genic Lin8-RNAi lines (Table I), indicating no significant impact of silenced cw-Inv on enzymes of primary carbon metabolism in source leaves.

Interestingly, reduced cw-Inv activity led to an approximately 50% reduction in starch content compared with that in wild-type plants (Table II), although activity of ADP-Glc pyrophosphorylase (AGPase) tended to be higher in the transgenic line (Table I). However, the increase was only significant for line 57, due to high variations. While the amounts of Glc and Suc remained unaltered, Fru levels increased in transgenic leaves compared with wild-type leaves (Table I). Electron transport and CO_2 assimilation rate remained unchanged in the transgenic lines (Fig. 2); hence, a lower rate of photosynthesis cannot account for the reduced starch accumulation. To further explore whether the lower amount of starch could be due to an increased respiration, the dark respiration rate was measured in one selected transgenic line (line 57) and compared with that in the wild type. This line was selected because it showed the strongest reduction in cw-Inv activity. The mean dark respiration rates were -0.49 ± 0.13 and -0.52 ± 0.10 μmol m^{-2} s^{-1} ($n = 6$) in the wild type and line 57, respectively, indicating no significant changes. An increased Suc export would be another explanation for the lower starch accumulation in the transgenic plants. Consistently, the Suc efflux rate through the petiole of detached leaves was increased in line 57 compared with wild-type plants (Fig. 3). Calculating Suc export rates per hour revealed a 2-fold higher rate in the transgenic line (67.2 μmol g^{-1} fresh weight h^{-1}) compared with the control (32.8 μmol g^{-1} fresh weight h^{-1}). This result indicates that cw-Inv activity might be involved in restricting Suc export from source leaves.

Xcv-Mediated Induction of Cw-Inv Activity Is Absent in Transgenic Plants

To investigate the role of cw-Inv in a compatible interaction, transgenic and wild-type plants were infected with *Xcv* strain 75-3, which is pathogenic to tomato. For comparison, leaves were infiltrated with 10 mM $MgCl_2$ (mock control). We used a low bacterial titer (5×10^4 colony-forming units [cfu] mL^{-1}) in our experiments to resemble "natural" conditions and to allow monitoring of bacterial growth and physiological changes under the same conditions.

Previously, we showed an induction of cw-Inv activity in tomato leaves at 72 h after *Xcv* infection with a high bacterial density (1×10^8 cfu mL^{-1}; Biemelt and Sonnewald, 2006). In this study, a progressive increase in cw-Inv activity was observed in wild-type plants starting from day 4 after infection. Maximum cw-Inv activity was reached after 8 d and remained high during the following 6 d (Fig. 4). This indicates that a lower bacterial inoculation titer caused a similar but delayed response, as described previously (Biemelt and Sonnewald, 2006). In contrast, the activity of cw-Inv was not or was only weakly induced in the transgenic lines following *Xcv* infection (Fig. 4).

Since changes in invertase activity should lead to altered Suc to hexoses ratios, the carbohydrate contents were measured in leaves of wild-type and transgenic

Table II. *Content of carbohydrates in source leaves of Lin8-RNAi plants compared with wild-type plants*

Amount of carbohydrates was determined from samples after 6 h of illumination. Data represent means ± SD of 15 samples taken from source leaves of four different plants. Asterisks indicate that Fru and starch levels in these lines were significantly different ($P = 0.001$) from levels in the wild type according to the *t* test.

Plant Line	Carbohydrates			
	Glc	Fru	Suc	Starch
	mmol m⁻²			
Wild type	1.38 ± 0.35	1.98 ± 0.33	0.60 ± 0.17	5.81 ± 1.44
Lin8-RNAi line 33	1.46 ± 0.31	3.09 ± 0.76*	0.62 ± 0.13	3.28 ± 0.84*
Lin8-RNAi line 50	1.37 ± 0.54	2.25 ± 0.75	0.73 ± 0.19	3.05 ± 0.60*
Lin8-RNAi line 57	1.76 ± 0.60	3.50 ± 0.80*	0.75 ± 0.30	3.33 ± 0.90*

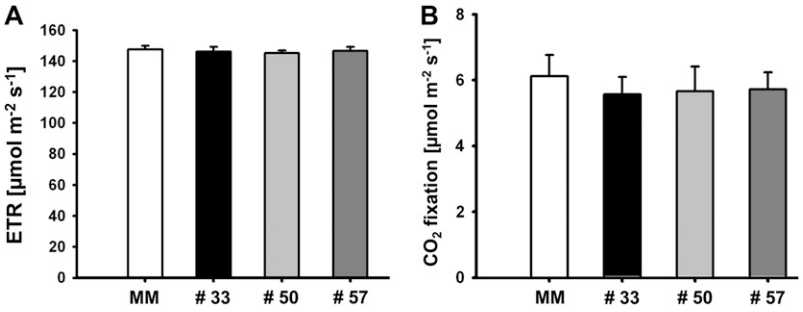

Figure 2. ETR and CO_2 assimilation rate in source leaves of Lin8-RNAi plants compared with wild-type plants. ETR (A) and CO_2 assimilation rate (B) were determined in wild-type plants (Moneymaker [MM]; white bars) and transgenic tomato plants (line 33, black bars; line 50, light gray bars; line 57, dark gray bars) under ambient conditions. Values are means ± SD of four replicates. Results of one representative experiment are shown.

plants following *Xcv* infections. The amount of total hexoses increased slightly in infected plants, but there was a high variation between different experiments, and no significant differences in carbohydrate content could be detected between wild-type and transgenic plants in response to infection (data not shown).

To prove that the different responses of cw-Inv in wild-type and transgenic plants caused an altered Suc to hexose ratio in the apoplast, the extracellular fluid was isolated from source leaves and amounts of hexoses and Suc were determined. For these experiments, we used high-titer inoculations to trigger a fast and strong response. There were no clear changes in the Suc to hexose ratio in the different genotypes before infection, although it was slightly higher in two transgenic lines (Table III). While the ratio remained almost unchanged in wild-type plants following *Xcv* infection, it clearly increased in all Lin8-RNAi lines, reflecting an accumulation of Suc relative to hexoses, which was most likely due to the absence of cw-Inv activity (Table III).

Lin8-RNAi Plants Show Less Severe Symptom Development following *Xcv* Infection But Unchanged Bacterial Growth

The infection experiments performed revealed that the development of disease symptoms was delayed in the Lin8-RNAi lines compared with the wild type. Thus, *Xcv*-inoculated wild-type leaves developed first symptoms about 8 d after infection, visible as water-soaked lesions and chlorotic spots. The infected leaves became necrotic after 12 d and were dead after 15 or 16 d (Fig. 5). In contrast, visible symptoms did not appear in the transgenic lines before day 10. After 16 d leaves became chlorotic (Fig. 5), and after 20 d they were necrotic (data not shown). There were little differences in the time course of symptom development between the different transgenic lines. Thus, lines 33 and 50 exhibited necrotic spots about 2 d earlier than line 57, in which symptom development was most clearly delayed.

A delayed symptom development was also observed after infection with a high bacterial titer (1×10^8 cfu mL^{-1}). Here, 3 d after *Xcv* infection, leaves of wild-type plants became necrotic and the leaf margins were curled downward, whereas the Lin8-RNAi plants showed no symptoms or only some necrotic spots at this time point (data not shown).

These results suggested that the transgenic lines with reduced cw-Inv activity might be less susceptible against *Xcv* infection. To further investigate this hypothesis, the bacterial growth in planta was monitored over a 16-d period. Besides the *Xcv* wild-type strain, we also included a T3SS-deficient mutant that is unable to deliver bacterial effector proteins and hence is unable to infect host plants. An enhanced growth of T3SS mutants in plants has been used as an indicator for reduced basal defense in recent studies (Hauck et al., 2003; Kim et al., 2005). As expected, the T3SS-deficient *Xcv* mutant did not cause disease in either plant type and showed only little growth in planta with no clear differences between the different genotypes (Fig. 6), but a kind of cell death occurred at the infiltration site, most likely due to wounding, as it was also observed after mock inoculation (Fig. 5).

Much to our surprise, growth of the *Xcv* wild-type strain was not impaired in the Lin8-RNAi plants (Fig. 6). Instead, bacterial growth reached the levels of wild-

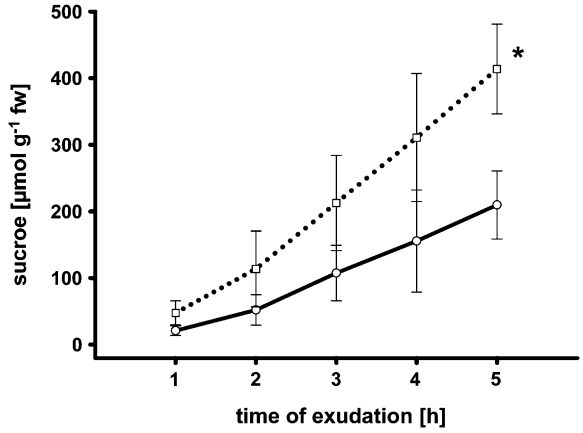

Figure 3. Suc efflux from source leaves in transgenic line 57 compared with the wild type. Phloem exudates were collected from one excised source leaf. Suc content in the exudate was determined at 1, 2, 3, 4, and 5 h after excision for the wild type (solid line) and transgenic line 57 (dotted line). Data are presented as means ± SD of at least five replicates. The asterisk indicates a statistical difference between transgenic line 57 and the wild type ($P = 0.05$).

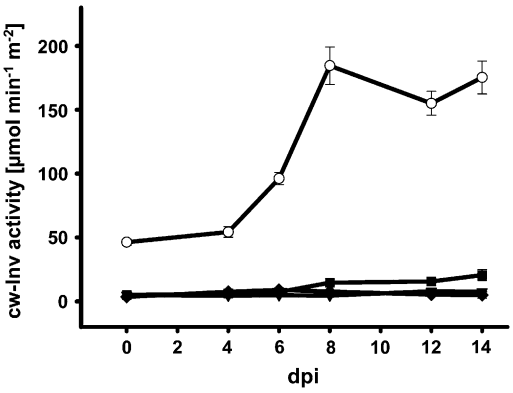

Figure 4. Activity of cw-Inv in tomato leaves following infection with *Xcv*. Leaves of the wild type (white circles) and three different Lin8-RNAi plants (black symbols: triangles, line 33; squares, line 50; diamonds, line 57) were inoculated with *Xcv* strain 75-3 at a concentration of 5×10^4 cfu mL^{-1}. Samples were taken before (0) and at 4, 6, 8, 12, and 14 d after infection (dpi). Values represent means ± SD ($n = 5$).

type plants and remained even longer in the stationary phase. While the bacterial growth rate dropped at day 16 in wild-type plants (consistent with the leaf death), it remained constant in all three Lin8-RNAi lines (Fig. 6).

Taken together, Lin8-RNAi plants showed a delayed appearance of disease symptoms when challenged with *Xcv*, but this was not associated with reduced bacterial growth.

In Lin8-RNAi Plants, Electron Transport Rate Is Maintained Longer and Chlorophyll Loss Is Delayed after *Xcv* Infection

The differences in symptom development prompted us to investigate how *Xcv* infection influences the photosynthetic capacity of wild-type and Lin8-RNAi plants. Down-regulation of photosynthetic gene expression and activity seems to be a general response of plants to pathogen attack and has been observed in a number of plant-pathogen interactions (Chou et al., 2000; Herbers et al., 2000; Berger et al., 2004; Scharte et al., 2005; Bonfig et al., 2006; Truman et al., 2006).

For these experiments, leaves were again inoculated with *Xcv* at low titer and with 10 mM $MgCl_2$ and chlorophyll fluorescence was measured under ambient growth conditions over a 16-d period. Chlorophyll fluorescence data were used to calculate the electron transport rate (ETR) of PSII.

ETR was indistinguishable between wild-type and Lin8-RNAi lines before infection (Figs. 2 and 7). During the course of the 16-d investigation, the ETR declined in mock-inoculated wild-type and transgenic lines starting at day 8 or 10 to 70% to 80% of the initial values (Fig. 7, A–D). This was most likely due to an age-dependent decrease in photosynthesis. Upon *Xcv* infection, the ETR continuously decreased in wild-type and Lin8-RNAi lines from day 8 onward. However, from day 10 after infection, the decrease was more pronounced in wild-type plants, in which the electron transport ceased at day 14. In contrast, in transgenic line 57, showing the slowest development of disease symptoms, the ETR decreased only to 71% and 40% of the initial values at days 14 and 16, respectively (Fig. 7D). This trend of delayed inhibition of ETR was also found for lines 33 and 50, although to a lower extent (Fig. 7, B and C). This indicates that the integrity of the photosynthetic apparatus was prolonged in cw-Inv-silenced plants.

The stronger decline of photosynthetic activity in wild-type plants after *Xcv* challenge was paralleled by a more steep reduction in chlorophyll content compared with the transgenic lines (Fig. 7, E–H). The loss of chlorophyll content was again most slow in transgenic line 57, which was consistent with the delayed symptom development.

Together, these data indicate that a reduced cw-Inv activity allowed maintaining photosynthetic capacity at higher levels upon bacterial infection, which could account for the delayed disease appearance and for sustained bacterial growth.

Expression of Photosynthetic, Senescence-Associated, and *PR* Genes Is Less Affected in Cw-Inv-Silenced Plants after *Xcv* Infection

To investigate whether the reduced photosynthetic capacity and the differences observed in chlorophyll accumulation between wild-type and transgenic plants following *Xcv* infection were paralleled by altered gene expression, transcriptional changes of the small subunit of Rubisco (*rbcS*) and of selected senescence markers were studied. Senescence-associated genes were monitored, since chlorophyll loss is an integral part of leaf senescence and pathogen infection promotes leaf senescence (Gan, 2007). For these studies, we used only Lin8-RNAi line 57, which showed the strongest differences compared with wild-type plants.

Similar to the decline in the ETR, there was a decrease in *rbcS* expression in mock-infected wild-type and transgenic plants during the 16-d period of investiga-

Table III. *Changes in Suc to hexose ratio in the extracellular fluid of wild-type and Lin8-RNAi plants*

Data represent means ± SD of at least six samples before (−) and 48 h after treatment with *Xcv* at 10^8 cfu mL^{-1} (+). Asterisks indicate significant differences ($P = 0.005$) from the wild type according to the *t* test.

Plant Line	*Xcv* 75–3	
	−	+
Wild type	1.37 ± 0.54	1.03 ± 0.32
Lin8-RNAi line 33	2.44 ± 1.00	6.16 ± 1.58*
Lin8-RNAi line 50	3.24 ± 1.77	10.51 ± 2.05*
Lin8-RNAi line 57	1.01 ± 0.42	3.63 ± 0.77*

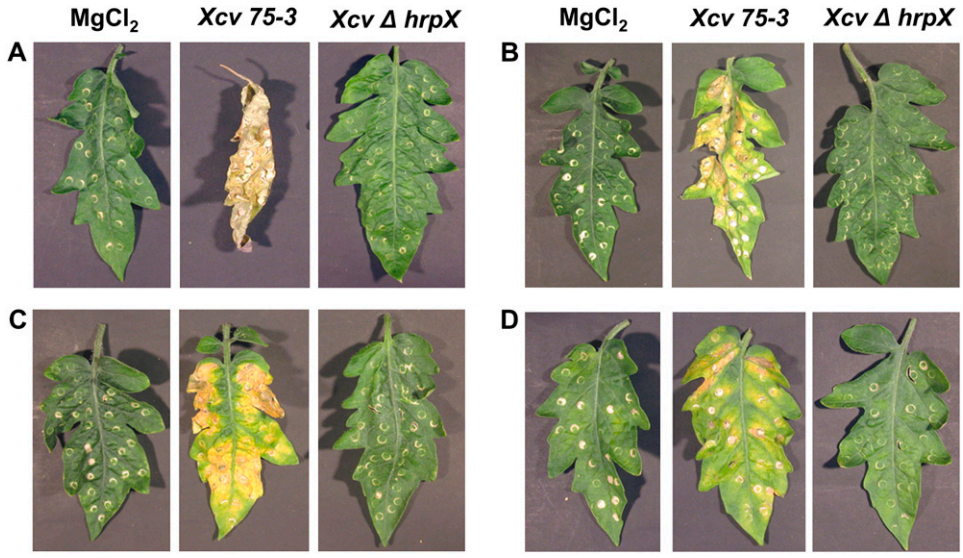

Figure 5. Development of disease symptoms upon treatment with *Xcv* in Lin8-RNAi plants. Source leaves were infiltrated with *Xcv* strain 75-3 or a T3SS-deficient (*Δ hrpX*) strain at a concentration of 5×10^4 cfu mL^{-1}. As a control, leaves were inoculated with 10 mM $MgCl_2$. Infected leaves were photographed at 16 d after infection. A, Moneymaker wild type. B, Lin8-RNAi line 33. C, Lin8-RNAi line 50. D, Lin8-RNAi line 57.

tion (Fig. 8). However, upon infection, the decrease was stronger compared with that in mock-infected plants and more pronounced in the wild type. Here, the accumulation of *rbcS* transcripts was clearly reduced already at 6 d after *Xcv* infection and was barely detectable after 14 d, whereas transcripts were still present in the Lin8-RNAi line. A similar tendency was found for the expression of two genes involved in photosynthetic electron transport, ferredoxin-NADP reductase and plastocyanin, in an independent experiment (Supplemental Fig. S2). To exclude a general effect on primary metabolism in response to *Xcv* infection, expression of cytosolic glyceraldehyde 3-phosphate dehydrogenase was analyzed. Here, no changes in gene expression could be detected between the different genotypes or in response to *Xcv* (Supplemental Fig. S2).

As marker for senescence, the cytosolic Gln synthetase (GS-1) and Glu dehydrogenase (GDH) were selected, which were shown to be involved in nitrogen mobilization of senescing tobacco leaves (Pageau et al., 2006). In addition, expression of *staygreen* (*Sgr-1*) was

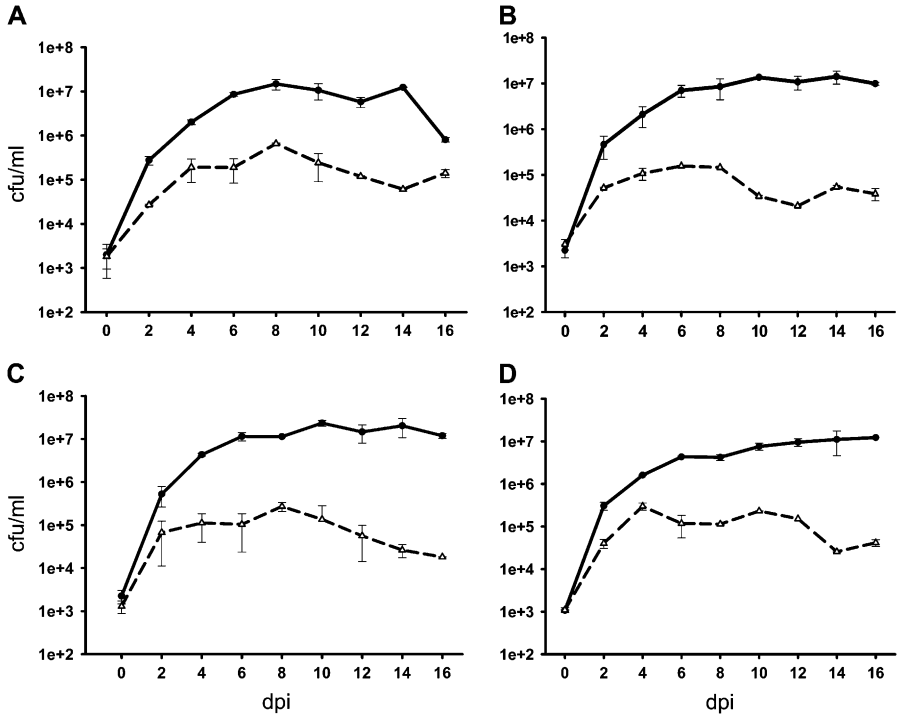

Figure 6. Bacterial growth in planta after infection of tomato leaves with *Xcv*. The in planta growth of the *Xcv* wild type (strain 75-3; solid lines) and the T3SS-deficient strain (*Δ hrpX*; dashed lines) was monitored over a 16-d period. Bacteria were infiltrated with 5×10^4 cfu mL^{-1}, and their growth was determined every second day. Values represent means of two samples taken from two infected leaves of two different plants. Error bars indicate SD. The experiment was repeated three times with similar results. A, Moneymaker wild type. B, Lin8-RNAi line 33. C, Lin8-RNAi line 50. D, Lin8-RNAi line 57.

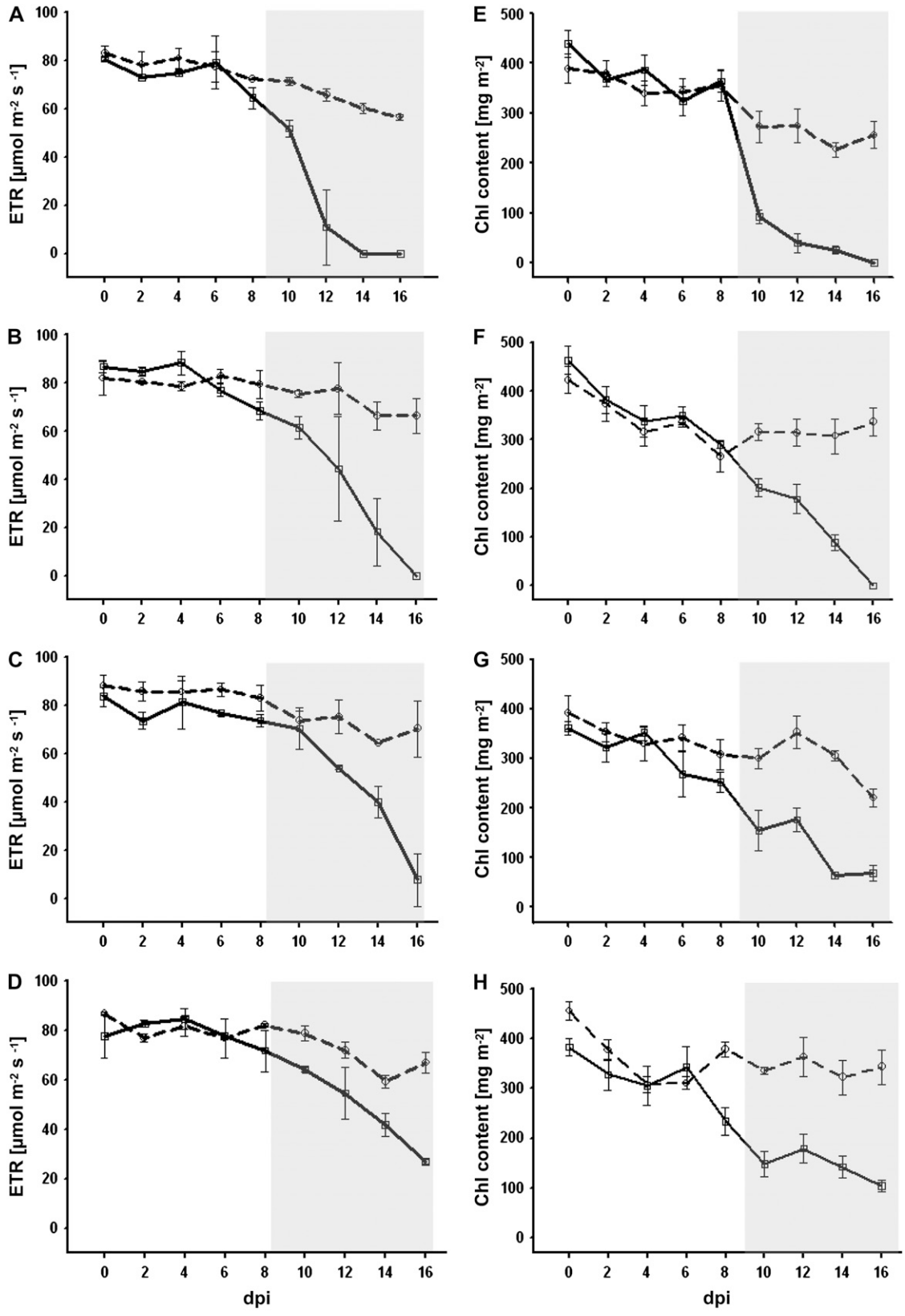

Figure 7. Changes in the ETR and the chlorophyll (Chl) content in transgenic tomato plants compared with the wild type. Wild-type (Moneymaker; A and E) and transgenic (line 33, B and F; line 50, C and G; line 57, D and H) tomato plants were inoculated with *Xcv* (solid lines) at 5×10^4 cfu mL^{-1}, and the effect on the ETR (A–D) and the chlorophyll content (E–H) was monitored over

analyzed, a recently identified senescence-inducible gene encoding a highly conserved chloroplastic protein (Park et al., 2007). As expected, expression of these senescence-associated genes increased during the time course of the experiment in mock-infiltrated plants, demonstrating the progressive senescence of noninfected leaves (Fig. 8). Compared with this, the transcript abundance increased to higher levels in *Xcv*-infected wild-type plants, with a clear accumulation at 14 and 16 d after infection. In contrast, there was only a slight induction in the expression of *GDH*, *GS-1*, and *Sgr* in the Lin8-RNAi line following bacterial infection in comparison with the mock-infected controls (Fig. 8). These results are in accordance with the observed differences in chlorophyll degradation as well as in symptom development between wild-type and cw-Inv-silenced plants.

In addition we analyzed the expression of different defense-related genes, namely, a basic β-1,3-glucanase (GluB), a chitinase (PR-Q), and the proteinase inhibitor Pin-II. GluB was shown to be up-regulated following infection with *Cladosporium fulvum* (van Kan et al., 1992). PR-Q transcripts were found to be induced by salicylic acid, soluble sugars, and potato virus Y infection (Herbers et al., 1996a, 1996b), whereas Pin-II is known to be regulated by wounding, jasmonic acid, and soluble sugars (Johnson and Ryan, 1990; Farmer and Ryan, 1992; Zhang et al., 2004).

As shown in Figure 8, GluB-, PR-Q-, and Pin-II-specific transcripts accumulated in tomato wild-type plants infected with *Xcv*. This induction was found to be abolished in cw-Inv-silenced plants.

DISCUSSION

Here, we mainly aimed at investigating the role of cw-Inv activity during the compatible interaction between tomato and *Xcv* as a model system.

An RNAi approach was used to silence Lin8 in transgenic tomato plants. Besides Lin8, the Lin6 isoform was also shown to be expressed in leaves and other tissues of tomato plants (Godt and Roitsch, 1997; Zamir and Fridman, 2003). Both sequences share high sequence identity and exhibit five stretches of at least 25 nucleotides with 100% identity. This was shown to be sufficient to generate small single-stranded RNAs, able to target the RNA-induced silencing complex to both target sequences (Le et al., 2006a, 2006b; Chen et al., 2008). Therefore, we reasoned that the Lin8-RNAi construct would be sufficient to silence both isoforms active in leaves. In fact, measuring the cw-Inv activity in different tissues of transgenic tomato plants revealed the strongest repression of cw-Inv activity in source leaves, which was reduced by about 90% compared with that in wild-type plants. In other tissues, the activity was decreased to a lesser extent (e.g. in sink leaves by about 50%) or was only slightly changed, indicating a preferential inhibition of leaf-specific isoforms Lin8 and Lin6, which was consistent with the published expression data (Godt and Roitsch, 1997; Fridman and Zamir, 2003). Importantly, the activities of neutral and soluble acidic invertases localized to the cytosol and vacuole, respectively, were not affected by the repression of cw-Inv (Table I).

In contrast to transgenic tobacco plants expressing the same RNAi construct (Essmann et al., 2008), low cw-Inv activity caused no significant alterations in primary metabolism of source leaves in transgenic tomato plants, as indicated by unchanged enzyme activities of SuSy and of glucokinases and fructokinases. Furthermore, under normal conditions, plant growth was not impaired, indicating that silencing of cw-Inv did not cause major perturbations in primary metabolism under these conditions.

Interestingly, our transgenic tomato plants accumulated more Fru and less starch during the day. This may be indicative of cytosolic metabolization of reimported Suc in the absence of cw-Inv. A similar observation was made in transgenic tobacco plants overexpressing a yeast invertase in the cytosol, which resulted in a 5- to 10-fold larger accumulation of Fru than Glc (Sonnewald et al., 1991).

The lower amount of starch was not brought about by an altered photosynthetic capacity or respiration rate. The activity of AGPase was even found to be higher in the transgenic lines. However, this might not result in a higher starch synthesis rate, since AGPase is strongly regulated by metabolites like 3-phosphoglyceric acid and pyrophosphate as well as by the redox state (Geigenberger et al., 2005). Our data rather suggest that the lower starch content in the transgenic lines is caused by a higher Suc efflux, indicating that cw-Inv restricts Suc export of source leaves and therefore might play a role in the regulation of source leaf metabolism.

Thus far, cw-Inv activity has been implicated in maintaining Suc unloading in sink tissues and ultimately in determining sink strength. This assumption is supported by the *miniature* mutant of maize (*Zea mays*), which is characterized by very small seeds due to the loss of cw-Inv activity (Miller and Chourey, 1992). The important role for cw-Inv in seed development could be confirmed by other studies, such as those in *Vicia faba* (Weber et al., 1996). Besides this, transgenic potato (*Solanum tuberosum*) tubers with enhanced cw-Inv activity also revealed a role for cw-Inv in the determination of sink strength (Sonnewald

Figure 7. (*Continued.*)
a 16-d period. Control leaves were infected with 10 mM $MgCl_2$ (dashed lines). ETR was determined at 350 μL L^{-1} CO_2 and illumination of 150 μmol m^{-2} s^{-1}. Values are means ± SD of four replicates. Results of one representative experiment are shown. Similar results were obtained in at least two experiments.

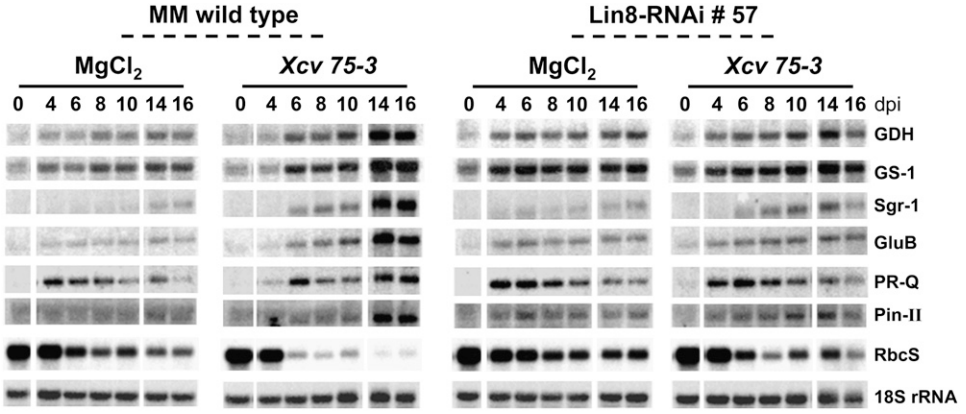

Figure 8. Expression of senescence and defense genes in Lin8-RNAi plants in response to *Xcv*. Total RNA was isolated from tomato leaves of wild-type plants and one transgenic plant (line 57) following infection with *Xcv* strain 75-3 (5×10^4 cfu mL^{-1}) or with 10 mM $MgCl_2$ as a control. Samples were taken before (0) and at 4, 6, 8, 10, 14, and 16 d after infection (dpi). Ten micrograms of total RNA was separated per each lane. Northern blots were hybridized with labeled [^{32}P]cDNA fragments of GDH, cytosolic GS-1, Sgr-1, GluB, PR-Q, Pin-II, RbcS, and 18S rRNA. MM, Moneymaker.

et al., 1997). However, a role for cw-Inv in regulating Suc export of source leaves has not been reported so far.

Enhanced cw-Inv activity and expression have been shown in response to different phytopathogens (Chou et al., 2000, Herbers et al., 2000; Swarbrick et al., 2006). Consistently, cw-Inv activity was found to be increased after infection of wild-type tomato plants with *Xcv*. This pathogen-induced induction of cw-Inv activity was abolished in Lin8-RNAi lines. However, increased invertase activity in the wild type did not result in significant changes in the amount of total soluble carbohydrates in response to bacterial infection (data not shown). This could be explained by the fact that extracellular sugars account for only a small percentage of total soluble sugars. For instance, the apoplastic Suc was found to represent only 0.6% to 0.9% of leaf Suc content in different species, such as *V. faba*, barley, maize, and spinach (*Spinacia oleracea*; Lohaus et al., 2001). Because of that, the apoplastic fluid was isolated and the Suc to hexose ratio was ascertained before and after *Xcv* infection of wild-type and transgenic plants. As expected, a clear increase in the Suc to hexose ratio was determined in the Lin8-RNAi lines following *Xcv* infection, whereas the ratio remained unchanged in wild-type plants. The latter observation may indicate that hexoses formed due to an induced cw-Inv activity in wild-type plants were immediately taken up by the cells and further metabolized; consequently, no increased content of hexoses could be found. In the transgenic plants, however, Suc accumulated relative to the hexoses due to the lack of cw-Inv activity, suggesting that it regulates hexose formation in the apoplast of source leaves.

The induction of cw-Inv is assumed to promote sink development, allowing the pathogen to withdraw carbohydrates for its nutrition. Besides this, hexoses formed by cw-Inv activity have been proposed to support defense responses by two mechanisms. On the one hand, they provide carbon skeletons and energy for the synthesis of phenolic compounds; on the other hand, they act as signals to induce the expression of defense-related genes (Herbers et al., 1996b). In this respect, a successful plant defense was reconciled with an early increase in cw-Inv activity and sugar accumulation (Scharte et al., 2005; Swarbrick et al., 2006; Essmann et al., 2008), whereas a late induction of cw-Inv is thought to support the nutrient supply of the pathogen and consequently disease (Seo et al., 2007). Hence, the relative competition for sugars between the pathogen and the host plant might determine the outcome of an infection.

Two scenarios could be envisaged concerning the response of cw-Inv-silenced plants to pathogen attack. The genetic modification could render the plants either more resistant because of a shortage of hexoses to feed the pathogen or more susceptible due to the absence of sugar signals, which are supposed to amplify plant defense. In fact, symptom development was delayed in transgenic plants compared with wild-type plants. However, the bacterial growth in planta was not reduced in the transgenic lines. This suggests that the loss of cw-Inv induction did not limit bacterial nutrition and propagation. In this respect, there are recent publications indicating that bacteria utilize a number of nutrients that are abundant in the apoplast, like sugars, organic acids, and amino acids (Tang et al., 2005; Rico and Preston, 2008).

The differences in the disease development of wild-type and transgenic plants upon *Xcv* challenge prompted us to investigate whether this would by paralleled by differences in photosynthetic capacity. Infected plants often exhibit a reduced rate of photosynthesis (for review, see Walters and McRoberts, 2006; Berger et al., 2007). Accordingly, a gradual decrease in ETR was measured in both genotypes following *Xcv* infection.

However, the decrease was steeper in wild-type plants than in the transgenic lines, particular during late stages of infection. A similar trend was also seen for the loss of chlorophyll. In addition, there was only a minor induction in the expression of senescence-associated genes following bacterial infection in the transgenic plants compared with wild-type plants. This was consistent with a delayed formation of chlorosis and necrosis in Lin8-RNAi lines. Together, these data suggest that inhibition of cw-Inv activity caused a delayed decrease in photosynthetic capacity and pathogen-induced senescence, thereby allowing the bacteria to maintain their growth for longer periods.

Two mechanisms have been suggested for the reduction of photosynthesis following pathogen infection: a direct effect of the pathogen on the expression of photosynthetic genes and an indirect effect mediated by sugar signals (Walters and McRoberts, 2006, and refs. therein). Supportive evidence for the direct effect comes from transcript-profiling experiments during the interaction between Arabidopsis and *Pseudomonas syringae* pv *tomato* (Truman et al., 2006). These authors found a widespread suppression of photosynthetic gene expression caused by bacterial effector proteins, indicating that bacterial infection itself influences photosynthesis. In agreement with these results, Mudgett and colleagues (Kim et al., 2008) recently demonstrated that the *Xcv* effector protein XopD contributes to a delayed development of tissue chlorosis and necrosis. Thus, the effector-mediated effect on photosynthesis was most likely responsible for the reduced rate of photosynthesis and the expression of corresponding genes observed in both wild-type and transgenic plants in our experiments.

However, the differences observed between wild-type and Lin8-RNAi lines are likely caused by cw-Inv-dependent sugar signals. Accumulation of soluble sugars, in particular hexoses, is known to repress photosynthetic gene expression, thereby providing an import mechanism to integrate environmental factors and internal signals to modulate growth and development (Koch, 1996; Smeekens, 2000; Rolland et al., 2006). Molecular data suggest the presence of hexokinase-dependent and -independent sensing mechanisms for Glc (Smeekens, 2000; Rolland et al., 2006). The hexokinase-dependent pathway has been implicated mainly in the down-regulation of photosynthetic gene expression (Rolland et al., 2006). Moreover, the analysis of transgenic tobacco plants expressing a yeast-derived invertase in different subcellular compartments revealed that sugar sensing occurs in the secretory membrane system (Herbers et al., 1996a). Thus, one could speculate that hexoses generated in the apoplast by an increased cw-Inv activity might trigger a signal cascade, causing a feedback inhibition of photosynthesis and a decreased expression of photosynthetic genes in wild-type plants. This view is supported by the reduced decline of photosynthesis and photosynthetic gene expression in Lin8-RNAi lines compared with the wild type.

Apart from regulating photosynthesis, sugars were also shown to up-regulate the expression of defense genes (Herbers et al., 1996b). Consistently, the expression of GluB-, PR-Q-, and Pin-II-specific transcripts was found in tomato wild-type plants infected with *Xcv*. This induction was not observed in Lin8-RNAi plants. In previous studies, it was demonstrated that both Pin-II and PR-Q are regulated by sugars, in addition to jasmonic acid and salicylic acid, respectively (Johnson and Ryan, 1990; Herbers et al., 1996b, 2000). Furthermore, sugar-mediated regulation of PR-Q was found to be independent of salicylic acid (Herbers et al., 2000). Therefore, it is tempting to speculate that the observed induction of Pin-II and PR-Q in wild-type tomato plants is caused by hexoses produced in the apoplast rather than the known hormone signals. This view is further supported by the observation that ectopic expression of a yeast-derived invertase in the apoplast of transgenic tobacco plants led to the induction of PR-Q expression (Herbers et al., 1996a).

Taken together, our results show that even though transgenic Lin8-RNAi plants developed delayed disease symptoms following infection with the virulent *Xcv*, this was not accompanied by reduced bacterial growth in planta. The delayed symptom production can be attributed to a slower reduction of photosynthetic capacity and a decelerated rate of pathogen-induced senescence. This is most likely due to the absence of hexose signals, which cannot be generated in the cw-Inv-silenced plants. In wild-type plants, however, increased formation of hexoses by induction of cw-Inv activity following *Xcv* infection might trigger the stronger down-regulation of photosynthesis and photosynthetic gene expression and the up-regulation of sugar-responsive *PR* genes. The higher photosynthetic rate obtained in the transgenic plants might enable the pathogen to maintain its growth longer and thereby seems to be advantageous for the bacterium. This is consistent with the observation that bacterial effector proteins suppress cw-Inv and thereby hexose-mediated signals (Biemelt and Sonnewald, 2006).

MATERIALS AND METHODS

Plasmid Construction and Plant Transformation

Standard procedures were carried out as described by Sambrook et al. (1989). Gateway technology was used to generate an RNAi construct. Primers were designed from the highly homologous sequence regions of Lin8 (accession no. AF506007) and Lin6 (accession no. AF506005), sharing 81.9% sequence identity, with the aim of silencing both genes using one RNAi construct. A 400-bp cDNA fragment of Lin8 was amplified by PCR using the primers Lin8-5′ (5′-CACCGGTACTGGAAATTGGG-3′) and Lin8-3′ (5′-ACAGGCCATTGA-ACCAATTG-3′; nucleotides 755–1,162 of *Lin8*) and tomato (*Solanum lycopersicum*) leaf cDNA as a template. cDNA synthesis was performed as described by Mustroph et al. (2007). The resulting PCR product was subcloned into pENTR/D (Invitrogen) to create an entry clone containing the *attL* recombination sites. Subsequently, the λ reconstruction reaction was performed to introduce the fragment into the destination vector pK7GWIWG2 (Karimi et al., 2002) containing the *attR* attachment sites according to the manufacturer's instructions. Thereby, the final construct, designated Lin8-RNAi, was obtained.

The construct was transformed into *Agrobacterium tumefaciens* strain CV58C1 carrying the pGV2260 virulence plasmid. Transformation of tomato plants (cv Moneymaker) was performed essentially as described by Ling et al. (1998) using tomato cotyledons and a tobacco (*Nicotiana tabacum*) cell suspension culture as a feeder layer.

Bacterial Strains

For this study, the wild type and a T3SS-deficient mutant (*Δ hrpX*) of *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) strain 75-3 were used (kindly provided by U. Bonas). Bacteria were grown at 28°C on NYG agar (1.5%, v/v) or NYG medium supplemented with 100 μg mL^{-1} rifampicin.

Plant Material, Growth Conditions, and Inoculation Experiments

Tomato plants were cultivated in a greenhouse with 16 h of supplemental light (150 μmol quanta m^{-2} s^{-1}) and 8 h of darkness. The temperature regime followed the day/night cycle with 22°C/20°C. The T1 generation of three selected transgenic lines was used for the primary characterization. More detailed investigations were performed with the T2 generation. To this end, seeds were sown in soil and siblings were screened for low invertase activity. Only plants with an activity 5 μmol min^{-1} m^{-2} or less were used for further investigations.

Six-week-old plants were used for infection experiments. One week before infection with *Xcv*, the temperature was increased to 25°C and 22°C during the light and dark periods, respectively. Bacterial cultures for inoculation were grown overnight in NYG medium containing 100 μg mL^{-1} rifampicin, centrifuged at 5,000*g* for 15 min at 4°C, and washed with 10 mM sterile $MgCl_2$. Subsequently, cells were resuspended in 10 mM $MgCl_2$ and adjusted to a final concentration of 5×10^4 or 1×10^8 cfu mL^{-1}.

Bacteria were hand-infiltrated at the abaxial side of fully mature leaves using a blunt-end-tip syringe. Leaf discs for biochemical and molecular analysis were sampled after inoculation of bacterial strains at the time points indicated in the figures and immediately frozen in liquid nitrogen.

In Planta Growth of *Xcv* Strains

To monitor the growth of *Xcv* in tomato plants, leaves were inoculated with a bacterial suspension of 5×10^4 cfu mL^{-1}. Bacterial levels in planta were determined by homogenizing leaf discs in sterile water, plating appropriate dilutions on NYG agar plates containing rifampicin (100 μg mL^{-1}), and counting the number of bacterial colonies.

RNA Isolation and Northern-Blot Analysis

Isolation of total RNA was performed as described by Logemann et al. (1987). For northern-blot analysis, 10 μg of total RNA was separated on 1.5% formaldehyde-containing agarose gels and blotted onto nylon membranes (GeneScreen; PerkinElmer) by capillary blotting overnight. The membranes were prehybridized and hybridized at 65°C. cDNA fragments of ferredoxin-NADP reductase (accession no. Y14032), preplastocyanin (accession no. X13934), GDH (accession no. U48695), cytosolic GS-1 (accession no. X95932), Sgr-1 (accession no. DQ100158), GluB (accession no. X74905), PR-Q (accession no. X54456), Pin-II (accession no. AY129402), RbcS (accession no. X02353), cytosolic glyceraldehyde 3-phosphate dehydrogenase (accession no. AF527779), and 18S rRNA (accession no. X51576) were used as probes and radioactively labeled with [^{32}P]dCTP by means of the High Prime Kit (Roche). After stringent washing, radioactive membranes were exposed to x-ray films (Kodak) overnight at −70°C.

Determination of Soluble Sugars and Starch

Leaf discs (0.5 cm^2) were extracted with 1.0 mL of 80% (v/v) ethanol and incubated at 80°C for 60 min. After centrifugation at 4°C for 5 min at 14,000 rpm, cleared supernatants were transferred into new tubes and evaporated to dryness at 40°C. The residue was resolved in 250 μL of water and used for the determination of soluble sugars. The pellet derived from the centrifugation step was used for the determination of starch. To this end, it was homogenized with 0.2 M KOH and incubated at 95°C for at least 1 h. The pH value was adjusted to 5.5 by adding 1 M acetic acid. Starch hydrolysis and determination of soluble sugars were performed as described by Hajirezaei et al. (2000).

Enzyme Extraction and Activity Assays

Samples of 10 to 20 mg of leaf material were homogenized with 50 mM Tris buffer, pH 6.8, containing 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 15% (v/v) glycerine, and 0.1 mM Pefabloc proteinase inhibitor. The extracts were centrifuged for 5 min at 15,000 rpm at 4°C. An aliquot of the resulting supernatant was desalted by centrifugation through Sephadex G-25 medium equilibrated in extraction buffer. The desalted extracts were used for the different assays. Activities of vacuolar and neutral invertase, glucokinase, and fructokinase were determined as described by Zrenner et al. (1995). The activity of AGPase was measured as described by Leidreiter et al. (1995). The activity of SuSy was determined by incubating the samples in 0.1 M HEPES buffer, pH 7.8, containing 0.17 M Suc and 10.6 mg mL^{-1} UDP for 15 min at 30°C. The reaction was stopped by heat inactivation at 95°C for 4 min. The amount of Fru formed was measured as described by Hajirezaei et al. (2000).

Protein concentrations were determined according to Bradford (1976). The remaining pellet was washed twice with 5 mM Tris buffer, pH 7.0, and centrifuged for 10 min at 15,000 rpm and 4°C. The activity of cw-Inv was determined by resolving the pellet in 50 mM sodium acetate buffer, pH 5.2, containing 0.1 M Suc and incubating the mixture at 37°C for 90 to 120 min. The mixture was neutralized by adding an aliquot of 1 M Tris-HCl, pH 8.0, and subsequently, the reaction was stopped by heat inactivation at 95°C for 5 min. The amount of Glc formed was measured as described by Hajirezaei et al. (2000).

Extraction of Apoplastic Fluid

For the extraction of apoplastic fluid, one or two tomato leaves (approximately 2 g fresh weight) were harvested, the midrib was removed, and the leaves were weighed and washed in water. After drying, leaves were vacuum infiltrated with 50 mM HEPES-KOH, pH 6.8, for 5 to 10 min. The leaf surface was dried, and leaves were wrapped in aluminum foil and inserted into a 50-mL Falcon tube with the tip cut down. The Falcon tube was placed in a centrifuge tube and centrifuged at 1,400*g* for 10 min at 4°C. An aliquot of 30 μL of the collected apoplastic fluid was used for the determination of soluble sugars as described above. The amount of chlorophyll was determined to control the purity of the preparation.

Determination of Suc Efflux

Suc efflux analysis was essentially carried out as described by Kronberg et al. (2007) with slight modifications. One mature source leaf was detached by cutting petioles close to the stem and immediately placed in 4 mL of 5 mM EDTA. Phloem exudates were sampled after 1, 2, 3, 4, and 5 h, and Suc content was determined as described above.

Measurements of Photosynthesis and Respiration

Photosynthetic parameters and respiration rate were determined with a combined gas exchange/chlorophyll fluorescence imaging system (GFS-3000 and Mini-Imaging PAM Chlorophyll Fluorometer; Walz). Parameters were monitored on an 8-cm^2 leaf area of fully infected leaves. Assimilation rate and ETR were calculated as described previously by Horst et al. (2008) at 350 μL L^{-1} CO_2 and illumination of 320 μmol m^{-2} s^{-1}.

To assess the respiration rate, plants were transferred to darkness for about 20 min. The CO_2 concentration of the air entering the leaf chamber and the temperature were adjusted to 320 μmol mol^{-1} and 25°C, respectively. Respiration rate was calculated from a 15-min period after it was equilibrated and remained at a constant level.

Determination of Chlorophyll Content

Chlorophyll content was determined after incubation of leaf discs (0.5 cm^2 diameter) in 80% ethanol at 80°C for 1 h, as described by Arnon (1949).

Statistical Analysis

To test for statistical significance, a modified Student's *t* test was used allowing statistical analysis of data sets with different variances (Welch, 1947).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF506005, AF506007, Y14032, X13934,

U48695, X95932, DQ100158, X74905, X54456, AY129402, X02353, AF527779, and X51576.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phenotype of Lin8-RNAi plants compared with the wild type.

Supplemental Figure S2. Expression of photosynthetic genes in Lin8-RNAi plants in response to *Xcv*.

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