

# Post-Translational Derepression of Invertase Activity in Source Leaves via Down-Regulation of Invertase Inhibitor Expression Is Part of the Plant Defense Response

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**ABSTRACT** There is increasing evidence that pathogens do not only elicit direct defense responses, but also cause pronounced changes in primary carbohydrate metabolism. Cell-wall-bound invertases belong to the key regulators of carbohydrate partitioning and source-sink relations. Whereas studies have focused so far only on the transcriptional induction of invertase genes in response to pathogen infection, the role of post-translational regulation of invertase activity has been neglected and was the focus of the present study. Expression analyses revealed that the high mRNA level of one out of three proteinaceous invertase inhibitors in source leaves of *Arabidopsis thaliana* is strongly repressed upon infection by a virulent strain of *Pseudomonas syringae* pv. tomato DC3000. This repression is paralleled by a decrease in invertase inhibitor activity. The physiological role of this regulatory mechanism is revealed by the finding that *in situ* invertase activity was detectable only upon infection by *P. syringae*. In contrast, a high invertase activity could be measured *in vitro* in crude and cell wall extracts prepared from both infected and non-infected leaves. The discrepancy between the *in situ* and *in vitro* invertase activity of control leaves and the high *in situ* invertase activity in infected leaves can be explained by the pathogen-dependent repression of invertase inhibitor expression and a concomitant reduction in invertase inhibitor activity. The functional importance of the release of invertase from post-translational inhibition for the defense response was substantiated by the application of the competitive chemical invertase inhibitor acarbose. Post-translational inhibition of extracellular invertase activity by infiltration of acarbose in leaves was shown to increase the susceptibility to *P. syringae*. The impact of invertase inhibition on spatial and temporal dynamics of the repression of photosynthesis and promotion of bacterial growth during pathogen infection supports a role for extracellular invertase in plant defense. The acarbose-mediated increase in susceptibility was also detectable in *sid2* and *cpr6* mutants and resulted in slightly elevated levels of salicylic acid, demonstrating that the effect is independent of the salicylic acid-regulated defense pathway. These findings provide an explanation for high extractable invertase activity found in source leaves that is kept inhibited *in situ* by post-translational interaction between invertase and the invertase inhibitor proteins. Upon pathogen infection, the invertase activity is released by repression of invertase inhibitor expression, thus linking the local induction of sink strength to the plant defense response.

**Key words:** Acarbose; *Arabidopsis*; defense responses; invertase inhibitor protein; invertase; plant-microbe interactions; *Pseudomonas syringae*.

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## INTRODUCTION

Invertases (EC 3.2.1.26) are the key enzymes that hydrolyze sucrose into glucose and fructose and play a central role in carbohydrate partitioning. They occur as a set of isoenzymes in many higher plants differing in pH optima, isoelectric points, and sub-cellular localization (see review by Roitsch and Gonzalez, 2004). The expression and activity of cell wall-bound invertase (cwlInV) are known to be up-regulated when plants are exposed to a variety of stress stimuli (Roitsch et al., 2003). In particular, during plant–pathogen interactions, there are several reports of induction of cwlInV expression (Benhamou et al., 1991; Chou et al., 2000; Fotopoulos et al., 2003; Berger et al., 2004; Swarbrick et al., 2006; Berger et al., 2007; Essmann et al., 2008; Kocal et al., 2008). One of the most intensively studied plant–pathogen interactions is the attack of the model plant *Arabidopsis thaliana* by the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000. For this interaction, a decrease in cwlInV activity has been reported (Bonfig et al., 2006). Silencing of cwlInV expression was shown to interfere with the growth of neither a bacterial pathogen in tomato (Kocal et al., 2008) nor a fungal pathogen in tobacco (Essmann, et al., 2008), respectively. Despite a number of reports on the regulation and function of invertase activity in various pathosystems, the physiological role of invertases in pathogen responses is not yet fully understood.

Regulation of invertase occurs at the level of expression during growth and development, by the hexose pool in plant tissues or by environmental stimuli (Pressey, 1994; Sampietro et al., 1980; Morris and Arthur, 1984; Krishnan and Pueppke, 1990; Sturm and Chrispeels, 1990; Krausgrill et al., 1998; Bonfig et al., 2006). Another control mechanism of invertase activity is the presence of endogenous inhibitor proteins. Although this post-translational regulation of invertases has been known for a long time (Schwimmer et al., 1961; Pressey, 1967), the physiological role is still not fully understood. Invertase inhibitors are found in a variety of plant species (Pressey, 1968, 1994; Krausgrill et al., 1998; Bate et al., 2004; Huang et al., 2007; Reca et al., 2008; Jin et al., 2009) and a functional approach revealed a role in senescence and fruit development of tomato (Jin et al., 2009). The first evidence for an invertase inhibitor in potato tubers was obtained from kinetic studies on invertase crude extracts (Pressey, 1967; Schwimmer et al., 1961) and it was suggested that this invertase inhibitor is an important regulator of invertase activity in potato tubers (Pressey, 1967). Moreover, it was assumed that during certain stages of development, cell wall invertase activity is modulated by invertase inhibitors, the latter operating as a regulatory switch (Krausgrill et al., 1998). Inhibitory proteins have a molecular weight of 16–20 kD and post translationally regulate invertase activity in a pH-dependent manner (Hothorn et al., 2003). Effectiveness of this inhibitor varied with pH, with an optimum of pH 4.5.

The reactivity of the inhibitor with various plant invertases (petunia, tomato, rhubarb, alfalfa, and sweet clover) ranged from negligible to total inhibition. In tobacco plants, the invertase inhibitor (Nt-CIF) was demonstrated to be highly thermostable and to retain full inhibitory activity when brought back to ambient temperatures (Hothorn et al., 2003).

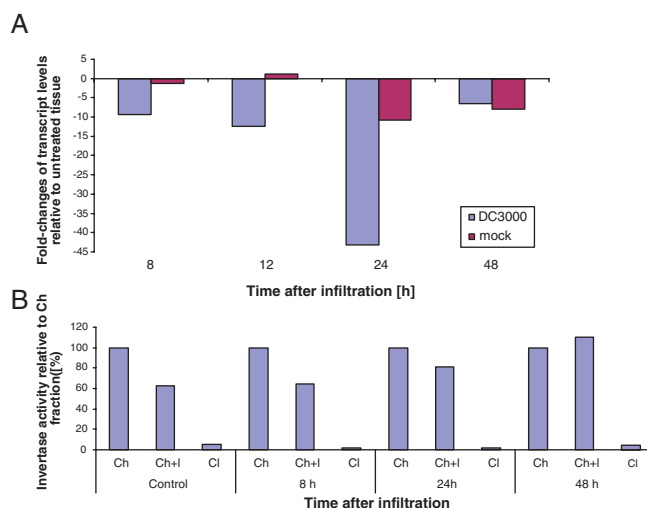
There are also certain chemicals known to act as invertase inhibitors; some of them are used for pharmaceutical applications. A thio-fructoside, Fru-S-ME, synthesized by transfructosylation of  $\beta$ -fructofuranosidase from sucrose to mercaptoethanol is hardly hydrolyzed by  $\beta$ -fructofuranosidases (from different organisms) and  $\alpha$ -glucosidases and therefore can be used as an invertase inhibitor (Kiso et al., 2003). Calystegines, hydroxylated nortropane derivatives found in many plant species, are strong glycosidase inhibitors (Asano et al., 2000; Richter et al., 2007). It was shown that calystegines could inhibit the invertase derived from *Calystegia sepium*, a member of the convolvulaceae family (Höke and Dräger, 2004), whereas the fungal acid invertase activity was not affected. Inhibitors of carbohydrate-hydrolyzing enzymes like the glucosidase inhibitor acarbose play an important role for the treatment of the so-called type II or insulin-dependent diabetes in humans (Hanozet et al., 1981; Brunkhorst, 2004; Kim et al., 2004). Acarbose is a pseudotetrasaccharide consisting of an unsaturated aminocyclitol moiety, a deoxyhexose, and a maltose and is synthesized in *Actinoplanes* sp. SE50/110 (Brunkhorst et al., 1999; Brunkhorst, 2004). It strongly inhibits glucoamylase,  $\alpha$ -amylase, and other retaining or inverting  $\alpha$ -glucoside-specific hydrolases and transferases as well as  $\beta$ -fructofuranosidases (Legler, 1990; Sinnott, 1990; Svensson and Sierks, 1992; Sigurskjold et al., 1994; Frandsen and Svensson, 1998; Höke and Dräger, 2004). Acarbose was shown to be a competitive inhibitor with a  $K_i$  of 1.5  $\mu$ M for the high-pI  $\alpha$ -glucosidase (Frandsen et al., 2000). In aphids, acarbose inhibited sucrase activity in gut homogenates and the production of mono- and oligosaccharides in the honeydew caused an increase in the hemolymph osmotic pressure for aphids reared on a diet hyperosmotic to the hemolymph (Karley et al., 2005).

Since plant–pathogen interaction studies have focused so far only on the transcriptional regulation of invertases and the invertase activity present in extracts, the possible role of proteinaceous invertase inhibitors in post-translational regulation of invertase activity *in situ* has been addressed in the model pathosystem *Arabidopsis thaliana* and *Pseudomonas syringae* pv. tomato DC3000. The expression and activity of invertase inhibitors have been determined and compared with the *in situ* and *in vitro* invertase activities. The functional relevance of the observed release of invertase from post-translational inhibition by invertase inhibitors for the plant defense response via repression of inhibitor expression has been substantiated by the use of the invertase inhibitor acarbose.

## RESULTS

### Expression of Invertase Inhibitor *AtC/VIF2* in Source Leaves Is Repressed in Response to Infection by *Pseudomonas syringae*

Cell wall and vacuolar invertases are known to be regulated by invertase inhibitor proteins (Huang et al., 2007). Since there is no report on the effect of pathogen infection on invertase inhibitor expression, we analyzed the transcriptional regulation of the endogenous invertase inhibitors of *Arabidopsis*. Three different genes have been annotated to encode invertase inhibitors in *A. thaliana*: At1g47960 encoding invertase inhibitor *AtC/VIF1* (Link et al., 2004), At5g64620 encoding invertase inhibitor *AtC/VIF2* (Link et al., 2004), and At3g17130 encoding a putative invertase inhibitor. The expression profile of invertase inhibitor genes was analyzed *in silico* to investigate the expression of invertase inhibitors in source leaves of *Arabidopsis*. Northern blot analysis was performed to re-evaluate these *in silico* data (data not shown). For invertase inhibitor *AtC/VIF1*, a low expression in leaves and flowers but high expression in siliques were predicted in genevestigator. In agreement with these data, very low and not specifically regulated *AtC/VIF1* expression was observed in leaves. Likewise, the expression level of the gene encoding the putative inhibitor At3g17130 was very low and could not be detected by Northern blot analysis. In contrast, according to genevestigator, *AtC/VIF2* is highly expressed in source leaves and its expression increases during the maturation of the leaves. The expression of this gene was predicted to be repressed by infection with *P. syringae*. In agreement with these *in silico* data, Northern blot analyses revealed that the expression of this gene in source leaves decreased at 24 and 48 hours past infection (hpi) upon infection with a virulent strain of *P. syringae* pv. tomato DC3000. A similar down-regulation of the steady-state level of mRNA was detected after wounding of leaves. To quantify the regulation of *AtC/VIF2* gene, a more comprehensive quantitative PCR analysis was carried out. Figure 1A shows that in pathogen-treated samples, transcript levels were diminished about nine-fold already 8 h past infection (hpi) and fell continuously, until expression was reduced more than 40-fold at 24 hpi. The apparent increase in transcript levels 48 hpi is due to a four-fold decrease in housekeeping gene expression in *P. syringae* DC3000-infected leaves, possibly because these leaves were already severely necrotic at this time point. Thus, CT values of *PEX4* ranged between 25.9 and 27.6 in all samples, with one exception: 48 h after infection with the pathogen, expression of *PEX4* was markedly lower, shown by a CT value of 29.6. Whereas expression of *AtC/VIF2* was not affected 8 hpi in mock inoculated samples, it decreased ~11-fold 24 h after infiltration of MgCl<sub>2</sub> infiltration. The clear decrease in *AtC/VIF2* expression after mock treatment can be explained by the infiltration procedure causing a wounding effect. Northern blot data from our group independently showed that *AtC/VIF2* expression is not detectable 24 and 48 h after MgCl<sub>2</sub> infiltration or wounding, while the gene is strongly expressed 8 h



**Figure 1.** Regulation of Invertase Inhibitor Expression and Activity by Infection with *P. syringae* DC3000.

**(A)** Expression of invertase inhibitor *AtC/VIF2* after mock infiltration (10 mM MgCl<sub>2</sub>) or pathogen infection (*P. syringae* DC3000, 10<sup>8</sup> cfu ml<sup>-1</sup>) in relation to untreated tissue, normalized against *AtPEX4* transcript levels.

**(B)** Activity of invertase inhibitors. The activity of invertase inhibitor proteins was measured as described in Methods. C<sub>l</sub>, fraction with lower molecular mass proteins containing invertase inhibitor proteins; C<sub>h</sub>, fraction with higher molecular mass proteins containing cell wall invertase. C<sub>h</sub> + C<sub>l</sub> = mixture of C<sub>h</sub> + C<sub>l</sub> with 10× excess of C<sub>l</sub>. Activity of the Ch-fractions of the control leaves are set to 100% and the data represented are mean values of three independent experiments.

past mock treatment or wounding. CT values of *AtC/VIF2* did not change significantly between 24 and 48 hpi both for MgCl<sub>2</sub> and pathogen-treated samples.

### Invertase Inhibitor Activity Is Reduced by Infection with *Pseudomonas syringae*

To test whether the repression of invertase inhibitor *AtC/VIF2* is reflected by a reduced invertase inhibitor activity, we established a new experimental protocol to directly assess the activity of the inhibitor proteins, since current protocols do not remove cytoplasmic invertases from the preparation (Kusch et al., 2009). Invertase inhibitors are low-molecular-weight proteins usually in the range of 16–20 kD, while cwInv are 70 kD in size. To separate these two proteins in a protein crude extract, we used Centricon® YM-30 centrifugal filter devices, where proteins of different molecular weight are separated by spinning the crude extract with a nominal molecular-weight limit of 30 kD. The protein fraction that should contain the invertase inhibitor was collected as flow-through consisting of lower-molecular-weight proteins from the crude extract (C<sub>l</sub>), while the fraction with higher-molecular-weight proteins (also containing cwInv) were retained in the upper part of the crude extract (C<sub>h</sub>). The invertase activity in the lower fraction (C<sub>l</sub>) was negligible at all time points, while in the upper fraction (C<sub>h</sub>), it ranged between 115.6 and 214.4 μg glucose g fw<sup>-1</sup> min<sup>-1</sup>

(Figure 1B). By adding the lower fraction ( $C_l$ ) to the invertase-containing fraction of the control extract ( $C_h$ ), we were able to detect clear changes in invertase inhibitor activity. The relative activity of the mixed extract immediately before infection with DC3000 was 37.3% lower than the invertase activity of the fraction  $C_h$  alone, after 8 h, the relative activity of the mixed extract was 36.0% lower compared with fraction  $C_h$ , after 24 h the decrease was 20.1%, and after 48 h no decrease was detectable; we even found an increase of 9.9%. These results demonstrate that the repression of invertase inhibitor *AtC/VIF2* expression upon pathogen infection results in a rapid decline in invertase inhibitor activity, indicating a high turnover of the inhibitor protein.

### Derepression of Invertase Activity in Source Leaves of *Arabidopsis* in Response to Infection by *Pseudomonas syringae*

Previously, we have shown that infection of leaves of *A. thaliana* with *P. syringae* results in a reduction in the invertase activity in cell wall extracts and in a weak and transient increase in *vacInv* activity in crude extracts (Bonfig et al., 2006). Since these invertase activity data obtained with crude extracts *in vitro* are in contrast to the observed regulation of the invertase inhibitor activity (see above), we have re-evaluated the changes in invertase activity *in situ*. Thus, the invertase activity present in leaves was visualized by a histochemical activity stain (Godt and Roitsch, 1997) in relation to pathogen infection. Figure 2 demonstrates that no invertase activity can be detected in control leaves (Figure 2A and 2C) or mock inoculated leaves (right leaf halves in Figure 2D and 2E). In contrast, a high level of sucrose-dependent invertase activity is present in leaves infected with *P. syringae* DC3000 visualized by a dark staining of the infected tissue (Figure 2B and left leaf halves in Figure 2D and 2E). The discrepancy between the *in vitro* and *in situ* invertase activities in control leaves and the high invertase activity upon pathogen infection are in agreement with the observed pathogen-



**Figure 2.** Effect of Infection by *P. syringae* DC3000 on *In Situ* Invertase Activity in Leaves Visualized by a Histochemical Activity Stain Complemented with Sucrose as Substrate for Invertase.

- (A) Control (untreated).  
 (B) After wounding.  
 (C) Control (after wounding, sucrose omitted).  
 (D, E) Left leaf half infiltrated with *P. syringae* DC3000, right leaf half mock infiltrated with 10 mM  $MgCl_2$ . Pictures were taken 48 h after the respective treatment.

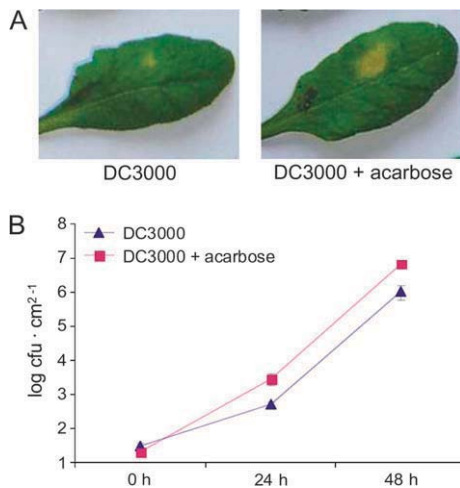
dependent repression of invertase inhibitor *AtC/VIF2*. Likewise, wounding was also shown to result in a highly elevated *in situ* invertase activity. A high level of *cwINV* and *vacINV* is present in healthy and uninfected leaves that is kept inactive through post-translational and non-covalent interaction with invertase inhibitor *AtC/VIF2*. Upon pathogen infection or in response to other stress-related stimuli, the invertase activity is released from post-translational inhibition via repression of invertase inhibitor expression leading to a de-repression of invertase activity. Hence, an increase in invertase activity in source leaves is achieved by depletion of the invertase inhibitor activity.

### Inhibition of *cwInv* Activity by Acarbose Increases Pathogen Susceptibility

To address the functional relevance of invertase de-repression upon pathogen infection, we devised an experimental approach to keep cell wall invertase activity inhibited post-translationally in the presence of the pathogen, despite a down-regulation of the endogenous invertase inhibitor. For this experimental approach, we selected the pseudotetra-saccharide acarbose that was previously shown to act as a competitive inhibitor of various  $\beta$ -fructosidases (Legler, 1990; Sinnott, 1990; Svensson and Sierks, 1992; Sigurskjold et al., 1994; Frandsen and Svensson, 1998; Höke and Dräger, 2004).

The effect of acarbose on *A. thaliana* invertase activity has been initially characterized *in vitro*. Addition of 10 mM acarbose to an assay with a cell wall extract resulted in an inhibition of invertase activity of 33%, while the *cwInv* was completely inhibited when 50 mM of the inhibitor was added (data not shown). Additionally, a concentration of 10 mM acarbose did not have any visible adverse effect when infiltrated in the leaves, while concentrations of 50 mM or more caused wilting. To rule out a direct effect on the growth of the pathogen, the effect of acarbose on *P. syringae* was tested in liquid culture. It was observed that acarbose had no direct effect on the growth of bacteria when added to the liquid growth media of the bacterium at a concentration of 10 mM (data not shown).

In order to assess the implication of inhibiting *cwInv* activity during pathogen infection, leaves of *A. thaliana* were infiltrated with a suspension of the virulent strain *P. syringae* pv. tomato DC3000 with or without 10 mM acarbose. The leaves were monitored up to 48 hpi for symptom development and growth of bacteria. Lesions in the infiltrated region of the leaf developed faster and stronger in leaves inoculated with the acarbose containing suspension (Figure 3A). The growth of *P. syringae* isolated from leaves infiltrated with acarbose containing bacterial suspension was five and six-fold higher after 24 and 48 hpi, respectively, than in leaves infiltrated with bacteria alone (Figure 3B). This result demonstrates that inhibition of extracellular invertase activity in the apoplast increases the sensitivity of *Arabidopsis* leaves towards infection by *P. syringae*. Thus, if the invertase activity present in source leaves is kept inhibited despite the down-regulation of the invertase inhibitor, the plant defense is weakened.



**Figure 3.** Effect of Invertase Inhibition by Acarbose on Bacterial Proliferation in Leaves of *A. thaliana*.

(A) Symptom development in leaves infiltrated with *P. syringae* DC3000 (left) or with bacterial suspension plus acarbose (right) 48 hpi.

(B) Proliferation of bacteria in leaves previously infected with *P. syringae* DC3000 and in leaves treated with bacterial suspension to which acarbose was added. Data represented are mean values of five independent experiments.

### Characterization of Carbohydrate Metabolism

Since invertase is a key regulatory enzyme in carbohydrate partitioning, we investigated the effect of invertase inhibition on different aspects of carbohydrate metabolism by monitoring photosynthesis and the expression of source and sink-related genes. Photosynthesis is one predominant metabolic process occurring in source leaves and has been shown to be affected by pathogen infection. Chlorophyll fluorescence imaging was used to monitor spatial and temporal changes in photosynthetic parameters in leaves infiltrated with *P. syringae* alone or in addition to acarbose. In agreement with stronger symptom development upon the combined treatment with pathogen and acarbose, the maximum quantum yield (Fv/Fm) and the optimal quantum yield (yield) of photosystem II decreased faster and stronger in the leaves infiltrated with the bacterial suspension containing the invertase inhibitor (Figure 4A).

The effect of pathogen and inhibitor treatment on the expression of genes related to source (*RbcS*) and sink metabolism (*cwINV1*) was also analyzed. Expression of *cwINV1* was not affected by mock treatment or by acarbose alone (Figure 4B), whereas *RbcS* showed a slight repression after 24 and 48 h of acarbose treatment. Pathogen alone or in the presence of acarbose resulted in stronger repression of *RbcS* and stronger induction of *cwINV1* transcripts, respectively, when analyzed for a period of 48 h (Figure 4B).

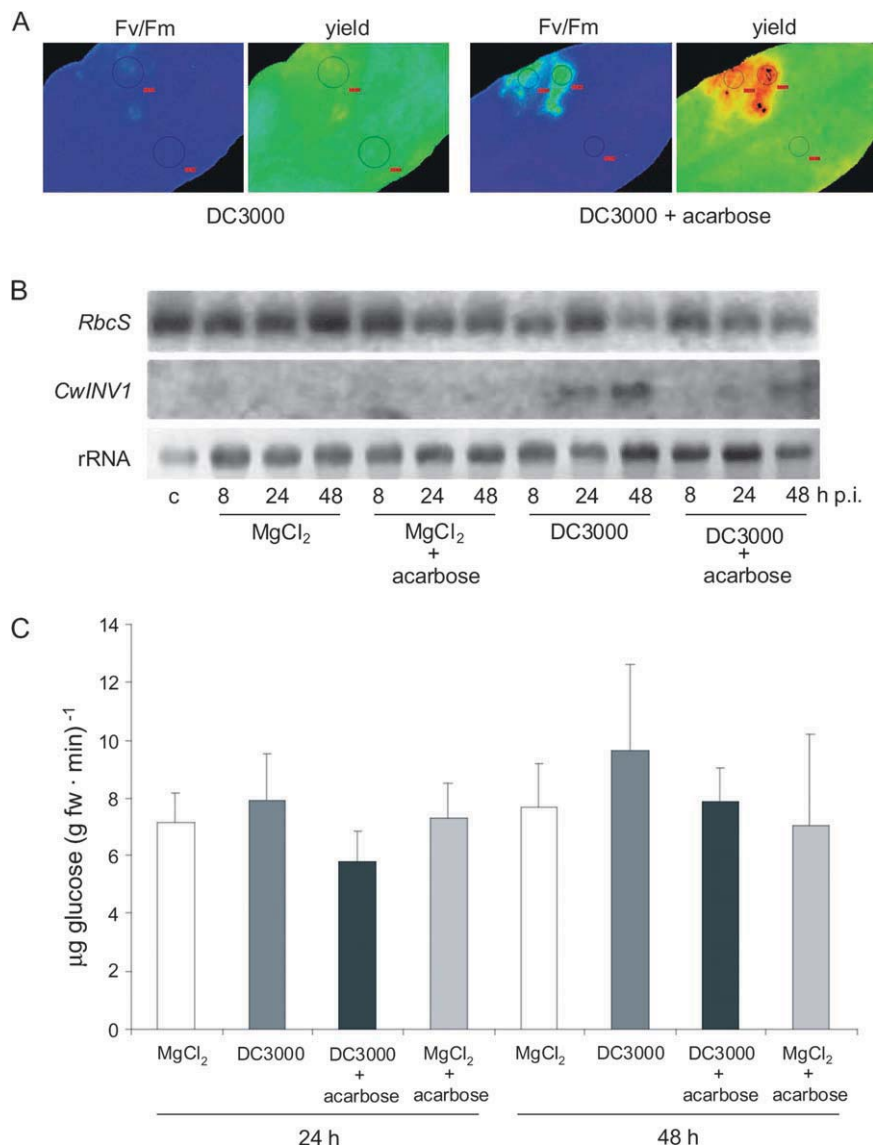
To analyze whether acarbose infiltration in leaves affects the *in vitro* activity of invertase activity extracts from infiltrated leaves were tested. No significant differences in the ex-

tractable cell wall invertase activity of mock inoculated leaves, leaves treated with acarbose alone, leaves infected by *P. syringae* alone, or leaves treated by a combination of acarbose and *P. syringae* could be detected 24 and 48 hpi (Figure 4C). Nevertheless, a tendency of lower invertase activity of extracts from leaves infiltrated with *P. syringae* and acarbose in comparison to leaves infected with *P. syringae* alone could always be observed. The finding that the extractable invertase activity is not significantly affected is in agreement with the non-covalent interaction of acarbose with invertase that is disrupted during extract preparation and also due to the competitive mode of action of acarbose.

### Characterization of Defense Responses

Since acarbose was shown not to inhibit bacterial growth *per se*, one possible explanation for the increased susceptibility of the acarbose-treated leaves to *P. syringae* could have been a negative impact of the acarbose treatment on plant defense responses. The salicylic acid (SA) pathway has been reported to be the major contributor to successful defense against *P. syringae*. Therefore, the effect of acarbose on the expression of SA-responsive genes and on the levels of SA was analyzed. Expression of the corresponding marker genes *PR1* and *PR2* was induced 24 and 48 h after *P. syringae* infection to similar levels in leaves with or without acarbose co-treatment (Figure 5A). We further analyzed SA levels in leaves inoculated with *P. syringae* alone and together with acarbose. Even though SA levels did not differ much between the respective treatments, induction of SA was overall higher when acarbose was infiltrated in combination with *P. syringae* (Figure 5B). These results together with the induction of *PR* gene expression data rule out that the increased susceptibility caused by acarbose is due to inhibition of the SA pathway.

To functionally investigate whether the SA pathway is involved in the acarbose-mediated increase of susceptibility, two different mutants in the SA-mediated defense pathway, namely *sid2*, which is defective in SA biosynthesis, and *cpr6*, which exhibits constitutive activation of the SA pathway, were analyzed. The *sid2* mutant is known to be more sensitive to *P. syringae* than the wild-type. The presence of acarbose further increased bacterial growth in this mutant (Figure 5C). The amount of bacteria in leaves co-treated with acarbose was around 600% higher than in leaves infiltrated with bacteria alone, which was similar to the acarbose effect in the wild-type. The *cpr6* mutant is known to be more resistant to *P. syringae* than the wild-type. This corresponds with lower overall growth of the bacteria in this mutant. However, co-infiltration with acarbose increased bacterial growth by 400% (Figure 5D). The findings that acarbose enhances susceptibility to *P. syringae* both in *Arabidopsis* mutants with defective and constitutive active SA pathway indicates that increased susceptibility of *Arabidopsis* towards *P. syringae* infection in response to invertase inhibition is mediated by a SA-independent mechanism.



**Figure 4.** Effect of Invertase Inhibition by Acarbose on Carbohydrate Metabolism during Pathogen Infection.

**(A)** Chlorophyll fluorescence imaging indicating maximum quantum yield (Fv/Fm) and effective quantum yield (yield) 24 and 48 h after infection with *P. syringae* DC3000 complemented or not with acarbose.

**(B)** Northern blot analysis of regulation of *RbcS* and *cwInv* transcripts. Total RNA was prepared from leaves treated with 10 mM MgCl<sub>2</sub>, MgCl<sub>2</sub> complemented with 10 mM acarbose, *P. syringae* DC3000, or *P. syringae* DC3000 complemented with 10 mM acarbose, sampled 8, 24, and 48 hpi. The experiment was repeated three times, with similar results.

**(C)** Extractable cell wall invertase activity. The *cwInv* activity was assayed in leaves infiltrated with 10 mM MgCl<sub>2</sub>, MgCl<sub>2</sub> with 10 mM acarbose, DC3000, and DC3000 with 10 mM acarbose, sampled 24 and 48 hpi. Data represented are an average of five independent experiments.

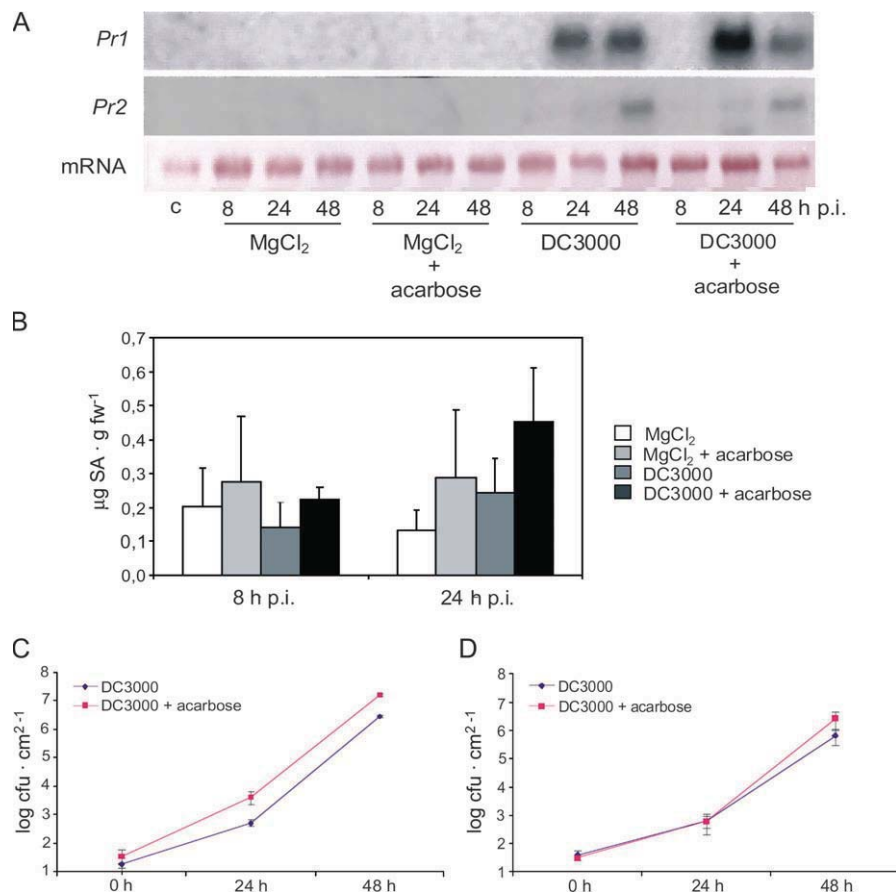
## DISCUSSION

Whereas the transcriptional regulation of invertases has been addressed in different pathosystems (Benhamou et al., 1991; Chou et al., 2000; Fotopoulos et al., 2003; Berger et al., 2004; Bonfig et al., 2006; Swarbrick et al., 2006), the present study focuses on the role of post-translational regulation of invertase activity by proteinaceous invertase inhib-

itors within the model pathosystem *A. thaliana* and *P. syringae* DC3000.

### Repression of Invertase Inhibitor Expression Is Part of the Defense Response

Although the presence of proteinaceous invertase inhibitors in plants has been reported for various species (Pressey, 1994; Krausgrill et al., 1998; Bate et al., 2004; Huang et al., 2007; Reca



**Figure 5.** Effect of Invertase Inhibition by Acarbose on Defense Responses during Pathogen Infection.

(A) Northern blot analysis of *PR1* and *PR2* transcripts. Total RNA was prepared from leaves treated with 10 mM MgCl<sub>2</sub>, MgCl<sub>2</sub> complemented with 10 mM acarbose, DC3000, and DC3000 complemented with 10 mM acarbose, sampled 8, 24, and 48 hpi. The experiment was repeated three times, with similar results.

(B) Salicylic acid accumulation was estimated in leaves of *A. thaliana* infiltrated with 10 mM MgCl<sub>2</sub>, MgCl<sub>2</sub> with 10 mM acarbose, DC3000, and DC3000 with 10 mM acarbose, sampled 8 and 24 hpi. Data are an average of three independent experiments.

(C) Bacterial growth in *sid2* plants. *P. syringae* was inoculated alone and together with acarbose and the number of bacterial colonies formed were counted 8, 24, and 48 hpi. Presented data are the mean value of three independent experiments.

(D) Bacterial growth in *cpr6* plants. *P. syringae* was inoculated alone and with acarbose and the number of bacterial colonies formed were counted 8, 24, and 48 hpi. Presented data are the mean value of three independent experiments.

et al., 2008; Jin et al., 2009), their physiological role is not yet fully understood and has not been addressed at all in the context of pathogen infection. Out of the three genes annotated to be invertase inhibitor proteins in *A. thaliana*, we found only invertase inhibitor *AtCIVIF2* (At5g64620) to be expressed in mature leaves, as assessed by *in silico*, Northern blot, and qPCR analysis. The transcript of this gene was down-regulated when leaves were infected by the pathogen, which is expected to result in de-repression of invertase activity during pathogen attack. This conclusion has been verified by developing a novel enzymatic assay to directly assess the invertase inhibitor activity present in the plant tissue. The activity of invertase inhibitor was analyzed in previous studies by heating a protein crude extract at high temperature, which destroys the invertase enzyme while the thermostable inhibitor remains active (Matsushita and Uritani, 1976; Hothorn et al., 2003). This

heat-killed extract was then added to invertase enzyme extract to assess the extent of inhibition. In the present study, the activity of invertase inhibitor protein was analyzed by separation of the invertase and invertase inhibitor proteins based on their differences in molecular weight, ~60 kD versus 17 kD, using molecular sieve columns with a 30-kD cut-off limit. The lower-molecular-weight protein fraction contained the invertase inhibitor proteins as they are around 17 kD in molecular weight (Krausgrill et al., 1998) and this fraction did not show apparent invertase activity. This fraction was mixed with the higher-molecular-weight fraction from control leaves. The resulting decrease in the invertase activity could be attributed to the activity of the invertase inhibitor present in the low-molecular-weight fraction. Analyzing the activity of invertase inhibitor activity clearly shows that the inhibitory activity decreased upon pathogen infection. The effect of decreased

invertase inhibitor activity on the *in situ* invertase activity present in leaves could be visualized by histochemical staining of invertase activity. These data demonstrate that the invertase inhibitor keeps the activity of invertase repressed in mature healthy leaves. During pathogen attack or through the impact of other stress-related stimuli such as wounding, the inhibitor activity will be repressed, leading to an increase in cell wall-bound invertase activity. It remains to be determined whether the reduction in inhibitor activity is further accelerated by rapid turnover of the inhibitor protein. The activity of *cwlInv* in source leaves of a resistant wild-type tobacco was shown to rapidly increase in a biphasic manner during an incompatible interaction with *Phytophthora nicotianae* (Essmann et al., 2008). The observed initial transient *cwlInv* activity peak within the first 4 h that precedes the appearance of *cwlInv* transcripts supports an activation of pre-existing *cwlInv* upon pathogen infection. The post-translational de-repression of invertase activity provides a regulatory mechanism for the rapid and localized induction of sink metabolism within the source leaf to satisfy the increased demand for energy for the activation of the cascade of defense reactions.

#### ***cwlInv* Activity Is Required for Plant Resistance**

The importance of de-repression of invertase activity for the defense response was substantiated by a functional approach using the pseudotetrasaccharide acarbose. Acarbose proved to be suitable to inhibit *Arabidopsis cwlInv* activity *in vitro* and *in situ*. Application of this competitive inhibitor to keep invertase post-translationally inhibited despite the pathogen-dependent repression of the endogenous inhibitor resulted in an increased susceptibility of the infected plant. Co-infiltration of acarbose and *P. syringae* into *Arabidopsis* leaves resulted in a more prominent growth of bacteria, in further reduction of maximum and effective photosynthetic quantum yield, in attenuated up-regulation of *cwlInv* and down-regulation of *RbcS* transcripts, and in a tendency of decreased extractable invertase activity compared with pathogen application alone. *P. syringae* is known to decrease the maximum and effective quantum yield analyzed by chlorophyll fluorescence imaging (Bonfig et al., 2006). These parameters of chlorophyll fluorescence, which are an indication of photosynthetic efficiency of the plants, have been shown to be affected in many plant–pathogen interactions (Chou et al., 2000; Lohaus et al., 2000; Berger et al., 2004; Swarbrick et al., 2006). Similarly, up-regulation of *cwlInv* and down-regulation of *RbcS* have also been shown during several plant–pathogen interactions (Ehness et al., 1997; Sinha et al., 2002; Bonfig et al., 2006). The antisense-mediated transcriptional silencing of *cwlInv* was shown not to interfere with the growth of a fungal pathogen in tobacco (Essmann et al., 2008) and a bacterial pathogen in tomato (Kocal et al., 2008), respectively. In contrast, post-translational inhibition of the *cwlInv* activity by acarbose resulted in more pronounced growth of the bacterial pathogen in *Arabidopsis* plants, although differences may be related to the different pathosys-

tems. The obtained results clearly demonstrate that the de-repression of cell wall invertase activity contributes to the plant defense response and interference with this regulatory mechanism increases the pathogen sensitivity.

#### **Inhibition of *cwlInv* Activity and Sensitivity of the Host Plant towards Pathogens Are Independent of the SA-Mediated Defense Pathway**

Attack by *P. syringae* is known to activate the SA-dependent pathways and expression of certain defense-related genes to render plants resistant to pathogens (Gomez-Gomez, 2004; Senda and Ogawa, 2004). Hence, we analyzed SA accumulation in plants after inoculation with the pathogen with and without acarbose. Acarbose alone induced SA accumulation in the leaves and SA levels were even higher when *P. syringae* was included in the infiltrated solution. In contrast to an earlier report, the present study indicates that pathogen infection alone does not result in an increase in SA content. This can be explained by the low concentration of bacteria used for the infection. Further, pathogen alone and also in combination with acarbose resulted in the expression of the defense-related genes *Pr1* and *Pr2*. In spite of expression of defense-related genes and accumulation of SA, the plants were more sensitive to the pathogen in the presence of acarbose, indicating clearly that inhibition of *cwlInv* activity contributes to the establishment of the bacteria in the plant. To further elucidate the involvement of the SA pathway, we used two mutants of the SA defense pathway—*sid2*, defective in SA synthesis (Nawrath and Metraux, 1999), and *cpr6*, a constitutive expresser of pathogenesis-related proteins (Clarke et al., 1998)—to study pathogen attack in the presence of invertase inhibitor. In both mutants, bacterial growth was higher when acarbose was infiltrated together with *P. syringae*, confirming our finding that activity of invertase is important for rendering the plant resistant to *P. syringae* and that it is independent of the SA pathway.

#### **Determination of Invertase Activity in Extracts May Overestimate the *In Situ* Activity of Source Tissues**

The present study demonstrates that the determination of invertase activity in extracts may overestimate the physiologically relevant invertase activity in the analyzed plant organ. During the extraction procedure, the invertase inhibitor protein is separated from *cwlInv* or may be diluted, resulting in a high *in vitro* enzyme activity. Additionally, the high sucrose concentration present in the enzymatic assay has been reported to protect the invertase from inhibition by invertase inhibitors. To get insight into the invertase activity in the plant tissue, the possible effect of proteinaceous and low-molecular-weight invertase inhibitors needs to be taken into consideration. The comparison of histochemical *in situ* invertase staining and determination of invertase activity in cell wall or crude extracts provides an experimental approach to assess whether post-translational regulatory mechanisms need to be accounted for.



The findings on the expression of the invertase inhibitor gene in source leaves also provides an explanation for results demonstrating a high constitutive invertase activity in healthy mature leaves even in the absence of a pathogen attack or stress. Although the activity of enzymes involved in sucrose catabolism was shown to be often extremely high, the physiological significance in particular of high extractable activity of *cwlnv* in source leaves has been a matter of a controversial debate for a long time (Kingston-Smith et al., 1999). The extracellular cleavage of the transport sugar sucrose is expected to interfere with phloem unloading (see discussion in Roitsch and Gonzalez, 2004) and to result in feedback inhibition of photosynthesis and induction of defense responses (von Schaewen et al., 1990). The results of the present study on the physiological relevance of a high expression of invertase inhibitors in source leaves of *Arabidopsis* is supported by the expression pattern of a tomato invertase inhibitor (Jin et al., 2009). The mRNA level of *INVINH1* of tomato increased as leaves progressed from sink to source stages. It can therefore be hypothesized that there has to be a strict endogenous regulation of *cwlnv* activity, which might get de-repressed once the cells are ruptured and the enzyme is extracted. Thus, this finding is not only relevant for the analyses of plant–pathogen interactions, but will be also of importance for the evaluation of the role of invertases during growth and development.

## METHODS

### Plants, Bacterial Strains, and Media

*Arabidopsis thaliana* Col-0 were used as wild-type and the mutants *cpr6* and *sid2* were in the Col-0 ecotype background. The bacterial strain *Pseudomonas syringae* pv. tomato DC3000 was provided by B. Staskawicz (Berkeley, CA, USA) and used for all pathogen experiments. *P. syringae* was cultured at 28°C in Kings B medium containing 50 mg l<sup>-1</sup> rifampicin. Bacto agar (Roth) at 1.0% (w/v) was added to LB medium for plate cultures.

To test the effect of acarbose on bacterial growth, the bacteria were cultured in a flask containing 10 ml Kings B medium with or without 10 mM acarbose. For testing the influence of acarbose on bacterial growth in plants, 10 mM acarbose was added to the bacterial suspension and infiltrated simultaneously with the bacteria.

### Growth of Plants, Plant Infection, and *In Vivo* Growth Curves

Plants were grown on soil in growth chambers (Binder) with a 9-h light period (light intensity 180 μmol m<sup>-2</sup> s<sup>-1</sup>) for 5–6 weeks. For inoculation, bacteria were re-suspended in 10 mM MgCl<sub>2</sub>, adjusted to an optical density of 0.2 at 600 nm (equivalent to 10<sup>8</sup> cfu ml<sup>-1</sup>), and were used in this concentration or diluted up to 10<sup>5</sup> cfu ml<sup>-1</sup>, depending on the experiment. Leaves were infiltrated with 20 μl of the bacterial suspension using a needleless syringe.

To determine bacterial growth in leaves of *Arabidopsis* plants, six leaves per plant were infiltrated with a bacterial suspension (with or without 10 mM acarbose) of 10<sup>5</sup> cfu ml<sup>-1</sup>. Bacteria in the leaves were sampled by taking four leaf discs using a cork borer (1.2-cm diameter), macerating the discs in 10 mM MgCl<sub>2</sub>, and plating appropriate dilutions on LB plates containing rifampicin. Population sizes were examined immediately after infiltration, 24 and 48 h; three replicates were taken for each sampling.

### Imaging of Chlorophyll Fluorescence

A special version of an Imaging-PAM Chlorophyll Fluorometer (Walz) was used to investigate spatio-temporal changes in photosynthetic parameters (Schreiber, 2004). For more detailed description of the measuring system, see Bonfig et al. (2006). With the Imaging-PAM, the current fluorescence yield (Ft) was continuously measured. In the absence of actinic illumination, in conjunction with the application of a saturation pulse, the dark-level fluorescence yield (Ft = Fo) and the maximum fluorescence yield (Fm) were determined, from which the maximum PS II quantum yield (Fv/Fm) was automatically calculated by the ImagingWin software (Walz). In the presence of actinic illumination, the current fluorescence yield (Ft = F) and the maximum light-adapted fluorescence (Fm') were determined, from which the effective PS II quantum yield (Y(II) = [Fm' - Ft]/Fm' = ΔF/Fm') was derived. Images of the fluorescence parameters were displayed with the help of a false color code ranging from 0.00 (black) to 1.00 (purple). After recording of kinetics, areas of interest (AOI) were defined (circles marked by red flags), over which the values of the selected fluorescence parameters were averaged.

### RNA Isolation, Northern Blot, and qRT-PCR Analysis

Total RNA was isolated from *Arabidopsis* leaves by grinding the leaf material and extracting RNA with Tritidy (Applichem) according to the manufacturer's instruction. Additionally, the RNA pellet was washed once with 3 M LiCl<sub>2</sub>. RNA was separated on 1.2% agarose gels and blotted to a nitrocellulose membrane (porablot NCL, Macherey-Nagel). Blots were hybridized with random primed <sup>32</sup>P-labeled DNA probes (Hexa-Label™ DNA Labeling Kit, Fermentas) overnight at 42°C. After washing with 2 SSC/0.1% SDS and 0.2 SSC/0.1% SDS, filters were exposed to a screen and scanned after 24–72 h with a PhosphorImager (BAS, Fuji). DNA of *RbcS* (At1g67090), *cwINV1* (At3g13790), *PR1* (At2g14610), *PR2* (At3g57260), *AtC/VIF1* (At1g47960), *AtC/VIF2* (At5g64620), and At3g17130 were used as probes.

For quantitative real-time PCR (qRT-PCR) analysis, total RNA was extracted from untreated leaves and leaves either mock-infiltrated with 10 mM MgCl<sub>2</sub> or infected with *P. syringae* DC3000 (10<sup>8</sup> cfu ml<sup>-1</sup>) with Total RNA Isolation™-reagent (Thermo Scientific). cDNA was synthesized from purified RNA using M-MuLV Reverse Transcriptase, Oligo(dT)18 primer and RiboLock™ RNase Inhibitor (Fermentas). PCR

experiments were performed with *AtC/VIF2*-specific primers (forward primer At5g64620\_F2-AGCATCAACCCTAATCTCAGC and reverse primer At5g64620\_R2-TTGCATACCAACGCC-GACC) and *PEX4*-specific primers (forward primer At5g25760\_F-TTCAGCTTGCTTTTCTGTTCC and reverse primer At5g25760\_R-CTGAGTCGCAGTTAAGAGG). In prior experiments and based on literature data (Czechowski et al., 2005), *AtPEX4* was found to be the most stably expressed housekeeping gene under the conditions tested. qRT-PCR was performed using the Light Cycler 480 SYBR Green I Master Mix (Roche) on a Light Cycler 480 instrument (Roche) following the manufacturer's protocols. PCR amplification was performed at 95°C for 10 min (pre-incubation) followed by 45 cycles with 95°C for 10 s (denaturation), 58°C for 15 s (annealing), and 72°C for 15 s (extension). Since PCR efficiency was equal for *AtC/VIF2* and *AtPEX4* (1.8), expression levels were calculated based on the DDeltaCT-Method (Pfaffl, 2004). The experiment was performed once. A preliminary qRT-PCR analysis with independent replicates had yielded similar results. Previously conducted independent Northern blot series showed the same differences in *AtC/VIF2* expression after mock treatment or pathogen infection (not shown).

#### **In Vitro Invertase Activity Analysis**

To assay invertase activity in *Arabidopsis* leaves, 0.5 g plant material was homogenized in liquid nitrogen in a mortar and re-suspended in 1 ml homogenization buffer (200 mM Hepes, 3 mM MgCl<sub>2</sub>, 1 mM EDTA, 2% glycerol, 0.1 mM PMSF, 1 mM benzamidine). The homogenate was mixed for 20 min at 4°C and centrifuged for 15 min at 10 500 g and 4°C. The supernatant was removed and used for soluble enzyme preparation. The pellet (cell-wall fraction) was washed three times with distilled water and re-suspended in 200 mM Hepes, 3 mM MgCl<sub>2</sub>, 15 mM EDTA, 2% glycerol, 0.1 mM PMSF, 1 mM benzamidine, 1 M NaCl. Cell wall invertase preparation was performed using 12.5 mM potassium phosphate buffer, pH 7.4 for 2–10 h at 4°C. Invertase activity at pH 4.5 was analyzed according to the method of Sung et al. (1989). The amount of liberated glucose was determined by addition of five-fold excess of GOD-POD reagent (0.1 M potassium phosphate buffer, pH 7, 0.8 U ml<sup>-1</sup> horseradish peroxidase, 10 U ml<sup>-1</sup> glucose oxidase from *Aspergillus niger*, 0.8 mg ml<sup>-1</sup> ABTS) and measurement of the absorbance was performed at 595 nm.

To assay acarbose-mediated invertase inhibition, an *in vitro* invertase activity test was performed. Acarbose (0, 5, 10, 15, 20, or 100 mM, respectively) was added to the reaction mixture (0.5 M potassium phosphate buffer, pH 4.5, and 3 mM of sucrose). Then, 30 µl of the cell wall fraction (prepared as aforementioned) was added and kept at 26°C for 30 min. The amount of liberated glucose was determined as described above.

#### **In Situ Staining of Invertase Activity**

For invertase activity staining, leaves or leaf halves were either squeezed to stimulate wound response or infiltrated with *P. syri* DC3000 (10<sup>6</sup> cfu ml<sup>-1</sup>) or MgCl<sub>2</sub> (mock). Leaves were

cut 48 hpi and washed with 50 mM sodium phosphate buffer (pH 5.5) (Applichem) for 20 min continuously shaking at RT. They were then placed in invertase staining solution consisting of 50 mM sodium phosphate buffer (pH 5.5), glucose-oxidase 20 U ml<sup>-1</sup> (from *Aspergillus niger* Type V; Applichem), 0.024% nitro blue tetrazoliumchloride (SERVA), 0.014% phenazine methosulfate (SERVA), and 20 mM sucrose (Applichem). Incubation was performed in the dark overnight at RT. For control, sucrose was omitted.

#### **Analysis of Repression of Invertase Activity by Invertase Inhibitor Protein Extracts**

To investigate the regulation of invertase by inhibitor proteins *in vivo*, invertase extracts (cell-wall fraction) were prepared as described above. After dialysis, invertase and invertase inhibitor were separated by spinning down the protein extract in Centrikon® YM-30 centrifugal filter devices (Millipore) for 40 min at 4500 g and 4°C. Invertase activity in the upper (C<sub>h</sub>) and the lower (C<sub>l</sub>) protein fraction and in a mixture of upper and lower (C<sub>h</sub> + C<sub>l</sub>, 10× excess of the lower fraction) protein fraction was analyzed as described above.

#### **Determination of Salicylic Acid**

Leaf SA levels were determined as described in Mishina and Zeier (2006). 200 mg frozen leaf tissue was homogenized with 600 µl of extraction buffer (H<sub>2</sub>O:1-propanol:HCl = 1:2:0.005), and 1 ml of methylene chloride as well as a methanolic solution of internal standards (100 ng D<sub>4</sub>-salicylic acid) was added. The mixture was shaken thoroughly and centrifuged at 14 000 rpm for phase separation. The lower, organic phase was removed, dried over Na<sub>2</sub>SO<sub>4</sub>, and treated with 2 µl of 2 M trimethylsilyldiazomethane in hexane (Sigma-Aldrich) for 5 min at room temperature for methylation of carboxy groups. The methylation reaction was stopped with 2 M acetic acid in hexane, and the sample was subjected to a vapor phase extraction procedure using a volatile collector trap packed with Super-Q absorbent (VCT-1/4X3-SPQ; Analytical Research Systems). The final evaporation temperature was set to 200°C and samples were eluted from the collector trap with 1 ml methylene chloride. Finally, the sample volume was reduced to 50 µl in a stream of nitrogen and the sample was subjected to GC/MS analysis. The sample mixture (2 µl) was separated on a gas chromatograph (GC 6890N; Agilent Technologies) equipped with a fused silica capillary column (DB-1; Fisons), and combined with a 5975 mass spectrometric detector (Agilent Technologies). For quantitative determination, peaks originating from selected ion chromatograms were integrated. The area of the substance peak was related to the peak area of the internal standard. An experimentally determined correction factor for the SA/D<sub>4</sub>-SA pair was considered.

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