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Alteration of the expression and activation of tomato invertases during *Botrytis cinerea* infection

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Abstract

Invertase is a key enzyme that hydrolyzes sucrose into glucose and fructose. In higher plants, invertases occur as a set of isoenzymes with different biochemical properties and sub-cellular locations. Although the expression and activity of invertase are known to be up-regulated when plants are exposed to stress-related stimuli, the interaction between the invertases and the necrotrophic pathogen *Botrytis cinerea* is not yet fully understood. Therefore, we have analyzed the expression pattern and activation of tomato invertases during an infection of *B. cinerea*. The expression of *Lin5* and *Lin6* is up-regulated during a response to *B. cinerea*, whereas other isoenzymes such as *Lin7* and *Lin8* are not significantly changed by *B. cinerea* in mature green fruit. In addition, the infection of *B. cinerea* results in increasing activity of vacuole and extracellular invertases. When the detached leaves were treated with methyl-jasmonic acid, the increased activities of vacuole and extracellular invertases were observed, and salicylic acid had no influence on the invertases activation. These findings indicate that carbohydrate partitioning via the activation of invertases might be involved in jasmonic acid-dependent defense responses during *B. cinerea* infections.

Keywords: Vacuolar invertase; Extracellular invertase; *Botrytis cinerea*; *In-silico* analysis.
Abbreviations: SA- Salicylic Acid; MeJA- Methyl Jasmonic Acid.

Introduction

In plants, algae, and prokaryotes (autotrophic prokaryotes), the overall process that converts carbon dioxide into organic compounds, especially sugar, using the energy from sunlight is called photosynthesis. Sugar production in plants is a vital process, and sugar status modulates and coordinates the internal regulators and physiological responses that govern growth and development, sink metabolism, and defense response (Koch, 1996; Roitsch, 1999; Smeekens, 2000). As a major transport form of photosynthetically assimilated carbon, sucrose is transported from source tissues to sink tissues via phloem and cleaved for utilization as a source of carbon and energy. Sucrose cleavage is catalyzed by invertases (β-fructofuranosidase, EC 3.2.1.26) or sucrose synthases (EC 2.4.1.13), which regulate the entry of sucrose into distinct biochemical pathways (Koch, 2004; Qi et al., 2007). Based on sub-cellular localization, pH optima, and isoelectric point, plant invertases can be classified into vacuolar invertase, extracellular invertase (cell-wall-bound invertase), and neutral/alkaline invertase (Roitsch and Gonzalez, 2004).

Vacuolar invertase and extracellular invertase belong to the acid invertase sub-family and have similar enzymatic and biochemical properties, although the physiological functions of these invertases are complex and depend upon tissues (Roitsch and Gonzalez, 2004). The expression and activity of extracellular invertases are known to be up-regulated when plants are exposed to a variety of stress-related stimuli such as different elicitor, wounding, and pathogen infections (Ehness et al., 1997; Godt and Roitsch, 1997; Roitsch et al., 2003; Bonfig et al., 2006; Hyun et al., 2009; Rumbos et al., 2011). The water deprivation in maize results in the strong enhancement of vacuolar invertase activity (Trouverie et al., 2003). In addition, it has been shown that the mRNA of vacuolar invertase was induced by wounding and virulent pathogens (Godt and Roitsch, 1997; Bonfig et al., 2006). These findings indicate that vacuolar invertase and extracellular invertase not only mobilize sucrose but also serve as a pivotal enzyme at the integration point of stress signals. Pathogen infection is a severe environmental stress factor for a plant. The analysis of plant resistance against biotrophic pathogens indicated that the induction of defense responses is accompanied by a fast increase of heterotrophic metabolism (Bonfig et al., 2006; Berger et al., 2007). Although this finding suggests that the increased heterotrophic metabolism could serve as metabolic energy to support appropriate defense responses, the interaction between invertases and necrotrophic pathogens is not yet fully understood. Therefore, in this study, we analyzed the expression profiles and regulation patterns of tomato invertases during responses to *Botrytis cinerea* to obtain a better understanding of the interaction between necrotrophic pathogens and plant heterotrophic metabolism. The up-regulated activities of vacuolar invertase and extracellular

Table 1. The list of probe sets for tomato invertase genes.

Invertase genes	Probe	Expect	Identities	Target length (bp)
TIV1	Les.2702.1.S1_a_at	0.0	98.90%	2397
Lin5	Les.3461.1.S1_at	0.0	98.20%	2002
Lin6	Les.3460.1.S1_at	0.0	98.20%	1924
Lin7	Les.3459.1.S1_at	0.0	99.60%	2050
Lin8	Les.3458.1.S1_at	0.0	99.20%	1913

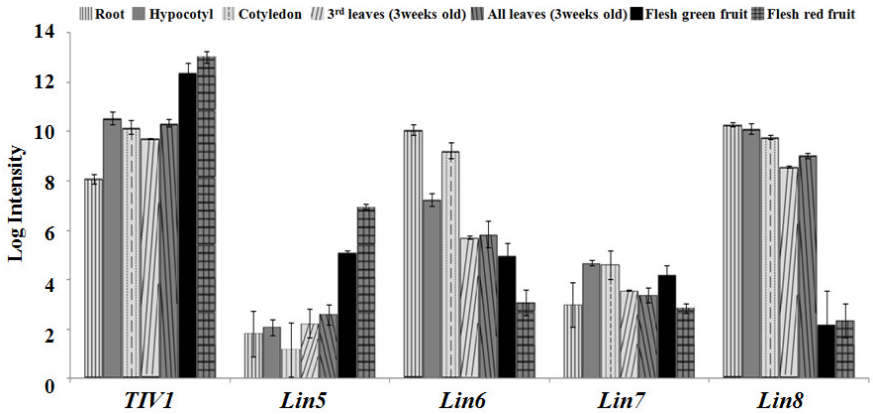

Fig 1. Transcript levels of invertase genes in different plant tissues at different development stages. The values and error bars indicate the mean and standard error, respectively, from three independent hybridizations.

invertase were observed in *B. cinerea* infected leaves. Most importantly, careful analysis of both invertase activities with signal molecules suggests that the increasing activities of both invertases are mediated by methyl-jasmonic acid (MeJA)-signaling.

Results and discussion

To estimate the expression level for each invertase gene, we conducted an analysis in the available Plant Expression Database (http://www.plexdb.org/index.php). mRNA sequences of tomato vacuolar invertase 1(*TIV1*) and extracellular invertases *Lin5*, *Lin6*, *Lin7,* and *Lin8* were used for screening probe sets (Table 1). The abundance of each probe represented and measured the gene expression levels in different tissue and *B. cinerea*-induced libraries. As shown in Fig. 1, *TIV1* was highly expressed in all tissues compared with extracellular invertases. *TIV1* transcript abundance was higher in red fruit than in other sink-tissue (roots). It has been shown that the expression of *TIV1* was increased during fruit ripening (Lu et al., 2010), indicating that TIV1 is involved in the sink metabolism of fruits. In the case of extracellular invertases, the different expression patterns from each gene were observed in different tissues. The strong expression of *Lin8* was observed in all tissues except fruits, whereas *Lin5* was highly expressed in fruits. In addition, *Lin6* abundance was higher in roots and cotyledon, but *Lin7* exhibited the similar level of expression in all tissues (Fig. 1). Similarly, tissue-specific distributions of mRNAs for *Lin5*, *Lin6,* and *Lin7* have been demonstrated by Godt and Roitsch (1997). Taken together, differential tissue-specific regulation of mRNAs for extracellular invertase isoenzymes indicates the different physiological roles of the different isoenzymes and suggests that they play important role for coordinated regulation of source-sink relations in different tissues. The transcript level of extracellular invertase is rapidly up-regulated under stress conditions and suggests essential role of apoplastic sucrose cleavage in mediating defense responses (Ehness et al., 1997; Godt and Roitsch, 1997; Roitsch et al., 2003; Bonfig et al., 2006; Hyun et al., 2009). When tomato fruit was infected with *B. cinerea*, we also observed the increased level of *Lin5* and *Lin6* transcript. By contrast, *B. cinerea* infection did not result in induction of *TIV1*, *Lin7,* and *Lin8* (Fig. 2). Analysis of *cis*-acting regulatory elements in the *Lin6* promoter region has shown that three W-box elements (GTCA) are located at close proximity at positions -1748, -1741, and -1732 (Proels and Roitsch, 2009). W-box is a binding site for WRKY transcription factors that are classified as zinc-finger proteins involved in plant defense signaling against pathogens, viruses, wounding, and drought stress (Ross et al., 2007; Kamal et al., 2010; Hyun and Kim, 2011). Therefore, the increased level of *Lin6* expression by *B. cinerea* infection may be related to plant defense signaling mediated by WRKYs. In addition, a specific up-regulation of GUS activity conferred by the *Lin5* promoter during responses to gibberellic acid, auxin, and abscisic acid was observed (Proels et al., 2003), suggesting that the induction of *Lin5* by *B. cinerea* infection might be involved in phytohormone-signaling pathways. To investigate whether the up-regulation of *Lin5* and *Lin6* expression by *B. cinerea* infection was reflected by an increase in invertase activity, the sink leaves were punctured, and 5 μL drop of the spore suspension (1×10^6 conidia mL^{-1}) of *B. cinerea* was placed over the wound. As shown in Fig. 3B, the activity of extracellular invertase was significantly increased by *B. cinerea* infection, indicating that the induction of *Lin5* and *Lin6* expression resulted in up-regulation of extracellular invertase activity. The treatment with mock (PDB medium) slightly increased the activity of extracellular and vacuolar invertase. This might be due to D-glucose, which can activate acid invertases (Roitsch et al., 1995), in the PDB medium (Fig. 3). In addition, the increased level of vacuolar invertase activity was also observed after treatment with *B. cinerea* (Fig. 3A), although *TIV1* did not respond to *B. cinerea* infection (Fig. 2). The difference between expression pattern and activity of vacuolar invertase indicated that TIV1 may not be a main factor

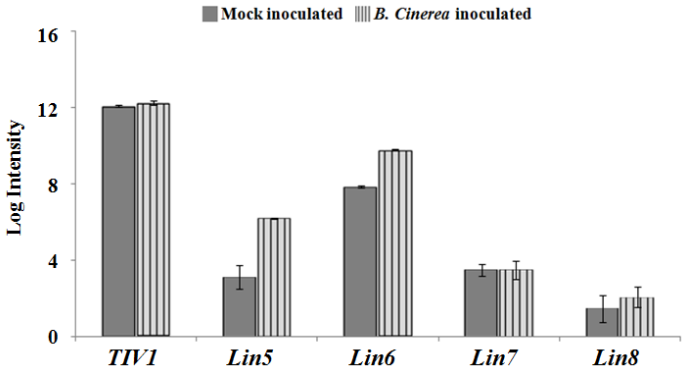

Fig 2. Expression of invertase genes during response to *B. cinerea* in tomato mature green fruit. The values and error bars indicate the mean and standard error, respectively, from three independent hybridizations.

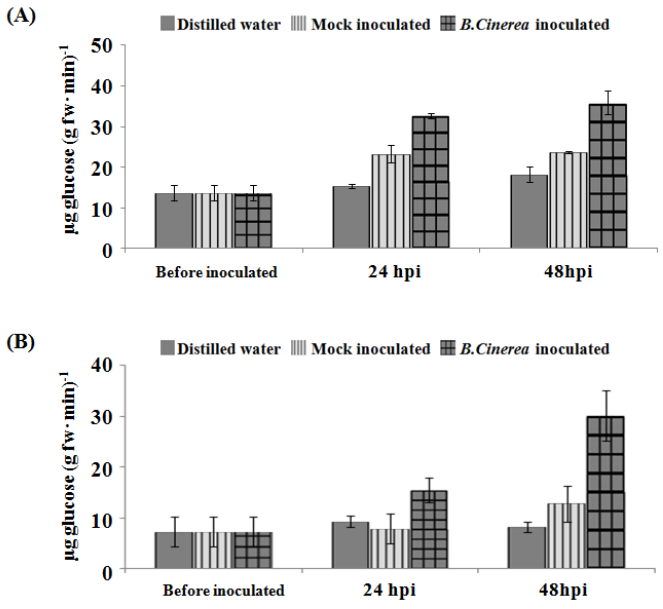

Fig 3. Effect of *B. cinerea* infection on the activation of vacuole invertase (A) and extracellular invertase (B). The invertase activity was assayed in leaves infected with 5 µL PDB medium, as a mock control, or same volume of spore suspension, sampled 24 and 48 hpi. Each bar represents the mean of ± S.D. of five replicates.

during a response to necrotrophic pathogens. Using comparative genomic analysis, it has been shown that *Arabidopsis* (Haouazine-Takvorian et al., 1997), rice (Ji et al., 2005), and poplar (Bocok et al., 2008) genomes contain at least two genes for vacuolar invertase isoenzymes, and this suggests the possibility of presence of vacuolar invertase isoenzymes in the tomato genome. The other possibility is that *B. cinerea*-induced vacuolar invertase activation may be independent of the amount of enzyme present. In fact, it has been shown that the activity of apple acid invertases is up-regulated by ABA treatment without changing these protein amounts (Pan et al., 2006). The high level of *TIV1* transcript in all tissue also supports that *B. cinerea* activates vacuolar invertase by a posttranslational mechanism. Among stress-specific phytohormones, SA and JA play distinct roles: JA has been predominantly implicated in necrotrophic resistances, while SA has been associated with biotrophic resistances (McDowell and Dangl, 2000; Glazebrook, 2005). To further test the hypothesis that the activation of acid invertases by *B. cinerea* infection is mediated by JA-signaling pathway, the detached leaves were treated with different concentrations of SA and MeJA. The treatment of MeJA resulted in the induction of vacuolar invertase and extracellular invertase activities (Fig. 4B), whereas neither invertase responded to SA treatment (Fig. 4A). This finding suggested that acid invertases are involved in JA-signaling pathway during response to *B. cinerea*.

Material and methods

Plant material and treatment of signal molecules

Tomato plants (*Lycopersicon esculentum* Mill. cv Moneymaker) were grown from seeds in soil in a greenhouse under 16-h day and 8-h night cycles, with 350 µE m^{-2} s^{-1} light intensity, at 23°C. For the treatment of signal molecules, the detached leaves of five-week-old plants were incubated with different concentrations of salicylic acid (SA, 200µM, 500µM and 1mM) and methyl-jasmonic acid (10µM, 50µM and 100µM). Twenty-four hours after treatment, samples were collected and quickly frozen in liquid N_2.

Botrytis cinerea inoculation

B. cinerea was grown on potato-dextrose-agar (PDA) plates at RT for two weeks in darkness. For inoculation, spore suspensions were prepared at a concentration of 1×10^6 conidia mL^{-1} in potato-dextrose-broth (PDB). Then, the sink leaves were punctured in two positions per leaf, and 5 µL drop of the spore suspension was placed over the wound. Plants were covered with transparent film in order to retain humidity. Samples were collected at the times indicated in each figure, quickly frozen in liquid N_2, and stored at -80°C until analysis.

In-silico analysis of tomato invertase genes expression

For the *in-silico* determination of the expression of tomato invertase genes during responses to *Botrytis cinerea* infection, the Plant Expression Database (PLEXdb http://www.plexdb.org/index.php) was screened, using tomato invertase mRNA sequences, by performing a bulk query. The list of probe sets of each invertase gene is presented in Table 1.

Determination of invertase activity

To assay the invertase activity in tomato leaves, 0.5 g of plant material was homogenized in liquid nitrogen and resuspended in 1 mL homogenization buffer (200 mM Hepes, 3 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 0.1 mM PMSF, and 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 transferred into new tube and used for vacuolar invertase preparation. The pellet was washed three times with distilled water and resuspended in the homogenization buffer with 1M NaCl. After centrifugation, the supernatant was taken for extracellular invertase preparation. Both enzyme preparations were dialyzed against 12.5 mM potassium phosphate buffer, pH 7.4 for 2 to 10 h at 4°C. Invertase activity at pH 4.5 and 6.8 was analyzed according to the method of Bonfig et al. (2006). The amount of liberated glucose was determined by the addition of a fivefold excess of GOD reagent

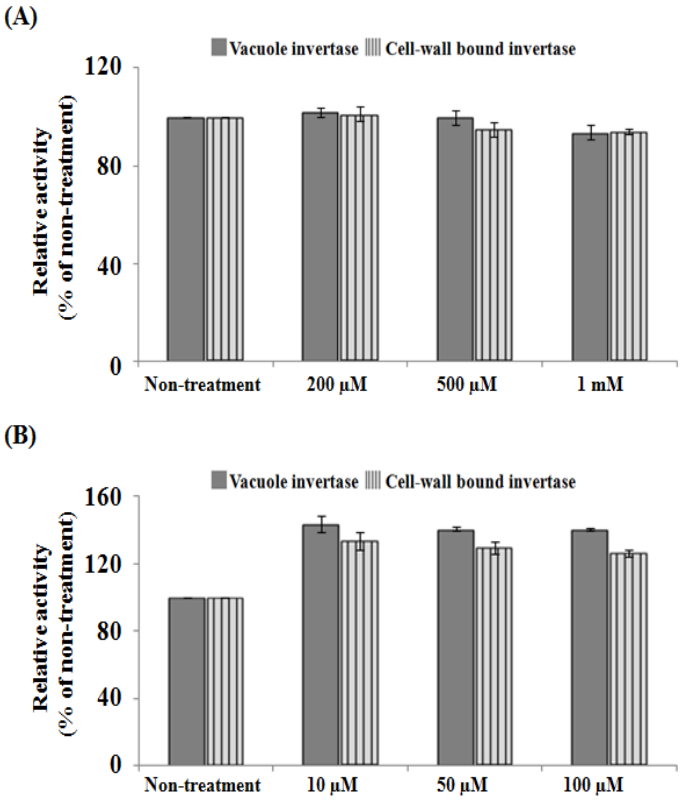

Fig 4. Effect of SA (A) or MeJA (B) on the activation of vacuole invertase and extracellular invertase. The detached leaves were incubated with different concentration of SA or MeJA. One day after treatment with SA or MeJA, the invertase activity was assayed. Each bar represents the mean of ± S.D. of five replicates.

(0.1 M potassium phosphate buffer, pH 7, 0.8 U mL^{-1} horseradish peroxidase, 10 U mL^{-1} glucose oxidase from *Aspergillus niger*, and 0.8 mg mL^{-1} ABTS) and measurement of the absorbance at 595 nm.

Conclusion

In this study, we addressed the alteration of heterotrophic metabolism by *B. cinerea* infection. The analysis of transcript abundance suggests the different physiological roles of the different isoenzymes. In addition, the investigation of invertase activity supports the hypothesis that vacuolar invertase and extracellular invertase play important roles in plant response and resistance against necrotrophic pathogens. Further investigations are needed to improve the molecular basis that contributes to the multiple regulation patterns of invertases. Based on various regulatory mechanisms that affect invertase expression and activation, it has to be assumed that invertases are regulated with complex networks of interplay among various signaling during heterotrophic metabolism.

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