

Expression of *Arabidopsis* sugar transport protein STP13 differentially affects glucose transport activity and basal resistance to *Botrytis cinerea*

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Abstract *Botrytis cinerea* is the causing agent of the grey mold disease in more than 200 crop species. While signaling pathways leading to the basal resistance against this fungus are well described, the role of the import of sugars into host cells remains to be investigated. In *Arabidopsis thaliana*, apoplastic hexose retrieval is mediated by the activity of sugar transport proteins (STPs). Expression analysis of the 14 STP genes revealed that only *STP13* was induced in leaves challenged with *B. cinerea*. *STP13*-modified plants were produced and assayed for their resistance to *B. cinerea* and glucose transport activity. We report that *STP13*-deficient plants exhibited an enhanced susceptibility and a reduced rate of glucose uptake. Conversely, plants with a high constitutive level of *STP13* protein displayed an improved capacity to absorb glucose and an enhanced resistance phenotype. The correlation between *STP13* transcripts, protein accumulation, glucose uptake rate and resistance level indicates that *STP13* contributes to the basal resistance to *B. cinerea* by limiting symptom development and points out the importance of the host intracellular sugar uptake in this process. We postulate that *STP13* would participate in the active resorption of hexoses to support the increased energy demand to trigger plant defense

reactions and to deprive the fungus by changing sugar fluxes toward host cells.

Keywords Sugar transporter protein · *Arabidopsis thaliana* · *Botrytis cinerea* · Basal resistance · Plant–pathogen interactions

Introduction

Botrytis cinerea is a necrotrophic ascomycete responsible for the grey mold disease (Williamson et al. 2007). This fungus kills host cells using a large arsenal of weapons that enables it to infect a broad range (>200) of host plants. Secretion of non-specific toxins, i.e. botrydial, and production of reactive oxygen species (ROS) are commonly associated features of the infection process (van Kan 2006; Choquer et al. 2007). *B. cinerea* also triggers an active oxidative burst produced by the host itself. In the case of necrotrophs, the subsequent plant cell death clearly promotes the pathogen growth (Govrin and Levine 2000). *B. cinerea* possesses multiple lytic enzymes, i.e. endopolygalacturonases, cutinases, cellulases, that degrade the cell wall and the cuticle (ten Have et al. 1998; van Kan 2006). Activities of cell wall-degrading enzymes allow the maceration of the plant biomass, generating an important source of nutrients for the fungus consumption.

Pathogens, whatever their type, develop at the expense of the sugars produced by plants and it is largely assumed that classical sources turn into sinks when colonized by pathogens (Biemelt and Sonnewald 2006; Lemoine et al. 2013). *B. cinerea* can infect either source or sink organs. The availability of carbohydrates may be an important issue for *B. cinerea* during every stage of the infection, e.g. germination, the penetration of the host surface, the killing

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of the host tissue and spreading. In most plants, sucrose is the main photo-assimilate translocated from sources to sinks. In the release phloem, sucrose can move simplistically or can be unloaded into the apoplast (van Bel 2003). Apoplastic sucrose enters the sink cells via sucrose transporters or alternatively, can be cleaved into hexoses (glucose and fructose) by the activity of cell wall invertases, consequently increasing the level of apoplastic free hexoses (Roitsch and Gonzalez 2004; Lemoine et al. 2013). Potentially, both the fungus and the host cells can exploit those sugars. In plant/birotrophic fungus interactions, the haustorial structure represents the site of nutrient exchanges where the pathogen can acquire sugars from the apoplast through the activity of fungal sugar transporters (Hall and Williams 2000; Voegelé and Mendgen 2011; Voegelé et al. 2001). Regarding *B. cinerea*, information on sugar acquisition mechanisms during pathogenesis is fragmentary. A fungal invertase has been detected in grape infected by *B. cinerea* (Ruiz and Ruffner 2002). A high affinity fructose transporter (BcFRT1) and a multigenic family of hexose transporter (BcHXTs) have been identified, suggesting that *B. cinerea* possesses a versatile hexose uptake system (Doehlemann et al. 2005; Dulermo et al. 2009).

Plant ability to retrieve extracellular sugars may be determinant for the final outcome of the interaction and may be part of the plant immune response to defend itself against pathogens (Roitsch et al. 2003). Plant innate immunity is triggered upon the perception of pathogen-associated molecular patterns (PAMPs), which are conserved components of pathogens, through pattern recognition receptors. The so-called PAMP-triggered immunity (PTI) has been extensively studied over the past years and excellent reviews have described the complex molecular perception and signaling mechanisms (Boller and Felix 2009; Monaghan and Zipfel 2012; Mengiste 2012; Thomma et al. 2011). PTI is characterized by common downstream signaling events, including ROS production, MAP kinases activation, hormone signaling and transcriptional reprogramming (Tsuda and Katagiri 2010). Hormone signaling mediates host resistance according to the pathogen lifestyle (Glazebrook 2005; Thomma et al. 1998, 2001). Classically, resistance to biotrophs is associated with the activation of a salicylic acid-dependent signaling pathway while jasmonic acid and ethylene are required for the basal resistance to necrotrophs, such as *B. cinerea* (Pieterse et al. 2012; Bari and Jones 2009). The roles of sugars in the PTI, via the coordinated activities of cell wall invertases and hexose transporters, have been postulated to satisfy the additional energy required to activate the cascade of defense reactions. Besides their roles as carbon and energy sources, sugars can modulate, as signaling molecules, many physiological processes including pathogenesis-related (PR) gene expression and can consequently participate actively

in the basal resistance to pathogens (Roitsch 1999; Bolouri Moghaddam and Van den Ende 2012; Herbers et al. 1996).

Modulation of host glucose transport activity has been described in several pathosystems. For example, *Pinus pinaster* suspension cells showed an increase in glucose uptake after inoculation with *B. cinerea* (Azevedo et al. 2006) whereas Bourque et al. (2002) reported an immediate inhibition of glucose absorption in tobacco cells elicited by cryptogein, a proteinaceous elicitor secreted by *Phytophthora cryptogea*. Host hexose uptake is mediated by the activity of monosaccharide transporters. In *Arabidopsis thaliana*, monosaccharide transporter (-like) gene superfamily (MST) comprises 53 genes (Büttner 2007). The retrieval of hexoses from the apoplasm across the plasma membrane is mediated by the sugar transport protein (STP) family, which is encoded by 14 highly homologous genes. STP is the best characterized family among MSTs and their substrate specificities, gene expression patterns, transcriptional regulation and functional roles are well described (Büttner 2010; Slewinski 2011). To date, little is known concerning the involvement of pre-existing or pathogen-induced *Arabidopsis* STPs in the changes of glucose absorption upon pathogen challenge. During the biotrophic interaction between *Erysiphe cichoracearum* and *A. thaliana*, the up-regulation of the plant cell-wall invertase (cwINV) *AtβFRUCT1* and the hexose transporter *AtSTP4* is correlated with increased invertase activity and glucose uptake, supporting the functional coordination of STPs and cwINVs in order to supply host tissues with hexoses (Fotopoulos et al. 2003). *STP4* is also transcriptionally induced by wounding, by treatments with bacterial elicitors from *Pseudomonas syringae* pv *tabaci* and after infection with necrotrophic fungi, e.g. *Fusarium oxysporum* and *Alternaria brassicicola* (Truernit et al. 1996). Global data collected from microarray experiments have showed that other *STP* genes are responsive to environmental stresses (Büttner 2010). For example, *STP13* is differentially regulated by various external factors or stresses, i.e. light, pathogens, drought, or chemicals, and is also induced in tissues undergoing programmed cell death (PCD) (Norholm et al. 2006).

In this study, we identified the Sugar Transporter Protein *STP13* as the only member of the *A. thaliana* *STP* family induced by the necrotrophic fungus *B. cinerea* isolate B05.10, at transcriptional and translational levels. Plants impaired in *STP13* expression exhibit a reduced glucose uptake rate and increased disease symptoms, indicating that *STP13* contributes to the basal resistance to *B. cinerea* by limiting the development of necrosis. High constitutive expression of *STP13* protein, before challenge, associated with an increased glucose uptake activity, leads to an enhanced resistance phenotype by preventing and slowing down the development of disease symptoms caused

by *B. cinerea*. Our results demonstrate that the modification of the host intracellular glucose uptake prior to infection affects *B. cinerea* resistance level. This work further suggests that the differential expression level of plant hexose transporters can probably change the balance of hexose fluxes toward either host living tissues or fungus cells, highlighting the importance of the competition for apoplastic hexoses at the plant/fungus interface.

Results

Expression of Arabidopsis *STP* genes upon *Botrytis cinerea* isolate B05.10 infection

Our goal was to identify Arabidopsis Sugar Transport Proteins (STP), which may play a role in defense mechanisms against the necrotrophic fungus *B. cinerea*. In order to find candidate genes with a *B. cinerea*-induced expression pattern, we monitored the expression of the 14 *STP* genes by qRT-PCR. The transcriptional-based screening of *STP* genes was carried out 48 h post-inoculation (hpi) when disease symptoms were visible and the JA-responsive marker gene *PDF1.2* was up-regulated, being conscious that an earlier time point might have allowed the identification of other candidates. We performed inoculation experiments in the dark to prevent the known diurnal and light-regulated expression of *STPs* (Büttner 2010; Stadler et al. 2003). Firstly, transcripts of *STP2*, *STP5*, *STP6*, *STP8*, *STP9*, *STP10*, *STP11* and *STP12* were not detected before and after infection, suggesting that these genes were not expressed to a detectable level in the leaves (data not shown). A clear amplification signal was obtained for only 6 out of the 14 *STP* genes: *STP1*, *STP3*, *STP4*, *STP7*, *STP13* and *STP14*. After inoculation of Arabidopsis leaves with *B. cinerea* isolate B05.10, the expression of *STP4*, *STP7* and *STP14* was not modified, while *STP1* and *STP3* were slightly down-regulated compared to mock-treated leaves. In contrast, the expression of *STP13* was clearly induced by *B. cinerea* at 48 hpi (Fig. 1).

Because of its unique expression pattern among *STPs*, we focused our study on the *B. cinerea*-responsive *STP13*, and monitored more precisely its kinetic of expression upon infection (Fig. 2). It appeared that the level of transcripts of *STP13* increased weakly after 48 and 72 h in mock-treated leaves, suggesting that *STP13* responded slightly to the stress caused by the experimental procedure, i.e. high humidity and extended darkness. However, after *B. cinerea* inoculation, *STP13* transcript level was maximal within 48 h post inoculation and was approximately 4 times higher compared to mock treatment, confirming the results shown in Fig. 1. No significant induction of *STP13* expression was observed 12 h (data not shown) and 24 h (Fig. 2)

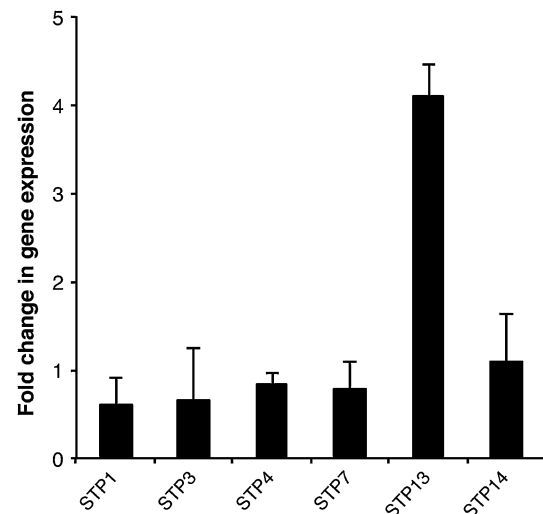


Fig. 1 Fold change in expression of 6 *STP* genes upon *B. cinerea* infection at 48 h post-inoculation. Five-week-old wild-type plants were sprayed with mock solution or *B. cinerea* suspension (5×10^4 conidia ml^{-1}) and at least three plants were harvested 48 h after treatment. *STPs* transcript levels were quantified by RT-qPCR. The gene expression of each *STP* was normalized to the plant reference gene *At4g26410* (Czechowski et al. 2005) and is relative to the corresponding mock treatment. Data are the mean (\pm SE) of 3 independent experiments

after *B. cinerea* inoculation while disease symptoms were absent.

Collectively, these data showed that *STP13* is the only *STP* positively regulated by *B. cinerea* during symptom development, suggesting that it may play a role in late plant defenses triggered in response to *B. cinerea*.

Characterization of *STP13*-modified plants

To determine the biological importance of *STP13*, the homozygous mutant line *stp13-2* (salk_021204), with a T-DNA insertion in the first intron of the *STP13* genomic sequence, was selected. We first monitored the expression of *STP13* in this mutant by RT-qPCR. *STP13* was not detected in healthy leaves and was no longer inducible after *B. cinerea* inoculation (Fig. 2). Two other homozygous insertion lines, with T-DNA in the third exon (*stp13-1*) and the promoter (*stp13-3*), were analyzed but not retained because both lines displayed a residual expression of *STP13* (data not shown). Conversely, a transgenic line expressing the complete *STP13* predicted coding sequence under the control of the *CaMV35S* promoter was produced (named *STP13OE-6*). As expected, *STP13OE-6* line displayed a high constitutive expression of *STP13* before and after infection (Fig. 2 and Fig. S1).

To verify the presence of the protein and to exclude a putative post-transcriptional regulation of *STP13*, we

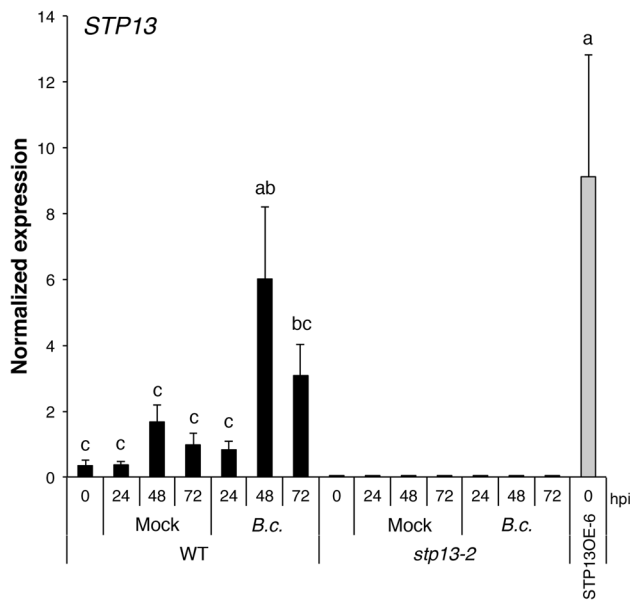


Fig. 2 *STP13* mRNA accumulation in wild-type (WT), *stp13-2* and STP13OE-6 plants. WT and *stp13-2* plants were sprayed with mock solution or *B. cinerea* suspension (5×10^4 conidia ml⁻¹). Healthy (0 hpi) or treated leaves from at least three plants were harvested at indicated time points. *STP13* transcript levels were quantified by RT-qPCR. Data are expressed as normalized expression (no unit) to the plant reference gene At4g26410 expression level (Czechowski et al. 2005) and are the mean (\pm SE) of 12 (WT), 5 (*stp13-2*) and 3 (STP13OE-6) independent experiments. Different letters represent groups that were significantly different as determined by an ANOVA with general scores followed by a multiple comparison test ($P < 0.05$). B.c.: *B. cinerea*. hpi: hours post-inoculation

examined its expression profile at the protein level by immunodetection. A specific peptide containing the last 27 C-terminal amino-acids of STP13 was produced and used to immunize rabbits in order to obtain a polyclonal antibody raised against this protein. The purified serum detected a single protein corresponding to STP13 in the microsomal fraction of yeast expressing STP13, while no signal was detected in WT yeast (data not shown). The purified serum was then tested on the microsomal fraction of WT *A. thaliana* leaves (Fig. 3). STP13 protein was constitutively present in non-stimulated WT leaves and undetectable in *stp13-2*, while the STP13OE-6 line exhibited an elevated constitutive expression of STP13 (Fig. 3a). Kinetic studies established that the STP13 protein accumulated slightly in WT mock-treated leaves and was induced at a high level within 48 hpi in response to *B. cinerea* (Fig. 3b). No induction of the STP13 protein was detected at 24 hpi compared to the mock treatment, which is consistent with transcript accumulation data (Fig. 2). In contrast to WT plants, no STP13-related signal was visible in the *stp13-2* mutant, neither in control nor in stimulated leaves,

confirming the specificity of the antibody and supporting that this T-DNA insertion line is a full knockout (Fig. 3c).

STP13 has been described as a high affinity H⁺/hexoses symporter (Norholm et al. 2006). To verify whether *stp13-2* and STP13OE-6 plants had modified rates of sugar uptake, glucose absorption was assayed using discs from healthy mature leaves that were floated on [¹⁴C]-glucose-containing solution. As shown in Fig. 4, the total glucose uptake by the over-expressor line was approximately 3 times higher than in the WT, while the *stp13-2* line had a rate of glucose transport that was reduced by 27 %. We used the protonophore carbonylcyanide m-chlorophenyl hydrazone (CCCP), which uncouples oxidative phosphorylation in mitochondria and collapses the transplasmalemma pH gradient, in order to discriminate the passive diffusion of radiolabelled glucose into the leaf discs and the active glucose uptake mediated by proton-driven glucose symporters. The total glucose uptake was inhibited by CCCP by 62, 59 and 71 % in WT, *stp13-2* and STP13OE-6 respectively, which is consistent with a proton-driven glucose transport mechanism (Fig. 4). We noticed an increase in the CCCP-insensitive glucose uptake by the STP13OE-6 line compared to the WT. This increase is probably caused by a higher quantity of proteins in the plasma membrane of STP13OE-6, making it more permeable to glucose. Interestingly, a lack of STP13 in *stp13-2* provokes a significant reduction (by approximately 30 %) of the active glucose uptake compared to the WT, indicating that STP13 contributes up to 30 % of the active glucose transport in WT healthy leaves. In agreement with the high level of STP13 protein detected in STP13OE-6 (Fig. 3a), the amount of glucose actively taken up in this line was more than 3 times higher than in WT (Fig. 4). This result demonstrates that the STP13 protein immuno-detected in the STP13OE-6 line was functional.

To check whether the STP13 induction in *B. cinerea*-treated leaves could correlate with an increase in glucose uptake, we tried to measure the STP13-mediated glucose uptake activity in leaf discs infected with *B. cinerea*. However, the accumulation of the STP13 protein is concomitant with the formation of necrosis. As a result, it was technically unachievable to obtain reliable results on such damaged tissues.

The STP13 protein level affects *B. cinerea* susceptibility

The availability of transgenic plants that exhibited differential expression of STP13 and altered glucose uptake rate prompted us to investigate the contribution of STP13 in the outcome of *B. cinerea* infection. To this end, STP13-modified plants were challenged with *B. cinerea* and tested for their susceptibility.

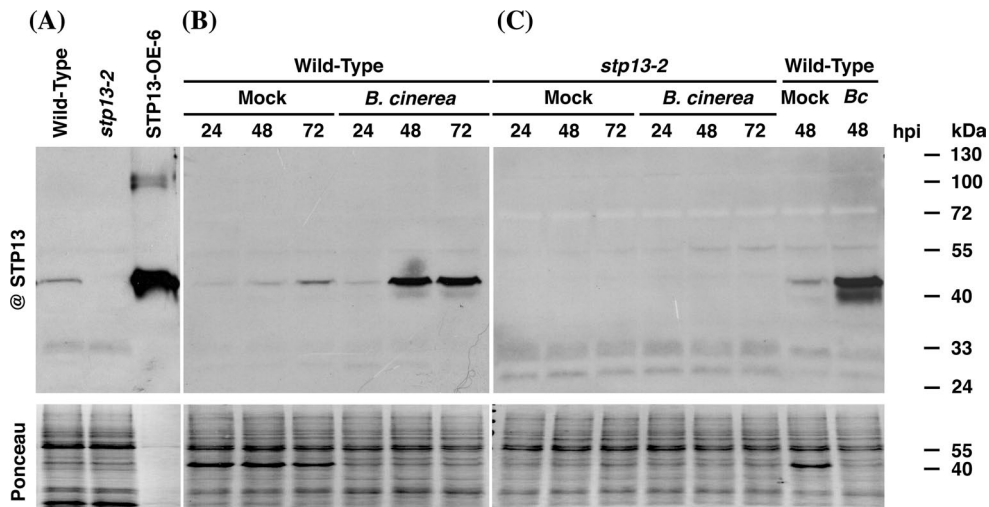


Fig. 3 Accumulation of STP13 protein in wild-type (WT), *stp13-2* and STP13OE-6 plants. Leaves were harvested and proteins extracted from the microsomal fraction were submitted to protein immunoblot analysis with a polyclonal antibody raised against the STP13 protein (@STP13). **a** STP13 protein accumulation in healthy leaves from WT, *stp13-2* and STP13OE-6 plants. Plants were placed and harvested in a controlled growth chamber with 60 % humidity on a 10-h photoperiod. Time course study of STP13 protein accumulation in WT (**b**) or *stp13-2* (**c**) leaves sprayed with mock solution or

B. cinerea suspension (5×10^4 conidia ml^{-1}). During inoculation experiments (*panel b* and *c*), mock and *B. cinerea*-treated plants were kept in the dark under saturating humidity (for 3 days). Thirty micrograms of total microsomal proteins have been loaded in each lane except for STP13OE-6 (2 μg). To visualize protein loading, blots were stained with a Ponceau S solution. *hpi* hours post-inoculation, *B.c.* *B. cinerea*, WT lanes (mock and *B. cinerea*) are used as a positive control of the *panel c*

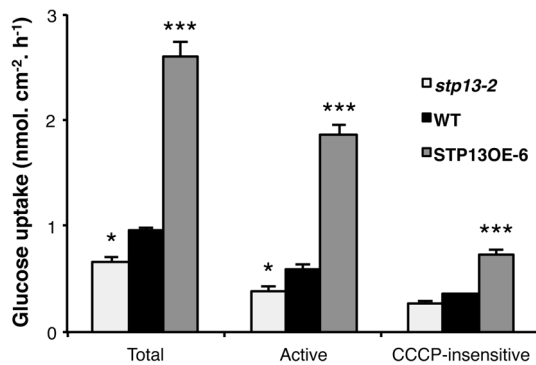


Fig. 4 Glucose uptake into wild-type (WT; *black bars*), *stp13-2* (*light grey bars*) and STP13OE-6 (*dark grey bars*) leaf discs. Discs were obtained from 5-week-old plants and total glucose uptake was measured after 45 min in equilibration buffer containing 0.2 mM of glucose (glucose buffer). Active glucose uptake results from the difference between total uptake and CCCP-insensitive uptake measured after incubation of leaf discs in glucose buffer containing CCCP (20 μM). Data are the mean ($\pm\text{SE}$) of 6 (WT) and 4 (*stp13-2* and STP13OE-6) independent experiments. Statistical analyses were undertaken independently for total, active and CCCP-insensitive glucose uptake. Stars represent groups that were significantly different from WT as determined by a one-way ANOVA followed by a multiple comparison test (* $P < 0.05$; *** $P < 0.001$)

Plants were drop-inoculated on leaves with a solution of conidia, and symptoms were observed 72 h after inoculation. We first used the average of the lesion diameters (LD) as an indicator for the susceptibility of each

genotype. As shown in Fig. 5a, WT plants were susceptible to *B. cinerea* isolate B05.10. Compared to WT plants, the *stp13-2* mutant showed a significant increase in LD, suggesting that this mutant is more susceptible than the WT, whereas STP13OE-6 plants displayed lesions with reduced size (decrease of LD by 26 %), indicating that these plants exhibited an enhanced resistance phenotype (Fig. 5a). Then, we looked at the distribution of the lesions according to their size (Fig. 5b). We determined three classes of necrotic lesions: small (LD < WT first quartile), medium (LD = WT interquartile range) and large (LD > WT third quartile). This scoring reflects the visual severity of symptoms. Compared to the WT, an increased proportion of large lesions and fewer small lesions were observed in the *stp13-2* mutant (Fig. 5b). Conversely, STP13OE-6 showed a twofold increase of the small lesion percentage, which represents 47 % of the total lesion number, whereas the number of large lesions was reduced (Fig. 5b). We introduced the cDNA corresponding to the STP13 mRNA into *stp13-2* under the control of the *CaMV35S* promoter. In the resulting plants (named C2-7), the construct was able to complement *stp13-2* mutation and to fully restore the WT resistance state, demonstrating that the susceptible phenotype observed in *stp13-2* was attributable to STP13 (Fig. S2).

Finally, we evaluated the capacity of *B. cinerea* to develop and provoke necrosis in each genotype. In these experiments, a very low density of conidia was

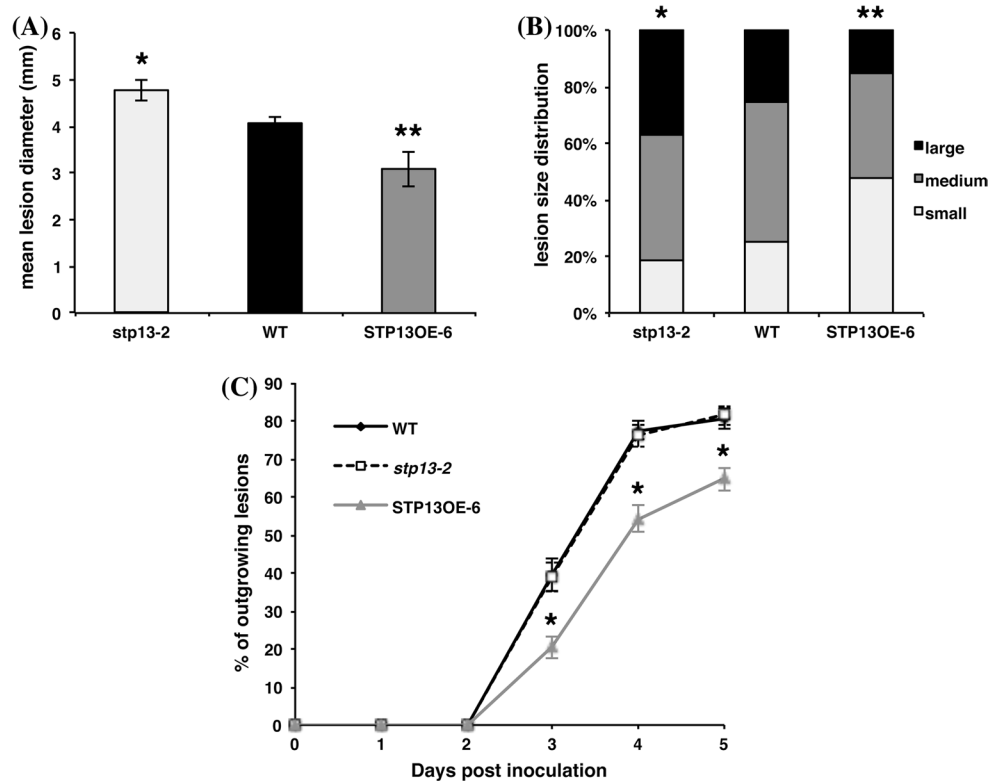


Fig. 5 Disease phenotype of wild-type (WT), *stp13-2* and STP13OE-6 plants infected with *B. cinerea*. Leaves of 5-week-old plants have been used for inoculation tests. **a** Lesion diameters observed on WT, *stp13-2* and STP13OE-6 plants 3 days after inoculation with 6 μ l droplets containing 10^4 conidia ml^{-1} . Data represent the mean (\pm SE) lesion diameter from 18 (WT and *stp13-2*) and 6 (STP13OE-6) experiments. In each experiment, at least five plants per genotype were infected with *B. cinerea*. Stars represent groups that were significantly different from the WT as determined by a permutation with general scores test (* $P < 0.025$; ** $P < 0.01$). **b** Lesion size distribution observed on WT, *stp13-2* and STP13OE-6 plants infected with *B. cinerea*. Plants were drop-inoculated and diameters of necrotic lesions (LD) were measured after 3 days. Lesions were grouped into 3 classes according to their size: small (LD < WT first quartile), medium (LD = WT interquartile range) and large

(LD > WT third quartile). The percentage of lesion size distribution from 18 (WT and *stp13-2*) and 6 (STP13OE-6) independent experiments is shown. Significant differences from the WT were determined by a Chi square test (* $P < 0.025$; ** $P < 0.01$). **c** Percentage of outgrowing lesions observed on WT, *stp13-2* and STP13OE-6 infected with *B. cinerea*. Plants were inoculated with 6 μ l droplets containing 10^3 conidia ml^{-1} . During 5 days, the number of necrotic lesions was counted and the ratio between outgrowing lesions and total inoculated spots was calculated. Data represent the mean (\pm SE) from six independent experiments. Statistical analyses have been realized independently for each day post-inoculation. Stars represent groups that were significantly different from WT as determined by a Kruskal–Wallis one-way analysis of variance followed by a multiple comparison test ($P < 0.05$)

drop-inoculated on leaves in order to extend the duration of the infection process. During 5 days, the number of necrotic lesions was counted and the ratio between visible necrosis and total inoculated spots was calculated in order to assess the percentage of outgrowing lesions. The appearance of new necrotic lesions represents the ability of *B. cinerea* to kill host cells and spread. In these experimental conditions, necrotic lesions started to be visible 3 days post-inoculation in WT plants (Fig. 5c). The infection process reaches its maximum within 4 days post inoculation, where approximately 80 % of successfully infected spots were recorded. The number of necrotic spots did not increase much after 4 days. In comparison with WT plants, the kinetic of the outgrowing lesion appearance did not change in the *stp13-2* mutant (Fig. 5c),

while the size of the necrotic lesions was previously shown to be larger in those plants (Fig. 5b). These results and the late induction of STP13 during *B. cinerea* infection suggest that STP13 may be involved in limiting the expansion of the symptoms but not in the formation of necrosis. By contrast, STP13OE-6 plants, which constitutively accumulated high amount of STP13 protein (Fig. 3a) and displayed a higher rate of glucose uptake (Fig. 4), were found to have a major reduction of the outgrowing lesion percentage (Fig. 5c). Compared to the WT, the overexpression of STP13 resulted in a drop of 50, 30 and 20 % in the number of visible necrotic spots at respectively 3, 4 and 5 days post-inoculation. These data indicated that the high expression of STP13 associated with an increased rate of glucose uptake prior to infection slowed down

the formation of necrotic lesions and reduced the disease severity by limiting the symptom development.

To summarize, STP13-modified plants with altered resistance levels revealed that STP13 contributes to the basal resistance to *B. cinerea* in WT plants. Furthermore, we evidenced that a high constitutive level of glucose uptake in the host before infection has a clear protective effect against *B. cinerea*.

Expression of the jasmonic acid-dependent defense gene *PDF1.2* and the camalexin biosynthetic gene *PAD3* in STP13-modified plants

To explain the modified resistance phenotype of *stp13-2* and STP13OE-6 mutants, we verified whether marker genes of the jasmonic acid-dependent signaling and the camalexin biosynthesis were affected in those transgenic lines. *B. cinerea* induces the expression of a subset of JA-dependent defense genes, including the antifungal defensin-like peptide *PDF1.2*, which are required for resistance (Glazebrook 2005). The role of camalexin in resistance to *B. cinerea* seems to be isolate-dependent (Kliebenstein et al. 2005; Ferrari et al. 2003; Rowe and Kliebenstein 2008). In response to several *B. cinerea* strains, including B05.10, the induction of *CYP79B2*, *CYP71A13*, and *CYP71B15* (*PAD3*), involved in the camalexin biosynthesis, is highly correlated with the accumulation of camalexin (Stefanato et al. 2009; Schuhegger et al. 2006; Arbelet et al. 2010). By contrast, Rowe et al. (2010) evidenced a lack of correlation with some *B. cinerea* isolates. Moreover, *pad3* mutant, which is deficient in camalexin accumulation, displays an increased sensitivity to the *B. cinerea* isolate B05.10, indicating that the amount of camalexin present in WT plants limits spreading (Stefanato et al. 2009).

The expression of *PDF1.2* and *PAD3* was tested after *B. cinerea* inoculation and used as specific markers of the JA signaling and camalexin biosynthetic pathways. In our experimental conditions, *PDF1.2* and *PAD3* were strongly induced at 48 hpi and are concomitantly expressed with *STP13* (Figs. 2, 6). This result validates our initial assumption that specific defenses were fully active 48 hpi. In *stp13-2*-infected plants, the pattern of transcript accumulation of *PAD3* was not affected in comparison with WT whereas the expression of *PDF1.2* was reduced at 72 hpi, which is consistent with the higher susceptibility of this line (Fig. 6 and table S2).

Because sugars are known to act as signaling molecules that can positively regulate gene expression (Herbers et al. 1996; Ehness et al. 1997; Roitsch et al. 2003; Bolouri Moghaddam and Van den Ende 2012; Rolland et al. 2006), we tested the hypothesis whether the improved resistance of STP13OE-6 line is attributable to a sugar-mediated

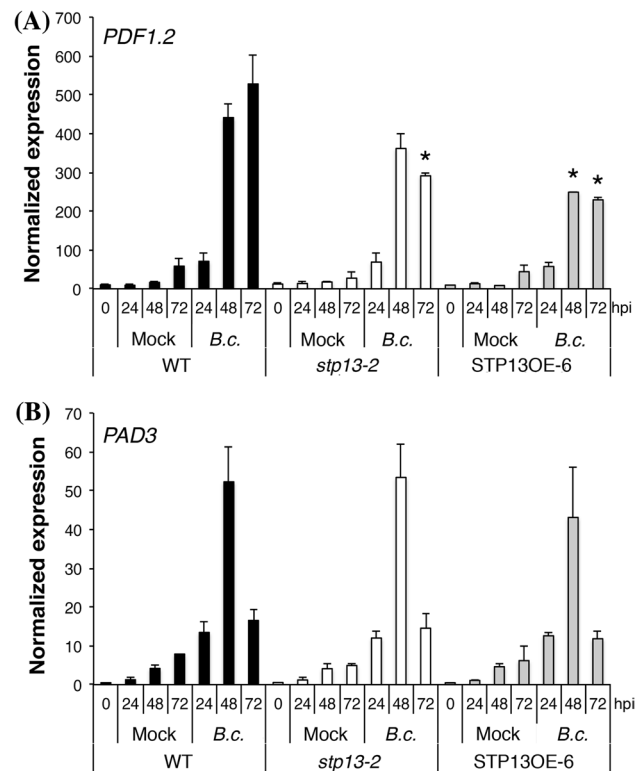


Fig. 6 *PDF1.2* and *PAD3* mRNA accumulation in wild-type (WT), *stp13-2* and STP13OE-6 plants. Plants were sprayed with mock solution or *B. cinerea* suspension (5×10^4 conidia ml^{-1}). Healthy (0 hpi) or treated leaves from at least three plants were harvested at indicated time points. *PDF1.2* (a) and *PAD3* (b) transcript levels were quantified by RT-qPCR. Data are expressed as normalized expression (no unit) to the plant reference gene *At4g26410* expression level (Czechowski et al. 2005). Data are mean \pm SE from two independent experiments. Stars represent groups that were significantly different from WT as determined by an ANOVA with general scores followed by a multiple comparison test ($P < 0.05$). *B.c.* *B. cinerea*, *hpi* hours post-inoculation

stimulation of defense genes. To this end, we monitored the expression level of *PDF1.2* and *PAD3* in STP13OE-6 plants (Fig. 6). The basal expression level (0 hpi) of *PDF1.2* and *PAD3* in STP13OE-6 did not differ from the WT, indicating that the higher amount of glucose uptake in STP13OE-6 did not result in the priming of these two genes. Unexpectedly, we noticed that the expression level of *PDF1.2* was reduced at 48 and 72 hpi compared to the WT (Fig. 6a and table S2), which could be explained by the limited expansion of the infection in the STP13OE-6 line (Fig. 5b). However, the expression of *PDF1.2* and *PAD3* was still highly inducible in response to *B. cinerea*, but did not display over-stimulation (Fig. 6a, b). According to these results, we can conclude that the enhanced resistance phenotype observed in the STP13OE-6 line cannot be supported by a higher induction of the expression of these defense genes.

Discussion

Our goal was to identify plant STPs that play a role in the basal resistance to the necrotrophic fungus *B. cinerea* isolate B05.10. In *A. thaliana*, the uptake of hexoses from the apoplast is mediated by STPs, which are encoded by 14 highly homologous genes. All the characterized STPs are proton symporters of the plasma membrane, showing specificities for a wide range of substrate (Büttner 2010). Most of them display a sink-specific expression pattern which correlates with their potential function in supporting sink demand for hexoses (Büttner 2010).

In healthy source leaves, the analysis of transcript accumulation of the 14 *STP* genes revealed that 6 of them are expressed in leaves, namely *STP1*, *STP3*, *STP4*, *STP7*, *STP13* and *STP14*. Our results confirmed the data obtained in different publications. It has been previously reported that *STP1* was strongly expressed in guard cells of different leaf types but also in other organs, i.e. roots, seeds and seedlings, having a major contribution to the absorption of monosaccharides from the surrounding environment (Stadler et al. 2003; Sherson et al. 2000; Yamada et al. 2011). *STP3* has been described as a low affinity monosaccharide transporter that is expressed in green tissues, and is up-regulated after wounding (Büttner et al. 2000). *STP4* is expressed primarily in sink tissues, i.e. roots and flowers but is also induced in leaves by various stress conditions, e.g. wounding, elicitors and pathogens (Truernit et al. 1996; Fotopoulos et al. 2003). *STP13* is found in source and sink tissues and is transcriptionally regulated by many abiotic or biotic stresses, like wounding, drought, high salinity, pathogens, or programmed cell death (Büttner 2010; Norholm et al. 2006; Yamada et al. 2011). *STP13* mediates the uptake of glucose, mannose, galactose and fructose at a significant rate (Norholm et al. 2006). A role for *STP13* in the uptake of monosaccharides in roots under high salinity has been shown (Yamada et al. 2011). *STP14* was found to be expressed in different tissues, mainly in leaves and siliques, but unlike other STPs, it has been identified as a plasma membrane galactose-specific transporter (Poschet et al. 2010). Concerning *STP7*, no uptake activities have been found so far for the common hexoses, suggesting it may be a pseudogene (Büttner 2010; Yamada et al. 2011).

The expression of *STP1*, *STP3*, *STP4*, *STP13* and *STP14* is also known to display a diurnal regulation (Büttner 2010; Stadler et al. 2003; Poschet et al. 2010). In order to minimize this effect, we decided to perform the inoculation experiments in the dark. Profiling of the transcriptional activities of the 14 *STPs* in *B. cinerea*-infected leaves showed that the expression of many STPs was not modified by *B. cinerea* challenge. Interestingly, none of the undetected STPs in healthy leaves were induced by *B. cinerea*, confirming that they may be involved in other physiological

processes, particularly in sink tissues. *STP4*, whose expression is correlated with glucose resorption during the interaction between the biotroph *E. cichoracearum* and *A. thaliana* leaves (Fotopoulos et al. 2003), is not affected by *B. cinerea*, indicating that different STPs could be activated depending on pathogens. We found that solely *STP13* was induced upon *B. cinerea* infection. The detailed analysis of *STP13* expression and the immunodetection of *STP13* showed that transcripts and proteins accumulated in *B. cinerea*-inoculated leaves. They followed the expression of late defense genes and the formation of necrotic lesions. Such disease symptoms are the consequence of the cell death of the host. This phenomenon occurs through fungal toxin activities and an oxidative burst generated by the pathogen and the host itself, which triggers an active or programmed cell death (Choquer et al. 2007). Our observations are in agreement with previous findings showing that *STP13* expression was upregulated in tissues undergoing PCD (Norholm et al. 2006).

The unique expression profile of *STP13* among STPs led us to undertake the functional analysis of this gene. To investigate the impact of *STP13* expression on *B. cinerea* resistance, we analyzed transgenic plants with modified levels of *STP13*. Under normal growth conditions on soil, a lack or an overexpression of *STP13* did not result in any obvious developmental or morphological changes (Fig. S3). Interestingly, Schofield et al. (2009) showed that *STP13*-overexpressing seedlings grown on soil-less media supplemented with 55 mM glucose and 9 mM nitrate had an increased biomass, higher endogenous sucrose levels and an improved capacity to use nitrogen, but observed no difference when plants were grown on soil.

Upon infection by *B. cinerea*, KO and overexpressor lines displayed clear contrasting phenotypes. Plants that did not accumulate *STP13* had an increased susceptibility to *B. cinerea* associated with a reduced basal capacity to absorb glucose. The significant residual glucose uptake activity observed in the *stp13-2* mutant suggests that the probable activity of other STPs may limit the effect of the mutation of a single STP (Fig. 4). However, the *stp13-2* line exhibited necrosis with increased size, providing genetic evidence that *STP13* contributes to the basal resistance but does not act in the prevention of symptom development caused by the highly virulent *B. cinerea* isolate B05.10. Since *STP13* is expressed simultaneously with necrosis, it would rather indicate that this protein plays a role in limiting the expansion of the symptoms. It should be noted that there is a large level of genetic diversity within *B. cinerea* strains (Kliebenstein et al. 2005; Rowe et al. 2010) and we can not exclude that the phenotype we observed could be different with other *B. cinerea* isolates. When challenged with the necrotroph *A. brassicicola*, no difference

in susceptibility was observed in *stp13* knockout mutants by Norholm et al. (2006) (data were not shown). It may be explained by the difference of virulence between these two necrotrophic fungi, *A. brassicicola* being poorly virulent compared to *B. cinerea* on *A. thaliana* leaves (Mukherjee et al. 2010).

In the present study, the importance of host hexose uptake has also been evidenced in STP13OE-6 plants. In this line, a high amount of STP13 prior to infection associated with a high level of glucose uptake rate clearly improved the plant resistance. It resulted in delaying the appearance and development of the symptoms. We showed that this protection against *B. cinerea* is not attributable to the priming of marker genes of the jasmonic acid (*PDF1.2*) and camalexin (*PAD3*) pathways, which are generally associated with basal levels of defense against necrotrophic fungi.

Collectively, our data suggest that STP13 could be involved in the resorption of monosaccharides released by damaged tissues in order to allow host living cells to compete with the pathogen for apoplastic hexoses. The role of the imported hexoses into host cells remains to be elucidated, but we hypothesize that host cells could use them as a fuel to support the increased energy demand to trigger plant defense reactions. The enhancement of host hexose resorption would imply a change in the direction of sugar fluxes toward host cells. It could consequently participate in the starvation of the fungus by limiting the availability of apoplastic hexoses. Such competition for apoplastic sugars is crucial for the final outcome of the interaction between the plant and the pathogen. *B. cinerea* has the functional equipment for apoplastic sugar uptake at the plant/pathogen interface (Lemoine et al. 2013). Dulermo et al. (2009) reported that *B. cinerea* possesses a multigenic flexible hexose uptake system (BcHXTs and BcFRT genes), allowing the fungus to re-allocate plant hexoses for subsequent conversion into mannitol through fungal metabolic pathways. The next challenge will be to understand the precise role of STP13 in this competition for nutrients between host cells and the invader, and more largely the regulatory role of sugar fluxes in the plant immunity.

In conclusion, we showed, using complementary approaches, a strong correlation between transcripts, protein detection, *in planta* glucose uptake rate and resistance level, which indicates that STP13 is involved in the basal resistance to *B. cinerea*. We and others have shown that STP13 contributes to the resorption of hexoses allowing plant cells to adapt to environmental changes, particularly during biotic and abiotic stresses. More investigations will be necessary to dissect the regulatory pathways leading to the expression of STP13 and the precise role of the sugar transport in the plant immunity and in the response to environmental stresses.

Materials and methods

Plant material and growth conditions

Seeds of *A. thaliana* (Columbia ecotype) were sown in an autoclaved mix of compost/vermiculite (3/1). Plants were grown in a controlled growth chamber at 23 °C (18 °C during the night), with 60 % humidity on a 10-h photoperiod. The *stp13-2* homozygous mutant line (salk_021204) was obtained from the SALK T-DNA insertion mutant collection (Alonso et al. 2003).

Culture of *Botrytis cinerea*, infection methods and disease scoring

B. cinerea isolate B05.10 (Staats and van Kan 2012) was grown on Difco potato dextrose agar (Becton–Dickinson). Conidia were harvested in sterile water, filtered through miracloth (EMD Chemicals) and were diluted in quarter-strength potato dextrose broth (PDB; Becton–Dickinson) for infection.

For the evaluation of plant susceptibility, 6 µl of conidia suspension (10^4 conidia ml⁻¹) were spotted on leaves of 5-week-old plants and lesion diameters (LD) were measured after 3 days. Three categories of necrotic lesions were determined. Small lesions : LD < 2,63 mm. Medium lesions : 2,63 mm < LD < 5,58 mm. Large lesions : LD > 5,58 mm. This range has been determined according to the lesion size distribution observed in the WT. Small, medium and large lesions corresponded to LD < WT first quartile, LD = WT interquartile range and LD > WT third quartile, respectively. For the analysis of the formation of necrosis, 6 µl of conidia suspension (10^3 conidia ml⁻¹) were deposited on leaves and the percentage of outgrowing lesions was recorded every day for 5 days. For the analysis of gene expression by RT-qPCR, the entire rosette was sprayed with the conidia suspension (5×10^4 conidia ml⁻¹) and mock-treated plants with quarter-strength PDB. Source leaves were harvested at the indicated time points. The inoculated plants were kept in the dark and under saturating humidity in a tray closed with a water-sprayed lid.

RNA extraction and RT-qPCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) and treated with DNase I (Sigma-Aldrich) prior to the retrotranscription. The cDNA was synthesized using M-MLV reverse transcriptase (Promega). Real-time quantitative PCR was performed using GoTaq PCR Mastermix (Promega). Target gene expression was normalized to the expression of the plant gene At4g26410, previously described as a stable reference gene (Czechowski et al. 2005; La Camera et al. 2011). The primers have been tested

for their efficiency and their specificity. The primers are listed in Table S1.

Cloning procedures and plant transformation

The cDNA made from RNA extracted from *B. cinerea*-treated Arabidopsis plants were used to amplify STP13 complete coding sequence (Phusion Taq Polymerase, Thermo Scientific). The following primers were used for the amplification: 5'-CACCATGACCGGAGGAGGA-3' and 5'-CACTTATTCACAAACGCAG-3'. PCR products were cloned into the pENTR-D-TOPO vector (Invitrogen), sequenced and LR-recombined with pB2GW7 destination vector (Karimi et al. 2002) to express *STP13* under the control of the *CaMV35S* promoter. This construct was electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMp90). Wild-type and *stp13-2* plants were transformed using the floral dipping method (Clough and Bent 1998) and transgenic homozygous T2 plant lines were selected according to their Basta resistance.

Glucose uptake into leaf discs

Discs of eight-millimeter diameter were cut from leaves of 5-week-old plants and placed lower epidermis down in petri dishes containing equilibration buffer (20 mM MES-KOH pH 5.8 and 1 mM CaCl₂) and left for 1 h under agitation. The discs were transferred in glucose buffer (20 mM MES pH 5.8, 1 mM CaCl₂, 0.2 mM glucose containing 0.1 μCi ml⁻¹ of [¹⁴C]-glucose) and incubated for 45 min. To measure CCCP-insensitive glucose uptake, CCCP (20 μM) was added in glucose buffer. After incubation, the discs were removed from glucose buffer, washed three times for 2 min in equilibration buffer, and dried on filter paper. The discs were incubated overnight in digestion buffer containing 36,4 % perchloric acid (w/v), 0,017 % triton X-100 (w/v) and 8,1 % hydrogen peroxide (w/v) at 55 °C. Samples were counted by liquid scintillation counting (Tri-Carb 2910 PR, PerkinElmer).

Western blot analysis

A microsomal fraction was prepared from *A. thaliana* leaves. Ground leaves were resuspended in 4 volumes of extraction buffer (50 mM HEPES pH 7.5, 500 mM sorbitol, 5 mM EDTA, 0.6 % PVPP 1 mM PMSF and 2 mM DTT), filtered through miracloth (EMD Chemicals) and centrifuged at 10,000g for 15 min at 4 °C. The supernatant was then centrifuged at 50,000g for 35 min at 4 °C. The pellet containing the microsomal fraction was resuspended in phosphate-sorbitol buffer (5 mM K phosphate pH 7.8, 330 mM sorbitol, 5 mM KCl, 1 mM EDTA, 0,5 mM DTT). Total microsomal proteins were separated by SDS-PAGE

(10 % acrylamide) and blotted on nitrocellulose membrane (Hybond ECL, GE Healthcare) for western blot analysis. To visualize protein loading, blots were stained with a Ponceau S solution (Sigma-Aldrich).

A specific peptide containing the last 27 C-terminal amino-acids of STP13 (DDHNDHEFVNGEKSNGKSNG-FDPSTRL) was synthesized (Proteogenix). After immunization of rabbits, the serum containing a polyclonal antibody raised against the STP13 protein was obtained and purified with the initial peptide (Proteogenix). Blots of total microsomal proteins were incubated with the purified STP13 antiserum and detection was realized using ECL Plus Western blotting Detection Reagents (GE Healthcare).

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