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A Ralstonia solanacearum Type III Effector Directs the Production of the Plant Signal Metabolite Trehalose-6-Phosphate

M. Poueymiro, A. C. Cazalé, J. M. François, J. L. Parrou, N. Peeters, S. Genin

INRA, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR441, Castanet-Tolosan, France; CNRS, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR2954, Castanet-Tolosan, France; Université de Toulouse, INSA, UPS, INP, LISBP, Toulouse, France; INRA, UMR792, Ingénierie des Systèmes Biologiques et des Procédés, Toulouse, France; CNRS, UMR5004, Toulouse, France

ABSTRACT The plant pathogen Ralstonia solanacearum possesses two genes encoding a trehalose-6-phosphate synthase (TPS), an enzyme of the trehalose biosynthetic pathway. One of these genes, named ripTPS, was found to encode a protein with an additional N-terminal domain which directs its translocation into host plant cells through the type 3 secretion system. RipTPS is a conserved effector in the R. solanacearum species complex, and homologues were also detected in other bacterial plant pathogens. Functional analysis of RipTPS demonstrated that this type 3 effector synthesizes trehalose-6-phosphate and identified residues essential for this enzymatic activity. Although trehalose-6-phosphate is a key signal molecule in plants that regulates sugar status and carbon assimilation, the disruption of ripTPS did not alter the virulence of R. solanacearum on plants. However, heterologous expression assays showed that this effector specifically elicits a hypersensitive-like response on tobacco that is independent of its enzymatic activity and is triggered by the C-terminal half of the protein. Recognition of this effector by the plant immune system is suggestive of a role during the infectious process.

IMPORTANCE Ralstonia solanacearum, the causal agent of bacterial wilt disease, infects more than two hundred plant species, including economically important crops. The type III secretion system plays a major role in the pathogenicity of this bacterium, and approximately 70 effector proteins have been shown to be translocated into host plant cells. This study provides the first description of a type III effector endowed with a trehalose-6-phosphate synthase enzymatic activity and illustrates a new mechanism by which the bacteria may manipulate the plant metabolism upon infection. In recent years, trehalose-6-phosphate has emerged as an essential signal molecule in plants, connecting plant metabolism and development. The finding that a bacterial pathogen could induce the production of trehalose-6-phosphate in plant cells further highlights the importance of this metabolite in multiple aspects of the molecular physiology of plants.
T3Es in *R. solanacearum* strain GMI1000, RipAA (AvrA) and RipP1, elicit a hypersensitive reaction (HR), a localized and programmed cell death at the sites of infection. The double inactivation of ripAA and ripP1 is sufficient to restore pathogenicity, showing that both determinants restrict the host range of *R. solanacearum* on *Nicotiana* spp. (18).

The molecular function of most *R. solanacearum* T3Es once inside plant cells remains elusive. Many T3Es do not have homology with proteins of known function, and relatively few functional studies have been conducted (10–12). There is evidence that RipP2 displays acetyltransferase activity (19), whereas the RipG (GALA) effector family was shown to interact with plant SKP1-like proteins, presumably acting as active components of the host ubiquitination machinery to subvert plant immunity (9, 20, 21).

In addition, *R. solanacearum* possesses a transcription activator-like effector named RipTAL that is likely to promote disease through the transcriptional activation of host genes, as shown for *Xanthomonas* spp. (22, 23).

In this study, we identified and characterized a novel T3E from *R. solanacearum* strain GMI1000 that displays homology to trehalose phosphate synthases, a class of enzymes that are ubiquitous in prokaryotes and eukaryotes. Complementation assays in yeast were used to demonstrate its biochemical activity and identify essential residues for enzymatic function. Interestingly, trehalose-6-phosphate has recently emerged as an essential signal molecule in plants, connecting plant metabolism and development (24). Heterologous expression assays also show that this T3E is specifically inducing an HR-like response in tobacco.

**RESULTS**

RipTPS encodes an effector protein homologous to trehalose phosphate synthases and is translocated into plant cells by the T3SS. The RSp0731 gene product displays homology with proteins of the glycosyltransferase superfamily that possess a trehalose-6-phosphate synthase (TPS) activity. TPS catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate, forming trehalose-6-phosphate (Tre6P) and UDP (Fig. 1).

In addition, *R. solanacearum* possesses a transcription activator-like effector named RipTAL that is likely to promote disease through the transcriptional activation of host genes, as shown for *Xanthomonas* spp. (22, 23).

In this study, we identified and characterized a novel T3E from *R. solanacearum* strain GMI1000 that displays homology to trehalose-6-phosphate synthases, a class of enzymes that are ubiquitous in prokaryotes and eukaryotes. Complementation assays in yeast were used to demonstrate its biochemical activity and identify essential residues for enzymatic function. Interestingly, trehalose-6-phosphate has recently emerged as an essential signal molecule in plants, connecting plant metabolism and development (24). Heterologous expression assays also show that this T3E is specifically inducing an HR-like response in tobacco.
A Tre6P Synthase Effector in \textit{R. solanacearum}

![Graph 2](image2)

**FIG 2** Bacterial TPS phylogeny. The accession numbers of the proteins aligned in this phylogeny can be found in the legend to Fig. S1 in the supplemental material. The outgroup \textit{Arabidopsis thaliana} TPS1 (accession number NP_177979) was added in this analysis. Sequences were aligned with MUSCLE and curated with GBLOCKS, and the phylogeny was reconstructed with PhyML. All analysis was run using the [http://phylogeny.fr](http://phylogeny.fr) webserver (58). Bootstrap values are indicated in red. Scale is number of substitutions per site.

(27). A chimeric protein fusion consisting of the 83 N-terminal residues from RSp0731 fused to CyaA was engineered on a plasmid vector and introduced into the wild-type strain GMI1000 and the T3SS (\textit{hrpV}) mutant derivative GMI1694. The cyclic AMP (cAMP) concentration was monitored in tobacco plant tissue following infiltration with these strains, and the results shown in Fig. 1C indicate that this value was 73-fold higher in the wild-type strain than in the T3SS mutant. This shows that the Rsp0731 N-terminal domain allows T3SS-dependent translocation into host cells; RSp0731 was therefore renamed RipTPS, in agreement with the new nomenclature (4).

\textit{ripTPS} is a conserved effector in the \textit{R. solanacearum} species complex, and homologues are present in other bacterial plant pathogens. Because the RipTPS effector is translocated into plant cells, we investigated the phylogenetic relationship of the protein with other bacterial TPS sequences. As the plant TPS is clearly an outgroup in this phylogenetic reconstruction (Fig. 2), it seems that the \textit{R. solanacearum} \textit{ripTPS} most probably originated from a duplication of a bacterial \textit{otsA} gene. Further comparative genomic analyses show that \textit{ripTPS} is widely conserved in strains of the \textit{R. solanacearum} species complex. Indeed, it is present in each of the four main phylogenotypes, with the exception of \textit{Ralstonia syzygii} and blood disease bacterium strains, which are undergoing a reductive genomic evolution (28) and presumably have lost the gene. All these RipTPS homologues display a high level of protein sequence identity (from 76 to 99\%) among the various strains. Interestingly, in most of the strains, \textit{ripTPS} is physically associated with the genome with another type III effector gene (\textit{ripAV}), contrary to the other (\textit{otsA}) TPS genes, which are physically associated with a trehalose-6-phosphate phosphatase (\textit{otsB})-related gene. In order to identify RipTPS effector homologues in other bacterial pathogens, we performed BLAST searches and detected proteins in \textit{Xanthomonas oryzae pv. oryzae} and \textit{Acidovorax citrulli} with a RipTPS architecture, i.e., a TPS domain and an approximately 100-aa N-terminal extension potentially involved in T3SS export (Fig. 2; also Fig. S1 in the supplemental material). This suggested that RipTPS-like effectors may be present in other vascular plant pathogens.

Functional complementation of a yeast \textit{tps}-defective mutant by \textit{R. solanacearum} \textit{ripTPS}. In \textit{Saccharomyces cerevisiae}, trehalose is synthesized by a TPS enzyme encoded by \textit{tps1} and a trehalose-6-phosphate phosphatase (TPP) encoded by \textit{tps2}. Inactivation of \textit{tps1} causes an absence of trehalose accumulation but also a growth arrest when glucose or fructose that is rapidly fermented is used as a carbon source (24). The expression of the \textit{E. coli} \textit{otsA} gene was shown to partially restore the growth of a yeast \textit{tps1} mutant in the presence of fermentable sugars (29). Since we also wanted to quantify Tre6P production, we decided to test for complementation by \textit{ripTPS} using the \textit{tps1} \textit{tps2} double mutant strain, which has the same growth defect on fructose as the single \textit{tps1} mutant, i.e., it is unable to grow on fructose but remains able to grow on galactose (30). When the \textit{tps1} \textit{tps2} mutant strain was transformed with \textit{ripTPS}, growth on fructose was restored to the wild-type level (Fig. 3). Similar results were observed with the expression of the \textit{E. coli} \textit{otsA} gene, as previously described (29). Complementation of the growth defect of the yeast \textit{tps1} \textit{tps2} mutant on fructose was a first indication that \textit{ripTPS} encodes a functional TPS.

\textit{RipTPS} catalyzes \textit{Tre6P} synthesis. We then used the double \textit{tps1} \textit{tps2} mutant to test for \textit{Tre6P} accumulation upon the expression of \textit{ripTPS}. In this double mutant, the overaccumulation of \textit{Tre6P} is easier to monitor in the absence of the \textit{tps} gene-encoded TPP activity. \textit{Tre6P} production was quantified in the \textit{tps1} \textit{tps2} mutant yeast strain complemented by \textit{ripTPS} after being triggered by a glucose pulse (31). Within 5 min after the pulse, \textit{Tre6P} synthesis reached a maximum in the RipTPS-expressing strains, whereas \textit{Tre6P} production was below the detection threshold in the \textit{tps1} \textit{tps2} mutant carrying the empty vector (Fig. 4, pVTU). Native and hemagglutinin (HA)-tagged versions of RipTPS and native \textit{E. coli} \textit{OtsA} were also generated and used to complement the \textit{tps1} \textit{tps2} mutant strain. As for \textit{OtsA}, the RipTPS-dependent
The black arrowhead indicates RipTPS (67 kDa). A corresponding Coomassie-stained gel was used as a loading control. Analysis of the expression of the HA-tagged TPS variants in the yeast protein served to detect any differences (at a $P < 0.05$) in the expression of the CA tag in strains expressing the empty vector or expressing E. coli OtsA with an HA tag or RipTPS with a tag. All samples were analyzed five times, generating biologically independent replicates. The $P$ value groupings indicate which strains cannot be distinguished (at a $P$ value of $<5\%$) using a nonparametric Wilcoxon matched-pairs signed-rank test, i.e., group a: the results for the empty vector pVTU and the three catalytic mutants cannot be distinguished and are all significantly different from the results for the other strains. (B) Western blot analysis of the expression of the HA-tagged TPS variants in the yeast protein extracts. A corresponding Coomassie-stained gel was used as a loading control. The black arrowhead indicates RipTPS (67 kDa).

The production of Tre6P was confirmed, although its catalytic activity was decreased due to the addition of the HA tag.

Based on the crystal structure of E. coli OtsA (26) and on the model generated for Magnaporthe grisea TPS1 (32), conserved residues in the glucose-6-phosphate binding domain were identified and shown to be essential for activity. Corresponding nucleotide changes were introduced into the full-length ripTPS gene to generate 3 amino acid substitutions at these key positions (Fig. 1B; also Fig. S1 in the supplemental material). As shown in Fig. 4, the tsp1 tsp2 mutant strains carrying the resulting ripTPS*V154V (ripTPS with a substitution of valine for tyrosine at position 154), ripTPS*W163S, or ripTPS*Y118S allele were all unable to synthesize Tre6P (the production of Tre6P was indistinguishable from that in the strain carrying the pVTU empty vector), showing the importance of these residues for TPS activity. Western blot analysis confirmed that the wild-type RipTPS and variant alleles were produced in equal amounts in yeast (Fig. 4B). A positive HA band at 58 kDa, as shown in Fig. 4B, suggests that, in yeast, there could be cleavage of the N-terminal extension of RipTPS-HA. As expected, none of the three mutated ripTPS alleles restored yeast growth on fructose when introduced into the tsp1 tsp2 mutant (Fig. 3).

RipTPS elicits an HR-like response on Nicotiana tabacum independent of TPS activity. In order to determine the contribution of ripTPS in R. solanacearum pathogenicity, a disruption mutant was created by inserting an interposon into the ripTPS coding sequence. The capacity of the corresponding mutant strain to cause disease was evaluated on several host plants, and no difference in symptom development was detected compared to that of the wild-type strain (see Fig. S2 in the supplemental material). Further inoculation tests on a panel of Arabidopsis ecotypes and competitive index experiments (13) on tomato and bean did not show any difference either (data not shown). We then wanted to evaluate the role of TPS activity during infection by R. solanacearum by testing available transgenic Arabidopsis plants expressing E. coli otsA (33) or ripTPS, generated by ourselves. Both otsA- and ripTPS-expressing plants had stunted growth and very low fertility (33; also our unpublished data), making the test of the contribution of TPS to disease difficult to evaluate.

We then investigated whether the expression of ripTPS could elicit defense responses in other plants. We used an Agrobacterium-mediated transient expression assay after infiltration of leaf mesophyll tissues using a vector carrying the full-length ripTPS coding sequence under the control of the 35S promoter. On Nicotiana tabacum, a strong HR-like necrotic response could be observed 48 h postinfiltration (Fig. 5B, a). This response could be specific to N. tabacum, since no reaction was observed after leaf infiltration of Nicotiana benthamiana or Nicotiana glutinosa (data not shown). The kinetics of this necrotic response on N. tabacum was comparable to the HR response elicited by the ripAA (avrA) avirulence gene product under the same experimental conditions (Fig. 5B, g) (18). Altogether, these data suggest that RipTPS is specifically recognized by the N. tabacum immune system.

Agrobacterium-mediated transient expression assays were also performed with strains expressing RipTPS carrying single point mutations in the essential catalytic sites described above. Agrobacterium tumefaciens strain GV3101::pMP90RK containing the pAM-PAT plasmids in which the ripTPS*V154V, ripTPS*W163S, and ripTPS*Y118S alleles were cloned induced an HR-like response similar to that induced by the strain carrying the wild-type ripTPS gene (Fig. 5B, b, c, and d). This demonstrates that the enzymatic activity of RipTPS is not required for the elicitation of the necrotic response on N. tabacum.

The C-terminal half of RipTPS is sufficient to trigger the HR-like response. Since plants contain TPS enzymes (34), which are related to prokaryotic TPS (Fig. 2), the finding that RipTPS elicited an HR-like response on a tobacco species was intriguing. We therefore performed Agrobacterium-mediated transient expression assays using the full-length E. coli otsA as performed for ripTPS. Although otsA is closely related to ripTPS (Fig. 2; also Fig. S1 in the supplemental material), we can see from the results shown in Fig. 5 (Fig. 5B, e) that Agrobacterium-mediated expression of otsA did not induce a necrotic response on N. tabacum.

The three functional domains of RipTPS (the T3SS translocation domain, the glucose-6-phosphate binding domain, and the...
UDP-glucose binding domain) (Fig. 5A) were cloned in a plant expression vector and used for transient expression assays. A necrotic response is observed only when the RipTPS C-terminal domain is present in the agroinfiltrated protein (Fig. 5D, a, h, and k). We can thus hypothesize that the *N. tabacum* immune system recognizes the C-terminal domain of RipTPS, containing the UDP-glucose binding domain. It is notable that, although the detection of the expressed proteins again proved difficult, the detection of agroinfiltrated recombinant proteins that failed to trigger necrosis was possible (Fig. 5E, see the small dots next to the HA-positive bands from infiltrations i and j). The expression of the different versions of recombinant RipTPS was confirmed by agroinfiltration in *N. benthamiana* leaves (see Fig. S3 in the supplemental material).

**DISCUSSION**

Trehalose is a nonreducing disaccharide found in many organisms, including eubacteria, fungi, insects, and plants. Although different biosynthetic routes exist in bacteria, a general pathway involving the successive action of TPS and TPP enzymes is conserved in fungi, plants, and many bacteria (35). Phylogenetic analyses have shown that there is a close evolutionary relationship between the TPS and TPP modules in these diverse organisms; in bacteria, the corresponding *otsA* and *otsB* genes are clustered and are generally transcribed from a single promoter (35). *ripTPS*, while being conserved in taxonomically distant *R. solanacearum* strains (4), is never associated with a TPP gene module. Instead, the neighboring gene to *ripTPS* is *ripAV*, which encodes a T3E of unknown function (4). The presence of an N-terminal T3SS secretion domain is also unique among bacterial TPS enzymes. For RipTPS, this N-terminal extension is a functional T3SS signal, and its presence in TPS enzymes of other plant-pathogenic bacteria makes them good candidates for also being secreted effectors. This unusual modular architecture of a T3SS-dependent TPS enzyme suggests that this effector evolved from a shuffling process that Stavrinides and colleagues have called “terminal reassortment” (36). This evolutionary mechanism has been described for several bacterial effector proteins in which the termini of the effector reassort with other genetic information to create new chimeric proteins with added secretion function.

There are increasing numbers of reports in the literature indicating that trehalose metabolism emerges in the establishment of virulence traits in distantly related microbial species (37). Either in prokaryotes or in several fungal species, trehalose-associated mechanisms have been shown to contribute to cell morphology, cell wall integrity, regulation of metabolism, and evasion of the host immune response (32, 37, 38). For example, a recent study...
has shown that the opportunistic pathogen *Pseudomonas aerugi-
nosa* has repurposed a specific trehalose biosynthesis pathway as a
virulence factor to increase its replication rate in the intercellular
leaf environment (39). However, there is not just one way to tam-
per with this signaling in host plants, as several distinct trehalose-
associated mechanisms operate to promote pathogenesis in species-specific manners (37).

This work provides evidence that RipTPS displays trehalose-6-
phosphate synthase activity and is translocated into plant cells by
the T3SS. Although Tre6P is present in trace abundance in plant
cells, its content is tightly regulated and not merely restricted to
trehalose synthesis (34). Several studies in recent years have
indeed unraveled the role of Tre6P as a key signal molecule that
regulates carbon assimilation and the sugar status in plants (34,
40–43). It is therefore tempting to speculate that the pathogen
directly induces Tre6P synthesis during the course of infection to
subvert plant cell metabolism through modulation of the endog-

phenomenon. Our results showed that this phenotype is independent of Tre6P
expression by additional T3E(s) in the wild-type GMI1000 strain.

avrA

is a specific target of the T3SS. Although Tre6P is present in trace abundance in plant
cells, its content is tightly regulated and not merely restricted to
the production of Tre6P in plant cells further highlights the impor-
tance of this metabolite in the molecular physiology of plants.

**MATERIALS AND METHODS**

Bacterial strains, yeast strains, plasmids, growth conditions, and plant
material. The bacterial and yeast strains and plasmids used for this study are
described in Table S1 in the supplemental material. *Escherichia coli*
cells were grown in Luria-Bertani medium at 37°C with 100 μg/ml ampi-
cillin or 15 μg/ml gentamycin. *Agrobacterium tumefaciens* strain GV3101::
PMP90RK cells were grown in YEB medium (5 g/liter beef extract, 1 g/liter
yeast extract, 5 g/liter peptone, 5 g/liter sucrose, 2 mM MgSO$_4$, pH 7.2) at
28°C with 100 μg/ml rifampicin, 50 μg/ml kanamycin, 15 μg/ml gentami-
cin, and 75 μg/ml carbencillin. Agar was added at 1.5% (wt/vol) for solid
medium. *R. solanacearum* strains GMI1000, GMI1694 (hrcV, a T3SS
mutant derivative), and GRS432 (ripTPS mutant) were grown in complete
medium B or minimal medium (MM) at 28°C with or without 40 μg/ml
spectinomycin (54). For liquid cultures, spectinomycin was reduced by
half. The *S. cerevisiae* tp1 tp2 mutant strain YSH652 (*MAT* leu2-3,112
ura3-1 trpl-1 his3-11,15 ade2-1 can1-100 tsp1::TRP1 tsp2::LEU2 GAL
SUC) and the control strain W303-1A (*MAT* leu2-3,112 ura3-1 trpl-1
his3-11,15 ade2-1 can1-100 GAL SUC) were grown at 30°C in SD-LWU
and SD medium (both from Clontech), respectively, supplemented with
galactose unless otherwise stated. The plants used in this study were
*Nicotiana tabacum* cv. Bottom special, *Nicotiana benthamiana*, *Solanum
melongena* cv. Zebrina), *tomato* (*Solanum lycopersicum* cv. Mar-
mande), and *geranium* (*Geranium sanguineum* var. Maverick Ecarlate),
grown in the greenhouse, and *Arabidopsis thaliana* grown in a growth
chamber at 22°C with a 9-h day length.

**DNA manipulations and genetic constructs.** Constructs were created
using the Gateway technology as recommended by the manufacturer (Life
Technologies, Carlsbad, CA). *ripTPS* was cloned from the genomic DNA of
*R. solanacearum* GMI1000 in pDONR207, creating pMP12 (with a stop
codon) and pMP15 (without a stop codon). Amplifications were
performed in two steps, using the cloning primers (731GW-ATG and
731GW-STOP or 731GW-NOSTOP) in the first PCR and then universal
primers (attB1 and attB2) in the second PCR, with Platinum Pfx (Life
Technologies), *Pfu* Ultra (*StrataGen*, Kirkland, WA), or Phusion (NEB,
Ipswich, MA). The primers used for the amplifications are provided in
Table S1 in the supplemental material. The three mutant alleles of *ripTPS*
were generated using the QuikChange XL site-directed mutagenesis kit (Agilent) on the pMP15 plasmid, creating plasmids pMP90 (Y154V), pMP91 (W163S), and pMP92 (D208G), respectively. Expression plasmids (pMP72, pMP93, pMP94, and pMP95) were created by LR Gateway cloning using pAM-PAT-P35S-GW-3xHA (L. D. Noel and J. E. Parker, unpublished data) and used to transform Agrobacterium tumefaciens GV3101::pMP90RK. The different constructs derived from RipTPS were amplified by PCR (RipTPS short-553, RipTPS short-346, and RipTPS short-653) and used to transform E. coli on the pMP15 plasmid, creating plasmids pMP90 (Y154V), pMP91 (W163S), and pMP92 (D208G). The resulting plasmids were used to transform A. tumefaciens. The expression levels of GUS (courtesy of L. Deslandes), OtsA (pMP74), and AvaR (pMP99) (18) were used as controls for the A. tumefaciens assay. OtsA from E. coli was amplified using primers otsA-fwd and otsA-rev (Table S1).

**Construction of an ripTPS mutant strain.** RipTPS was disrupted by the insertion of an Ω cassette conferring resistance to spectinomycin as previously described (55). A 2.2-kb DNA fragment comprising the ripTPS coding sequence was cloned after PCR amplification using primers GRS432-fwd and GRS432-Rev. The Ω cassette was inserted into the unique EcoRI restriction site within the coding sequence, resulting in plasmid pMP2. This construct was linearized by Ncol and introduced into R. solanacearum GMI1000 by natural transformation. The mutant strain GRS432 generated after selection for a single recombination event using the pDONR207 vector (pMP71, pMP35, pACC758, and pMP87) and then in pAM-PAT-P35S-GW-3xHA (pMP73, pACC678, pACC766, and pMP89), and the resulting plasmids were used to transform A. tumefaciens. The expression levels of GUS (courtesy of L. Deslandes), OtsA (pMP74), and AvaR (pMP99) (18) were used as controls for the A. tumefaciens assay. OtsA from E. coli was amplified using primers otsA-fwd and otsA-rev (Table S1).

**Adenylate cyclase assay.** A DNA fragment comprising 509 bp upstream from the ripTPS start codon and the region encoding the first 83 amino acids was PCR amplified (primers BglII735 and HindIII1753) (see Table S1 in the supplemental material) and cloned into the pSC266 vector (6) HindIII-BglII restriction sites to create a translational fusion with the cyaA gene. The resulting plasmid, named pGG7, was introduced into the GM1100 and GMI11694 strains by natural transformation to select for a single recombination event, leading to genomic integration. Natural transformation of R. solanacearum strains was performed after growth in minimal medium supplemented with 2% glycerol as described previously (54).

The adenylate cyclase assay was performed as previously described (6). Briefly, strain GM1100 and derivatives carrying plasmid pGG7 were infiltrated into Nicotiana tabacum leaves at an optical density (OD) of 1. Plants were sampled 7 h later with a cork borer. Leaf disks were transferred into Eppendorf tubes and immediately frozen in liquid nitrogen. For cyclic AMP (cAMP) extraction, samples in Eppendorf tubes were kept frozen while grinding by shaking with 2-mm tungsten beads in a Qiagen MM300 mixer mill for two runs of 2 min at 30 Hz. Samples were stored at −80°C before cAMP quantification. Protein concentration was assessed by Bio-Rad protein assay kit. Cyclic AMP levels were monitored with a cAMP enzyme immunoassay kit (Biokraft; Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

**Complementation of the tps1 tps2 yeast mutant.** To express ripTPS and its derivatives in yeast, the Gateway-compatible yeast episomic vector pVTU102-GW containing the yeast alcohol dehydrogenase (ADH1) gene promoter (56) was used. The full-length ripTPS gene was cloned, resulting in the yeast expression plasmid named pMP31, which was used to transform the tps1 tps2 yeast mutant strain (31). Similarly, the catalytic mutants of ripTPS were cloned in pVTU102-GW (pMP96, pMP97, and pMP98). To create the HA-tagged versions of these vectors, a PCR fragment obtained from pMP72 amplification with primers TPSHA-fwd and TPSHA-rev (see Table S1 in the supplemental material) was inserted into the Xhol-HindIII site. The resulting plasmids (pMP104, pMP105, pMP106, and pMP107) were used to transform the tps1 tps2 yeast mutant strain. As a positive control, the full-length otsA gene from E. coli was cloned in the pVTU102-GW vector with an HA tag in the Sall-HindIII site (pMP108), using primers otsAHA-fwd and otsAHA-rev (Table S1).

The yeast strains were grown on SD-LWU containing 2% galactose and 40 mg/liter adenine. For the fructose growth assay, 10-fold dilution series from exponential growing cells were spotted onto plates containing either fructose or galactose.

To measure the production of Tre6P, the extraction of yeast metabolites following a glucose pulse was carried out as described previously (31). The measured production of Tre6P (mM Tre6P/OD unit) was normalized by attributing a value of 100 to the sample with maximal value. All the samples were analyzed five times, generating independent biologically replicates. To analyze the differences between strains, we used the non-parametric Wilcoxon matched-pairs signed-rank test with a one-tailed P value. This was performed using the GraphPad Prism 5.0 software package.

**Pathogenicity assays.** Arabidopsis (Col-0) plants were grown for 4 to 5 weeks on Jiffy plugs (Jiffy Products International BV, Netherlands). Geranium (Geranium sanguineum var. Maverick Ecarlate), tomato (cv. Marmande), and eggplant (cv. Zebrina) were grown in greenhouse facilities in potting mixture. For all plant inoculations, the roots were soaked with a bacterial suspension at 107 CFU/ml of GM1100 or GRS432 (the ripTPS mutant). Solanaceae were then placed at 28°C (light) and 27°C (night) with a 12-h photoperiod. For Arabidopsis, the temperatures were 27°C (light) and 26°C (night). The progression of the disease was reported by scoring the plants on a scale from 1 to 4 based on 25% to 100% of leaves being wilted; to perform the survival analysis (see Fig. S2 in the supplemental material), all scores equal or higher than 2 (50% wilted leaves) were set as 1 (or dead) when the lower scores were 0 (or alive). The experiments were repeated at least 3 times independently. The statistical analysis was performed as described previously (21).

**Agrobacterium-mediated expression of proteins in plant cells.** A. tumefaciens strains carrying plasmid-borne ripTPS constructs were grown overnight in YEB medium. Cells were pelleted at 4,000 rpm, resuspended in infiltration medium (10 mM MgCl2, 10 mM morpholine ethanesulfonic acid, and 150 μM acetosyringone), and incubated for 2 h at room temperature. Resuspended cells were infiltrated into leaves of 4-week-old Nicotiana spp. plants at an optical density at 600 nm (OD600) of 0.6 with a 1-ml needleless syringe. The infiltrated plants were incubated in growth chambers for 16 h at 20°C. After infiltration (48 to 72 h), leaf disks taken in infiltrated areas were ground with a mixer mill (MM400; Retsch, Haan, Germany) and homogenized in Laemmli buffer in order to be analyzed by Western blotting to control for the expression levels. Rat anti-HA antibody (1:5,000) (number 11867423001; Roche) was incubated overnight in Tris-buffered saline (TBS)—0.1% Tween with 1% bovine serum albumin (BSA), and then goat anti-rat antibody coupled to alkaline phosphatase (1:10,000) (A8438; Sigma) was added after three washes of 10 min each in TBS-Tween, and the mixture was incubated for 1 h. Incubation was followed by two washes in TBS-Tween and then nitroblue tetrazolium (NBT)-BCIP (5-bromo-4-chloro-3-indolylphosphate)-based staining. The expression of the RipTPS variants was followed in N. benthamiana in parallel to the expression in N. tabacum because the low expression level in N. tabacum was limiting for Western blot analysis. After SDS-PAGE electrophoresis, Coomassie staining was performed to check for equal protein loading (57).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl/doi:10.1128/mBio.02065-14/-/DCSupplemental.

Figure S1, EPS file, 0.8 MB.
Figure S2, EPS file, 0.2 MB.
Figure S3, EPS file, 0.04 MB.
Table S1, XLSX file, 0.02 MB.
Figure S1, EPS file, 0.8 MB.
Figure S2, EPS file, 0.2 MB.
Figure S3, EPS file, 0.04 MB.
Table S1, XLSX file, 0.02 MB.

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