***Streptomyces turgidiscabies* Possesses a Functional Cytokinin Biosynthetic Pathway and Produces Leafy Galls**

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***Streptomyces turgidiscabies*, a cause of potato scab, possesses a mobilizable pathogenicity island containing multiple virulence genes and a cytokinin biosynthetic pathway. These biosynthetic genes are homologous and collinear with the *fas* operon in *Rhodococcus fascians*. Reverse-transcriptase polymerase chain reaction of *S. turgidiscabies* demonstrated that all six genes were transcribed in oat bran broth with and without glucose, though transcription was partially repressed by glucose. The supernatant of *S. turgidiscabies* cultures had cytokinin activity in callus initiation and differentiation assays. *Arabidopsis* and tobacco plants inoculated with a thaxtomin-deficient mutant (Δnos) produced leafy galls, indistinguishable from those produced by *R. fascians*. Deletion of the *ipt* gene in the pathway eliminated gall phenotype. Other symptoms on tobacco included production of hairy roots and de novo meristems.**

Additional keywords: cytokinin biosynthesis.

Of the hundreds of *Streptomyces* spp. that have been described, relatively few cause plant disease. In fact, this genus is best known for its ability to produce valuable secondary metabolites, including antibiotic, anti-tumor, and nematicidal compounds. Because these filamentous, gram-positive bacteria have a complex life cycle, members of this genus are genetic models for prokaryote development. Potato scab is the most economically important plant disease caused by *Streptomyces* spp., though species that cause this disease are neither host nor tissue specific (Loria et al. 1997). Of the species known to cause potato scab, *Streptomyces scabies* (syn. *S. scabiei*) was the first described and appears to be the most ancient of those characterized (Lambert and Loria 1989). It has a global distribution, presumably due to vegetative propagation of the potato crop, and has been the subject of most of the disease control research over the past 100 years. However, new pathogenic species repeatedly have emerged in agricultural systems and this phenomenon has led to the hypothesis that the genetic determinants for plant pathogenesis are being shared among *Streptomyces* spp. that are adapted to specific ecological niches. This hypothesis is supported by data showing conservation of virulence genes and associated insertion elements (IS) in multiple strains of phylogenetically diverse pathogenic species (Bukhalid et al. 1998; Healy et al. 1999, 2000).

S. turgidiscabies emerged as a virulent pathogen in Japan within the last 50 years (Miyajima et al. 1998; Takeuchi et al. 1996). This species was characterized based on isolates col-

lected from potato scab lesions with a distinctly erumpent phenotype. The 21 isolates used for characterization were almost identical based on morphological and physiological characteristics, suggesting that the pathogenic strains in the *S. turgidiscabies* population are clonal. Genetic analysis confirmed the homogeneity of the population. Population studies showed that this new, clonal, pathogenic species occurred in the eastern portion of the island of Hokkaido, whereas a genetically diverse population of *S. scabies* occurred on the western portion of the island (Takeuchi et al. 1996). These data suggested that *S. turgidiscabies* might have emerged as the result of horizontal transfer of virulence genes from the more ancient species *S. scabies*.

Discovery of a mobile pathogenicity island (PAI) in the emergent pathogen *S. turgidiscabies* demonstrated that PAIs exist in gram-positive plant pathogens and provided a likely mechanism for the emergence of new pathogenic species in the genus *Streptomyces* (Kers et al. 2005). Multiple virulence genes reside on a contiguous 660-kb mobilizable fragment in *S. turgidiscabies* (Kers et al. 2005). Virulence genes on this fragment are conserved in *S. acidiscabies* and *S. scabies*, including the *nec1* gene and the biosynthetic pathway for the nitrated dipeptide phytotoxin, thaxtomin (Bukhalid et al. 1998; Healy and Lambert 1991; Healy et al. 2000; Kers et al. 2004; King et al. 1989). These two loci lie approximately 325 kb apart and the mobilized fragment has open reading frames (ORFs) with variable G+C content and representatives of multiple IS families (Kers et al. 2005).

Interestingly, a plant fasciation (*fas*) operon, homologous to the six-gene *fas* operon in *Rhodococcus fascians*, lies adjacent to the thaxtomin biosynthetic pathway in *S. turgidiscabies* (Kers et al. 2005). The *fas* operon is absent from *S. scabies* and *S. acidiscabies*, suggesting that this operon was introduced to the *S. turgidiscabies* PAI after it was acquired from *S. scabies*. Five of the six *fas* genes are collinear in these two pathogens; *fas6* appears to have undergone a rearrangement on the *S. turgidiscabies* chromosome. *R. fascians* is a gall-producing, gram-positive, plant-pathogenic bacterium. Though primarily studied as a model system for molecular plant-microbe interactions (Goethals et al. 2001), *R. fascians* has emerged as a pathogen on ornamental plants in the United States (M. Putnam, *personal communication*). Leafy galls that develop on stems and shoots of monocot and dicot stock plants following propagation can result in total loss of affected cultivars. The structure of the cytokinins produced by *R. fascians* has not been determined, but the *fas* operon is required for pathogenicity (Eason et al. 1996).

Cytokinin biosynthesis is a virulence determinant in other gall-producing bacteria, such as *Erwinia herbicola* pv. *gypsophylae* (Lichter et al. 1995), *Pseudomonas syringae* pv. *sa-*

vastanoi (Macdonald et al. 1986), and *Agrobacterium tumefaciens* (Sakakibara et al. 2005). Like *R. fascians*, these bacteria possess genes that encode an isopentenyltransferase (*ipt*), an enzyme, which catalyzes the first dedicated step in cytokinin biosynthesis. However, none possess the other genes in the *fas* operon. In fact, until the *fas* operon was discovered in *S. turgidiscabies* (Kers et al. 2005), it was not known to exist outside of *R. fascians*. Here, we provide data that demonstrate expression of the *fas* genes and *fas*-dependent cytokinin production in *S. turgidiscabies*. We also demonstrate that *S. turgidiscabies* produces leafy galls on tobacco and *Arabidopsis* and de novo meristem production on tobacco, in a manner indistinguishable from *R. fascians* (de O. Manes et al. 2001, 2004; Vereecke et al. 2000).

RESULTS

Bioinformatic analysis of the *fas* operon in *S. turgidiscabies*.

Five of the six *S. turgidiscabies* genes, *fas1* to 5, are colinear with the six-gene *fas* operon of *R. fascians* (Fig. 1). These genes overlap or are separated by small intergenic regions, suggesting that they constitute an operon, as they do in *R. fascians*. Upstream of *fas1* in *S. turgidiscabies*, we discovered two methyltransferase genes (*mtr1* and *mtr2*). A role for these genes in cytokinin biosynthesis has not been demonstrated in *R. fascians*. A transmembrane transporter homolog, which is not conserved in *R. fascians*, and *fas6* lie directly upstream of *mtr2*. Interestingly, the *fas* region is bordered by two IS110 family transposable elements in *S. turgidiscabies*.

The six *S. turgidiscabies* *fas* genes are highly similar (67 to 84%) to their cognate *R. fascians* homologs (Table 1). The *fas1*

gene is homologous to P450 cytochromes (Table 1), which play a critical role in biosynthetic and biodegradative reactions in actinomycetes. Both the *R. fascians* *fas1* (Crespi et al. 1994) and the *S. turgidiscabies* *fas1* contain the heme-binding loop and conserved cystine residue typical of these enzymes. The *fas2* gene encodes a ferredoxin-pyruvate decarboxylase α subunit homolog and *fas3* encodes a pyruvate decarboxylase β subunit homolog. These proteins are likely to be responsible for the oxidation of pyruvate as demonstrated in *R. fascians* (Goethals et. al. 2001). The *fas4* gene encodes an isopentenyl transferase (*ipt*) that catalyzes the first dedicated step in cytokinin biosynthesis. An amino acid sequence alignment revealed that *S. turgidiscabies* *ipt* shares the consensus pattern GxTxGK [ST]xxxxx [VLI]xxxxxxx[VLI][VLI]xxDxxQx{57,60}[VLI][VII]xGG[ST] predicted by Kakimoto (2001) for all kind of isopentenyltransferases. In this consensus pattern, “x” denotes any amino acid residue, “[]” any one of the amino acid residues in these brackets, and “{m, n}” m to n amino acid residues. The ATP/GTP binding motif GxxxxGK[TS] is conserved at the amino terminal region of the *Streptomyces* *ipt* (Fig. 2). The *fas5* gene is homologous to cytokinin oxidases in plants, including *Arabidopsis thaliana*, that are involved in cytokinin degradation. The *fas6* gene encodes a lysine decarboxylase homolog and contains the conserved motif (PGGXGTXXE) found in this protein family (Jeon et al. 2006). This gene functions as a virulence determinant in *R. fascians* (Goethals et al. 2001).

The *S. turgidiscabies* *fas* genes are expressed and *ipt* encodes a functional protein.

To determine whether the *fas* operon is transcribed in *S. turgidiscabies*, we monitored expression of the all of the *fas* genes using reverse-transcriptase polymerase chain reaction

Fig. 1. Genetic organization of the *fas* loci in *Streptomyces turgidiscabies* (St) and *Rhodococcus fascians* (Rf).

Table 1. List of genes present in Fas operon, length, G+C content, similarity and identity to *Rhodococcus fascians*

Gene	Length (amino acid)	G+C (%)	Identity (%)	Similarity (%)
<i>fas 1</i> : Cytochrome P450	400	69	65	77
<i>fas 2</i> : Ferredoxin-pyruvate decarboxylase α	295	66	61	71
<i>fas 3</i> : Pyruvate decarboxylase β	310	66	72	84
<i>fas 4</i> : Isopentenyltransferase (cytokinin synthesis)	257	63	49	68
<i>fas 5</i> : Cytokinin oxidase	445	66	54	67
<i>fas 6</i> : Lysine decarboxylase	207	67	65	76
<i>mtr1</i> : Methyltransferase 1	284	65	...	...
<i>mtr2</i> : Methyltransferase 2	280	66	...	...
Transmembrane transporter	489	69	...	...
<i>araC</i> : AraC type transcriptional regulator	212	56	31	53

(RT-PCR). *S. turgidiscabies* was grown in oat bran broth (OBB) and OBB amended with 10% glucose; OBB supports production of the phytotoxin thaxtomin, whereas the addition of glucose represses it. Expression of all of the *fas* genes (*fas1* to 6) and two methyl transferase genes (*mtr1* and 2) was detected in both media (Fig. 3). However, the transcript level was lower in glucose-supplemented OBB than in OBB.

Transcriptional analysis suggested that the *fas* operon is functional in *S. turgidiscabies*. To confirm this prediction, we evaluated the ability of the *S. turgidiscabies* IPT protein to complement the osmosensor *sln1* mutant (TM182-CRE) in *Saccharomyces cerevisiae* (Inoue et al. 2001). The SLN1 deleted strain, which carries PTP2-gene under the control of Gal-inducible promoter and cytokinin receptor CRE1, is conditionally lethal. Viability depends on the presence of galactose or cytokinin in the growth medium (Haberer and Kieber 2002). The *Streptomyces turgidiscabies ipt* was cloned into the high-copy yeast expression vector p423TEF (Mumberg et al. 1995) and transferred to TM182-CRE. Transformed yeast (TM182-CRE/IPT) colonies were able to grow on SD medium (SD-URA-LEU-HIS) without galactose or cytokinin (Fig. 4). The yeast transformed with the empty vector (TM182-CRE/p423TEF) was able to grow only on SD+Gal-URA-LEU-HIS medium (with galactose), confirming the functionality of the *S. turgidiscabies* IPT, whereas original yeast strain TM182-CRE was unable to grow without histidine in the media.

***S. turgidiscabies* produces a cytokinin.**

Because the structure of the cytokinin produced by *R. fascians* is not known, we determined whether *S. turgidiscabies* culture supernatants have cytokinin activity using a callus initiation bioassay. Root segments of the *Arabidopsis* (ecotype Landsberg) wild type (WT) and the cytokinin-sensitive mutants *ckh1* and *ckh2* (Kubo and Kakimoto 2000) were grown on tissue culture medium supplemented with kinetin or *S. turgidiscabies* culture supernatants. The synthetic cytokinin, ki-

Fig. 3. Expression of the *fas* genes (*fas1*–6) and the methyltransferase genes (*mtr1*–2), based on reverse-transcriptase polymerase chain reaction analysis. The cDNA were synthesized from RNA extracted from *Streptomyces turgidiscabies* grown in oat bran broth medium with (+) or without (–) glucose. Expression of *murX* was monitored as a control for cDNA transcription and RNA quality.

Fig. 4. Complementation of the osmosensor *Sln1* yeast mutant (TM182-CRE) by the *Streptomyces turgidiscabies ipt* gene. **A** and **B**, Independent transformant (TM182-CRE/IPT) yeast clones carrying *ipt* gene growing on SD-URA-LEU-HIS medium with or without galactose. **C**, Original yeast strain TM182-CRE was unable to grow without histidine in the media. **D**, Yeast clone carrying empty vector p423TEF grows in absence of histidine but only in presence of galactose (plate on left).

Fig. 2. Alignment of the amino acid sequence of Fas4 (IPT) from *Streptomyces turgidiscabies* with the IPT sequences in *Arabidopsis thaliana*, *Rhodococcus fascians*, and *Agrobacterium tumefaciens*. A consensus sequence for IPT is provided and the conserved ATP/GTP binding motif GxxxxGK[TS] is boxed.

netin, induced green calli without formation of root primordial structures in the WT *Arabidopsis*, as did the cytokinin-sensitive mutants at lower kinetin concentrations. Tissue grown on media supplemented with *Streptomyces* culture supernatant (50 μ l/ml) produced rapidly proliferating green calli (Fig. 5). Response of the plant tissue to the supernatant was very similar to kinetin at 100 ng/ml. The mutants *ckh1* and *ckh2* were more responsive to Kinetin treatments than the WT, as expected. Likewise, these cytokinin mutants responded more strongly to the supernatant from *S. turgidiscabies* cultures. Although root primordia-like structures were produced from callus grown in the presence of kinetin, they were not produced in calli grown in the presence of culture supernatant (Fig. 5). Calli produced from WT *Arabidopsis* initiated organogenesis when cultured on *S. turgidiscabies* supernatant.

S. turgidiscabies infection causes leafy galls and induces de novo meristems.

Streptomyces spp. that cause potato scab have never been reported to produce leaf galls or similar symptoms. However, the functionality of the *fas* genes in *S. turgidiscabies* suggested that this scab pathogen might have the ability to produce symptoms similar to those produced by *R. fascians*. An *S. turgidiscabies* *Anos* mutant, which is deficient in thaxtomin production (Kers et al. 2004), was used in plant bioassays to avoid the necrogenic affects of this toxin. Log-phase cultures grown in OBB were used as inoculum. Symptoms produced on plants varied greatly, depending on the plant species, the site of inoculation, and the age of the plant. Infection of germinating tobacco seedlings resulted in hairy, bushy roots with little growth inhibition (Fig. 6A). However, when 2-week-old plants of tobacco were inoculated, multiple buds formed in the shoot meristem region, where bacteria growth was visible (Fig. 6B). Inoculation of the axillary meristems of 3-week-old tobacco resulted in the formation of calli or multiple buds (Fig. 6C) at the infected leaf axils within 7 to 10 days. By 4 weeks, these calli developed into gall-like structures with small, thick green

leaves (Fig. 6D). Growth of the pathogen was visible in the leaf axils and plant growth was greatly reduced relative to non-inoculated plants. When tobacco plants were decapitated to suppress apical dominance, before inoculating the leaf axils, profuse bud proliferation occurred at the site of inoculation (Fig. 6E). Multiple bud formation occurred at inoculated apical meristems with or without decapitation. De novo meristem formation and shoot regeneration occurred on inoculated leaf petioles and stems (Fig. 6F). An *S. turgidiscabies* *Anos* Δ *ipt* mutant did not produce galls or cause shoot regeneration or de novo meristem production (Fig. 6G). A parallel infection study was carried out with *A. thaliana* (ecotype Columbia); leafy galls were formed at the shoot meristem and both shoot and root growth was greatly reduced relative to noninoculated plants (Fig. 7A and B). Interestingly, infected plants produced larger and more numerous trichomes than did control plants (Fig. 7C and D).

DISCUSSION

Based on amino acid similarity, G+C content, and gene organization, it is clear that the *fas* genes in *R. fascians* and *S. turgidiscabies* have a common ancestor (Fig. 1; Table 1). The *fas* genes lie on a mobilizable PAI in *S. turgidiscabies* (Kers et al. 2005). This PAI is conserved in *S. scabies* and *S. acidiscabies* but it lacks the *fas* operon in these two species. It seems likely that the *fas* operon was incorporated into the PAI after it was mobilized into *S. turgidiscabies*. The *fas* genes are bordered by IS110 family transposable elements in *S. turgidiscabies*, suggesting that mobilization may have occurred via a transposition event (Fig. 1). The *fas* operon resides on a linear plasmid in *R. fascians* and on a mobilizable PAI in *S. turgidiscabies*, in both cases providing opportunities for mobilization into other bacteria. Interestingly, a gall-producing *Streptomyces* sp. emerged as an economically important pathogen of cucurbits in Japan within the last 25 years (Yoshida and Kobayashi 1991) and in Costa Rica in the last 10 years (data not presented). Analysis of this emergent pathogen for the presence of the *fas* operon on a PAI would be a logical next step in understanding the evolution of pathogenicity in the genus *Streptomyces*.

The *fas* operon has not been described from bacteria other than *R. fascians* and *S. turgidiscabies*. The existence of this operon in gram-positive bacteria but not in gall-producing gram-negative bacteria, such as *E. herbicola* pv. *gypsophila*, *Pseudomonas syringae* pv. *savastanoi*, and *Agrobacterium tumefaciens* is a matter for speculation. Interestingly, the *ipt* genes in these bacteria are located on plasmids, suggesting that the genes required to synthesize cytokinins are mobilized frequently. In fact, cytokinin biosynthesis appears to be a trait that is relatively common in plant-associated bacteria. For example, there is evidence for cytokinin production by *Frankia* sp. (Stevens and Berry 1988), *Bacillus subtilis* (Arkhipova et al. 2005), and *Azotobacter vinelandii* (Taller and Wong 1989), indicating that cytokinins can confer a fitness advantage in plant associations. None of these bacteria produce galls, though *Frankia* and *Azotobacter* spp. produce nodules.

Transcription of all six of the *S. turgidiscabies* *fas* genes occurred in OBB and OBB amended with glucose but not in CRM (Pigac and Schrempf 1995), Terrific Broth, or Luria Broth (data not provided). Plant components in OBB are required for transcription of the *fas* locus, but transcription is not completely repressed in the presence of glucose (Fig. 3). Thaxtomin production also occurs in OBB but is repressed by glucose. In contrast, the production of cytokinin via the *fas* operon is tightly controlled in *R. fascians* and requires induction by extracts of infected plant tissue; no induction occurs with healthy plant tissue (Temmerman et al. 2000). The *att* locus,

Fig. 5. Callus initiation from *Arabidopsis* root segments. Root segments of wild-type Landsberg (WT) and two cytokinin-sensitive mutants, *ckh1* and *ckh2*, were cultured on Murashige and Skoog agar +2,4-D (200 mg/liter) media containing kinetin (0, 25, or 100 mg/liter) or *Streptomyces turgidiscabies* (St) culture supernatant (50 μ l/ml). Organogenesis induced in WT callus is indicated by an arrow.

which is in the vicinity of the *fas* operon on the *R. fascians* plasmid pFiD188, is essential for the formation of these inducing factors (Maes et al. 2001). An AraC-type regulatory gene, *fasR*, regulates *fas* gene expression in *R. fascians*. Deletion mutants of *fasR* are blocked for *fas* gene transcription, cytokinin production, and virulence. There is an AraC family regulator on the *S. turgidiscabies* PAI upstream of the *fas* genes and in the vicinity of the thaxtomin biosynthetic genes. Unlike the *fas* and *mtr* genes, this gene is conserved in *S. scabies* and *S. acidiscabies*. It is possible that this AraC is involved in regulation of the *fas* genes in *S. turgidiscabies*, however, it appears to have been present on the PAI prior to the introgression of the *fas* operon.

S. turgidiscabies produces all of the cytokinin-dependent symptoms produced by *R. fascians*, including leafy galls on

tobacco and *Arabidopsis* that are indistinguishable from those produced by *R. fascians* (de O. Manes et al. 2004; Goethals et al. 2001). Leafy gall production has been studied extensively in *R. fascians*. Dormant axillary meristems become active and generate shoots, but growth of these shoots is inhibited (Vereecke et al. 2000). At the same time, cortical cells in stems and petioles divide to form new shoot primordia (de O. Manes et al. 2001) and cells neighboring the vascular tissue in leaves give rise to de novo formed shoots (Vereecke et al. 2000). The repetition of these processes ultimately leads to the formation of a dense gall of meristematic tissue. No other gall-forming bacteria produce leafy galls, suggesting that the properties or metabolism of the cytokinins produced via the *fas* operon may be different from those produced *R. fascians*. The fact that *S. turgidiscabies* also produces leafy galls is consistent with this

Fig. 6. Symptoms of tobacco infected by the *fas* operon containing *Streptomyces turgidiscabies* *Anos* mutant. **A**, Tobacco seedlings infected with *S. turgidiscabies* (*Anos*) (St) and noninoculated plants (control). **B**, Multiple buds at the point of infection in the shoot meristem region. **C**, Leafy gall formation at infected leaf axils. **D**, Initiation of calli, bud proliferation, and shoot regeneration for over 4 weeks, ultimately resulting in a gall with small, thick green leaves at the point of inoculation, and noninoculated plant (control). **E**, Multiple buds at inoculated apical meristem. **F**, De novo meristem formation on inoculated leaf petioles and stems. **G**, Phenotype of *S. turgidiscabies* *Anos* (St) and *S. turgidiscabies* *Anos* *Δipt* (St/IPT) mutants, and noninoculated plant (control).

hypothesis. We noticed another plant phenotype that is characteristic of excess cytokinin production; trichomes were abnormally large and numerous on *Arabidopsis* plants infected with *S. turgidiscabies* (Greenboim-Wainberg et al. 2005).

In plants, cytokinin works with auxin to regulate many developmental processes, including vascular differentiation, lateral root initiation, shoot and root apical dominance and root gravitropism, and de novo shoot formation (Aloni et al. 2006; Mahonen et al. 2006). Gall production requires both auxin and cytokinin, and gall-producing gram-negative bacteria produce both hormones (Jameson et al. 2000). *S. scabies* produces indole 3-acetic acid (IAA) (Manulis et al. 1994), though the genetics and regulation of biosynthesis are not known; however, auxin production by *S. turgidiscabies* has not been evaluated. Production of IAA by *R. fascians* recently was documented. Interestingly, production is highly induced by infected-plant extracts (Vandeputte et al. 2005).

Because *S. turgidiscabies* has never been reported to produce galls on plants in nature, what effects might cytokinin biosynthesis have on the interaction of *S. turgidiscabies* and potato? This species originally was isolated from scab lesions on potato. In the original description of *S. turgidiscabies*, it was noted that this species caused larger, more “turgid” lesions than did

S. scabies. It is tempting to speculate that this large, erumpent lesion is due to cytokinin production. Perhaps reproduction of the pathogen is greater in lesions or pathogen dispersal is more effective in these large, erumpent lesions. An increase in cytokinin levels has been correlated with accumulation of metabolites in fungal host–pathogen interactions (Angra-Sharma and Sharma 1999). Walters and McRoberts propose a mechanism by which cytokinin-induced increases in invertase activity occurring early on in the host–pathogen interaction result in nutrient mobilization toward the infection site in biotrophic interactions (Walters and McRoberts 2006). However, the role of cytokinins in this plant–microbe interaction could be multifaceted because cytokinin can act as an apoptotic inducer in plants (Carimi et al. 2003) and act as a signaling molecule for antibiotic production in *Streptomyces* spp. (Yang et al. 2006).

MATERIALS AND METHODS

Strains, plasmids, and culture conditions.

A Δnos mutant strain of *S. turgidiscabies* Car8 that produces trace quantities (approximately 3.0% of WT) of thaxtomin (Kers et al. 2004, 2005) was used in this study to minimize the necrotic phenotype effect of this phytotoxin in plant infection

Fig. 7. Symptoms of the *fas* operon containing *Streptomyces turgidiscabies* (Δnos) on *Arabidopsis* (ecotype Columbia). **A**, Inoculated (Δnos) and noninoculated (control) plants. **B**, Small, thick, green leaves form a leafy gall at apical meristem on inoculated plant. Growth of *Streptomyces* on plant tissue is indicated by arrow. **C**, Trichome production on inoculated plant. **D**, Uninoculated control plant.

studies. *S. turgidiscabies* strains were grown routinely in the International Streptomyces Project (ISP) medium 2 and ISP medium 4 (BD Biosciences, San Jose, CA, U.S.A.) as described previously (Kers et al. 2004, 2005). Spore suspensions of *S. turgidiscabies* Car8 strains were prepared as described by Kieser and associates (2000). The strains were grown for 2 days in OBB (Goyer et al. 1998) at 28°C. Mycelium was recovered from cultures by spinning at 150 rpm and used in plant virulence assays. DNA was extracted from *Streptomyces* cultures grown in CRM medium (Pigac and Schrepf 1995) for 24 to 48 h at 30°C using a modification of the procedure of Rao and associates (1987). For RNA extraction, 10⁵ spores were inoculated in OBB or OBB + 10% glucose media and grown for 4 days at 26°C. Mycelium growth was not effected by glucose.

Bioinformatic analysis.

Putative ORFs and protein coding regions were identified by database similarity searching, using BLAST (National Center for Biotechnology Information) as described earlier (Kers et al. 2005). These sequence data have been submitted to the GenBank database (accession numbers AY7079 to AY7083). DNA and protein sequences were mapped and aligned using Vector NTI Suite 8.0 (InforMax, Frederick, MD, U.S.A.).

Disruption of the *ipt* gene.

A cosmid library of *S. turgidiscabies* was mutagenized for DNA sequencing as described previously (Kers et al. 2005). A *S. turgidiscabies* cosmid (Cos891) with a Tn5 kanamycin cassette inserted in the *ipt* (*fas4*) gene, 331 bp downstream of start codon, was selected. A 7,835-bp *EcoRV* fragment of Cos891 containing disrupted *fas* operon was ligated into the *EcoRV* site of pOJ260 (*apra*^R). Ligated product was transformed to *Escherichia coli* DH5 α and colonies were selected with apramycin. The pOJ260 containing the disrupted *fas* operon construct then was propagated in *E. coli* ET12567 containing the helper plasmid pUZ8002 and conjugated to *S. turgidiscabies*. Colonies were selected on kanamycin media.

Yeast strains, growth conditions, and complementation assay.

Yeast strain TM182 (MAT α leu2 ura3 his3 *sln1::hisG*+ pSSP25) is deficient in the *SLN1* gene, which encodes an osmosensing histidine kinase; this mutation is lethal in media containing glucose. This *sln1* mutant carrying a plasmid containing the P_{GALI}-*PTP2* promoter fusion gene grows normally in media containing galactose (Maeda et al. 1994). A plasmid (p415CYC-CRE1) carrying a plant cytokinin receptor CRE1 was introduced to the *sln1* mutant (TM182-CRE) and suppressed conditional lethality in the presence of cytokinin in the media (Inoue et al. 2001). This modified strain TM182-CRE can grow in the absence of galactose only when cytokinin is present in the media. For our study, strains TM182 carrying a plasmid p415CYC-CRE1 (TM182-CRE) were kindly supplied by Dr. Tatsuo Kakimoto (Osaka University, Japan). Yeast strains were grown on synthetic dropout (SD) medium (0.67% Bacto-yeast nitrogen base and 0.065% -His/-Leu/-Ura DO supplement) (Clontech, Palo Alto, CA, U.S.A.) supplemented with 2% galactose or glucose for cytokinin assay, where appropriate, with Leu at 30 and His at 20 μ g/ml. The entire coding region of *ipt* was PCR amplified and cloned into the yeast expression vector p423TEF (P_{TEF2} promoter, *HIS3*⁺ marker) at the *Bam*H1 and *Clal* sites, generating p423TEF-IPT (amp^R). After sequence confirmation, plasmids were transformed to the yeast strain TM182-CRE by the lithium acetate method (Ausubel et al. 1998). Complementation of the His auxotrophy was used to select for transformants carrying p423TEF vector and p423TEF-IPT constructs. In galactose-supplemented me-

dia, the yeast transformant carrying the empty vector (TM182-CRE/p423TEF) grows in the absence of histidine, whereas the original yeast strain TM182-CRE was unable to grow without histidine in the media. Yeast clones (TM182-CRE/IPT) carrying the *ipt* gene grow in medium without galactose and without histidine (SD-URA-LEU-HIS).

RNA extraction and RT-PCR.

Streptomyces strains were grown to log phase (4 days) in shaken liquid media at 26°C. Bacterial growth was halted using a stop solution (95:5 ethanol/acid phenol) at a 5:1 culture to stop solution ratio. Samples were mixed thoroughly and centrifuged, and the supernatant was decanted to recover mycelial pellets. RNA was extracted from the mycelium using RNeasy kit (cat. no. 74104) (Qiagen, Valencia, CA, U.S.A.) and was reverse transcribed using the SuperscriptIII first-stand synthesis system kit (Invitrogen, San Diego, CA, U.S.A.). Expression of the genes present in the *fas* locus was confirmed by amplifying the cDNA using gene-specific primers. The *murX* gene, involved in peptidoglycan biosynthesis, was used as a control in these analyses.

Plant bioassays.

For virulence assays, *Arabidopsis thaliana* (ecotype Columbia) and *Nicotiana tabacum* plants were produced in vitro from seed on Murashige and Skoog agar medium (MS) with 2% sucrose (Murashige and Skoog 1962) in Magenta boxes. Plants were grown at 21 $\pm$ 2°C with a 16-h day length and were inoculated with an *S. turgidiscabies* mycelium culture. Bacterial cultures were grown for 2 days in OBB prior to inoculation.

Callus initiation.

A. thaliana plants (ecotype Landsberg) and the cytokinin-sensitive mutants *ckh1* and *ckh2* (Kubo and Kakimoto 2000) were grown for 7 days on MS with 1% sucrose. Root segments were aseptically excised and placed on MS containing 2,4-D (200 mg/liter) and sucrose (2%). This medium was supplemented with kinetin (25 or 100 mg/liter) or *S. turgidiscabies* culture supernatant at 50 μ l/ml; cultures were grown in OBB medium for 4 days before the supernatant was harvested by centrifugation and filter sterilized. Plant tissue cultures were incubated for 21 days at 21 $\pm$ 2°C with a 16-h light and calli size and color were noted.

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