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A conserved mechanism for nitrile metabolism in bacteria and plants

Andrew J. M. Howden, C. Jill Harrison and Gail M. Preston*
Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK

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*For correspondence (fax + 44 1865 275074; e-mail gail.preston@plants.ox.ac.uk).

Summary

***Pseudomonas fluorescens* SBW25 is a plant growth-promoting bacterium that efficiently colonises the leaf surfaces and rhizosphere of a range of plants. Previous studies have identified a putative plant-induced nitrilase gene (*pinA*) in *P. fluorescens* SBW25 that is expressed in the rhizosphere of sugar beet plants. Nitrilase enzymes have been characterised in plants, bacteria and fungi and are thought to be important in detoxification of nitriles, utilisation of nitrogen and synthesis of plant hormones. We reveal that *pinA* is a NIT4-type nitrilase that catalyses the hydrolysis of β-cyano-L-alanine, a nitrile common in the plant environment and an intermediate in the cyanide detoxification pathway in plants. In plants cyanide is converted to β-cyano-L-alanine, which is subsequently detoxified to aspartic acid and ammonia by NIT4. In *P. fluorescens* SBW25 *pinA* is induced in the presence of β-cyano-L-alanine, and the β-cyano-L-alanine precursors cyanide and cysteine. *pinA* allows *P. fluorescens* SBW25 to use β-cyano-L-alanine as a nitrogen source and to tolerate toxic concentrations of this nitrile. In addition, *pinA* is shown to complement a *NIT4* mutation in *Arabidopsis thaliana*, enabling plants to grow in concentrations of β-cyano-L-alanine that would otherwise prove lethal. Interestingly, over-expression of *pinA* in wild-type *A. thaliana* not only resulted in increased growth in high concentrations of β-cyano-L-alanine, but also resulted in increased root elongation in the absence of exogenous β-cyano-L-alanine, demonstrating that β-cyano-L-alanine nitrilase activity can have a significant effect on root physiology and root development.**

Keywords: Nitrilase, β-cyano-L-alanine, *Pseudomonas fluorescens, NIT4,* cyanide.

Introduction

Bacteria belonging to the genus *Pseudomonas* are abundant in the plant environment and are found colonising the surfaces of plant leaves and roots as well as growing endophytically inside plant tissues (Preston, 2004). *Pseudomonas* are metabolically diverse, can tolerate an array of biotic and abiotic stresses and can successfully outcompete other microbes in the plant environment (Hirano and Upper, 2000). However, the mechanisms by which these bacteria achieve competitive fitness remain to a large extent unknown. *In vivo* expression technology (IVET) has been used to identify traits that contribute to the ecological success of the plant growth-promoting rhizobacterium *Pseudomonas fluorescens* SBW25 (*Pf*SBW25), by identifying bacterial genes that are specifically expressed in the plant environment (Gal *et al.*, 2003; Rainey, 1999). *Pf*SBW25 was originally isolated from the surface of a sugar beet leaf (Bailey *et al.*, 1995), but has since been shown to be able to colonise the rhizosphere and leaf surfaces of a wide range of plants, and has been used as a model strain to identify traits involved in environmental competence, plant colonisation and promotion of plant growth (Giddens *et al.*, 2007; Jones *et al.*, 2007; Naseby *et al.*, 2001). The IVET screen carried out by Gal *et al.* (2003) identified a putative nitrilase gene (*pinA*, PFLU2708), which is expressed in the rhizosphere of sugar beet plants, but is not expressed during growth in standard bacteriological media. Nitrilases are thought to be important in nitrile detoxification, nitrogen utilisation and plant hormone synthesis (O'Reilly and Turner, 2003; Pace and Brenner, 2001). Thus, *pinA* represents an interesting candidate when considering the success of *Pf*SBW25 in the plant environment and which traits may be important in the plant growth-promoting properties of *Pf*SBW25. The aim of the research presented in this paper was therefore

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to describe the biochemical function of *pinA* and its potential role in plant colonisation.

Nitrilases catalyse the hydrolysis of nitrile compounds (R-CN) to the corresponding carboxylic acid and ammonia (O'Reilly and Turner, 2003). These enzymes have been characterised in plants, bacteria and fungi, and nitrilase homologues exist in the genomes of animals and yeast (Brenner, 2002; Pace and Brenner, 2001). The widespread occurrence of nitrilases across kingdoms is not surprising, since nitrile compounds are common in the environment as intermediates in chemical biosynthesis and degradation and as contaminants from industrial and farming processes (Legras *et al.*, 1990). In plants nitriles are found in a number of pathways including the biosynthesis of cyanolipids and cyanogenic glycosides, the breakdown of glucosinolates and the detoxification of cyanide (Halkier and Gershenzon, 2006; Legras *et al.*, 1990). Thus, the ability to hydrolyse nitrile compounds may be a useful trait, especially for organisms living in nitrile-rich habitats.

While much is known about plant nitrilases, the biological role of many microbial nitrilases remains unknown. Microbes have been shown to synthesise and degrade nitrile compounds during cyanogenesis and metabolism (Vannesland *et al.*, 1981; Yoshikawa *et al.*, 2000), but the majority of microbial nitrilases have been studied from an industrial standpoint, with the aim of discovering novel pathways for carboxylic acid synthesis or nitrile degradation (Mathew *et al.*, 1988; Singh *et al.*, 2006). Preliminary sequence analyses revealed that *pinA* showed higher similarity to plant nitrilases, such as those of *Arabidopsis thaliana*, than to any functionally characterised microbial nitrilase (Gal *et al.*, 2003). *A. thaliana* has four nitrilases (NIT1, 2, 3 and 4). NIT1, 2 and 3 are thought to be important in auxin biosynthesis and have been shown to hydrolyse the arylacetonitrile, indole acetonitrile (IAN), to the plant hormone indole acetic acid (IAA) (Bartel and Fink, 1994; Bartling *et al.*, 1992). NIT4 hydrolyses β-cyano-L-alanine [Ala(CN)] to aspartic acid and ammonia (Piotrowski *et al.*, 2001). β-Cyano-L-alanine is synthesised as a intermediate during cyanide detoxification and is also produced as a defensive compound against herbivory (Ressler *et al.*, 1997; Vannesland *et al.*, 1981). NIT4 may represent an important detoxification mechanism in *A. thaliana*, hydrolysing Ala(CN) to non-toxic products.

The sequence similarity between *pinA* and plant nitrilases raises the question of whether *pinA* hydrolyses arylacetonitriles or Ala(CN) in the plant environment. To test this we have characterised the function of *pinA* using a combination of bioinformatic and phylogenetic analyses, bacterial growth studies, biochemical assays and heterologous expression studies. We report that *pinA* is a NIT4-type nitrilase which catalyses the hydrolysis of Ala(CN) and confers tolerance to toxic levels of Ala(CN). *pinA* expression is shown to be induced on *A. thaliana* seedlings and also by Ala(CN) and the precursors for Ala(CN) synthesis, cysteine and cyanide. We reveal that NIT4-type enzymes are found in a range of plant-associated bacteria, suggesting that hydrolysis of Ala(CN) is an important trait for bacteria colonising the plant environment. Finally, we demonstrate that *pinA* can be used to functionally complement a *NIT4* mutant of *A. thaliana.*

Results

pinA *is similar to plant nitrilases that hydrolyse arylacetonitriles and Ala(CN)*

Sequences of characterised and putative nitrilases from plants, bacteria, animals and fungi were subjected to bioinformatic and phylogenetic studies to determine the level of similarity between *pinA* and other nitrilases, to identify *pinA* homologues that have yet to be characterised and to assess whether nitrilase activity and substrate range are clade specific (Podar *et al.*, 2005; Robertson *et al.*, 2004). BLAST analyses of *pinA* against the NCBI sequence database had previously revealed that *pinA* showed significant similarity to genes from other genome-sequenced *P. fluorescens* strains and to plant nitrilases such as the *A. thaliana* nitrilases NIT1, -2, -3 and -4 (Gal *et al.*, 2003). Pairwise alignment of *pinA* with other nitrilases using Emboss Align confirmed that *pinA* shows a high level of amino acid identity to PFL_2909 from *P. fluorescens* Pf-5 (82.8%), PflO1_2571 from *P. fluorescens* PfO-1 (84.7%) and PSEEN_3513 from *Pseudomonas entomophila* L48 (81.8%) (Table S1 in Supporting Information). A moderate level of identity was observed between *pinA* and plant nitrilases that hydrolyse arylacetonitriles and Ala(CN) (for example 44.2% identity to NIT4A from *Nicotiana tabacum*). *pinA* showed a lower level of identity to enzymes that hydrolyse aromatic nitriles, such as the nitrilase from *Klebsiella pneumoniae* (26.6%), or aliphatic nitriles, such as the nitrilase from *Rhodococcus rhodochrous* K22 (24.1%).

Amino acid sequences of 55 nitrilases and three aliphatic amidase enzymes were aligned for phylogenetic analysis. The phylogenetic tree generated is shown in Figure 1 and the complete alignment file is provided in Figure S1. The grouping of enzymes in this tree compares favourably with the limited published nitrilase phylogeny data (O'Reilly and Turner, 2003; Piotrowski *et al.*, 2001). *Pf*SBW25 *pinA* groups closely with PflO1_2571, PFL_2909 and PSEEN_3513. This group of enzymes is part of a much larger group which contains all the plant nitrilases as well as representatives from bacteria, nematodes and fungi. Enzymes hydrolysing aromatic and aliphatic nitriles and cyanide fall outside this group, and in some cases form subgroups according to substrate. Interestingly, the tree suggests that substrate specificity has evolved independently many times in bacteria and in plants.

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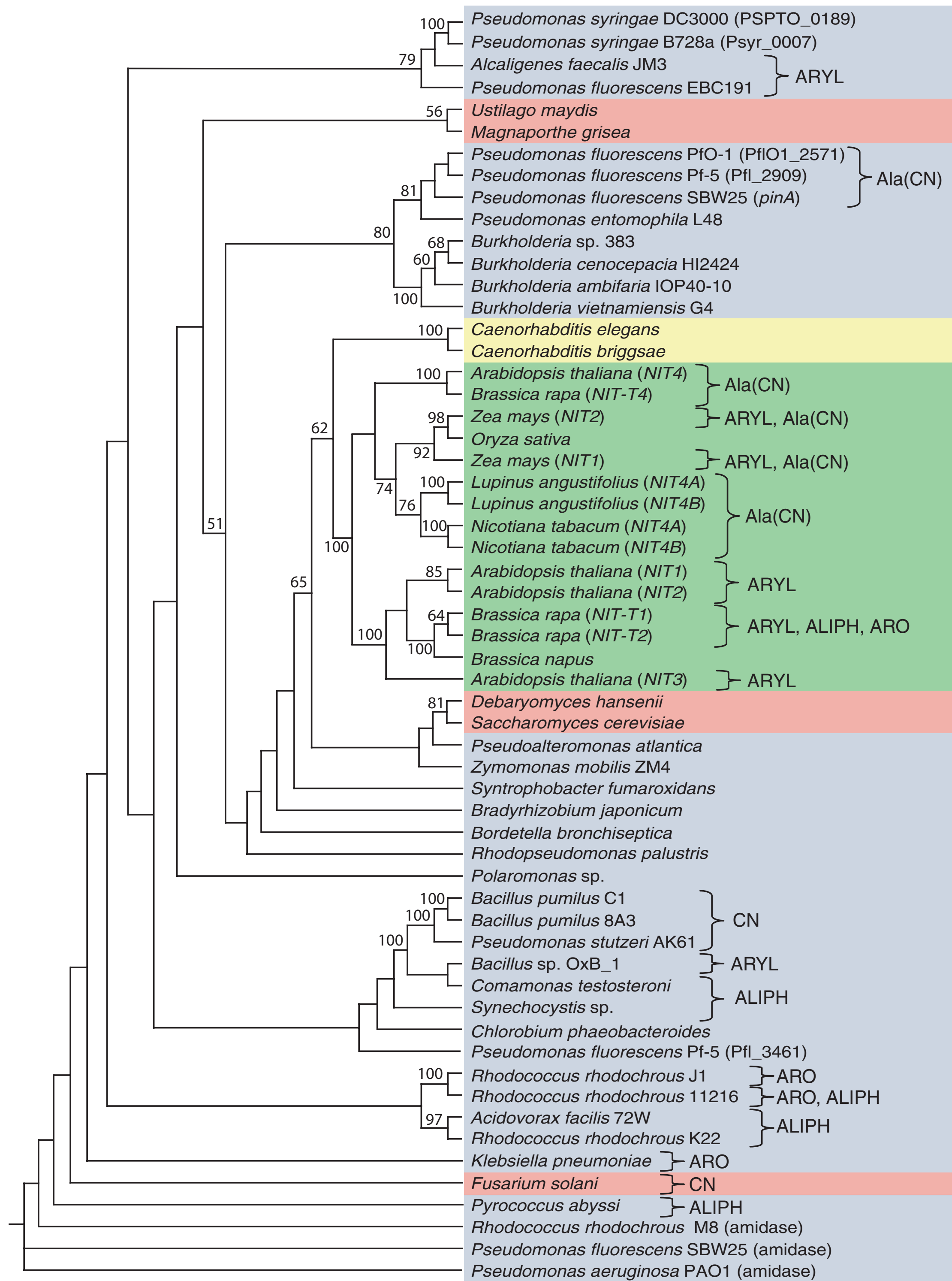

Figure 1. Strict consensus tree of characterised and putative nitrilases.
The tree was generated by parsimony analysis and is supported by a bootstrap analysis with 500 replicates. All nitrilase sequences included have the catalytic triad of amino acids (glutamic acid, lysine and cysteine) that is found in all members of the nitrilase superfamily, and the cysteine, tryptophan and glutamic acid motif conserved at the cysteine residue of the catalytic triad (Pace and Brenner, 2001). Included in the tree are aliphatic amidase enzymes from *Pseudomonas aeruginosa* PAO1, *Rhodococcus rhodochrous* and *Pseudomonas fluorescens* SBW25. The tree is rooted using the *P. aeruginosa* amidase sequence. Shading corresponds to plant genes (green), bacterial genes (blue), fungal genes (pink) and animal genes (yellow). Gene names, where known, are shown in parentheses. The tree is annotated with the substrate or substrate class for each enzyme, where known (ARO, aromatic; ALIPH, aliphatic; ARYL, arylacetonitriles; Ala(CN), β-cyano-L-alanine; CN, cyanide). The alignment file used to generate this tree and Genbank accession numbers for enzyme sequences are provided in Figure S1.

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pinA *allows bacteria to use Ala(CN) as a nitrogen source and to tolerate toxic concentrations of Ala(CN)*

In bacteria, nitrilase activity can be detected by examining growth in media in which the sole nitrogen source is a nitrile compound. Bacteria will only grow if they can hydrolyse the nitrile group to liberate ammonia, which can subsequently be used as a nitrogen supply. *Pf*SBW25 was tested for growth in M9 supplemented with nitriles representing each class of nitrile compound (aromatic, aliphatic and arylacetonitriles). Of those nitriles tested, *Pf*SBW25 could only use Ala(CN) (an aliphatic nitrile) as a nitrogen source. The nitrilase mutant *Pf*SBW25 Δ*pinA* was significantly impaired in growth in media in which Ala(CN) was the nitrogen source, but growth could be restored to wild-type levels when the mutant was complemented with plasmid pBBR1MCS5-*pinA*, which constitutively expresses *pinA* (Figure 2a). Deleting *pinA* did not confer a general fitness effect since wild-type *Pf*SBW25, *Pf*SBW25 Δ*pinA* and *Pf*SBW25 Δ*pinA* (pBBR1MCS5-*pinA*) grew very similarly in M9 with ammonia as the nitrogen source (Figure S2). Since *pinA* homologues were also found in *P. fluorescens* Pf-5, *P. fluorescens* PfO-1 and *P. entomophila* L48 these strains were also tested for their ability to use nitrile compounds as a nitrogen source. *P. fluorescens* Pf-5 and *P. fluorescens* PfO-1 were both able to use Ala(CN) as a nitrogen source (data not shown), suggesting that Ala(CN) hydrolysing activity is conserved within these strains. No Ala(CN)-hydrolysing activity was detected in *P. entomophila* L48.

Nitrilases are hypothesised to be important in nitrile detoxification (Piotrowski and Volmer, 2006; Piotrowski *et al.*, 2001). Thus, Ala(CN) was tested for its toxicity to *Pf*SBW25, *Pf*SBW25 Δ*pinA* and *Pf*SBW25 Δ*pinA* (pBBR1MCS5-*pinA*). *Pf*SBW25 and *Pf*SBW25 Δ*pinA* (pBBR1MCS5-*pinA*) were able to grow at all concentrations of Ala(CN), and achieved the greatest growth at 200 mM Ala(CN). The growth of *Pf*SBW25 Δ*pinA* was impaired at Ala(CN) concentrations of 25 mM and above, with growth completely inhibited at 200 mM Ala(CN) (Figure 2b). Toxicity data for the full range of Ala(CN) concentrations tested are shown in Figure S3.

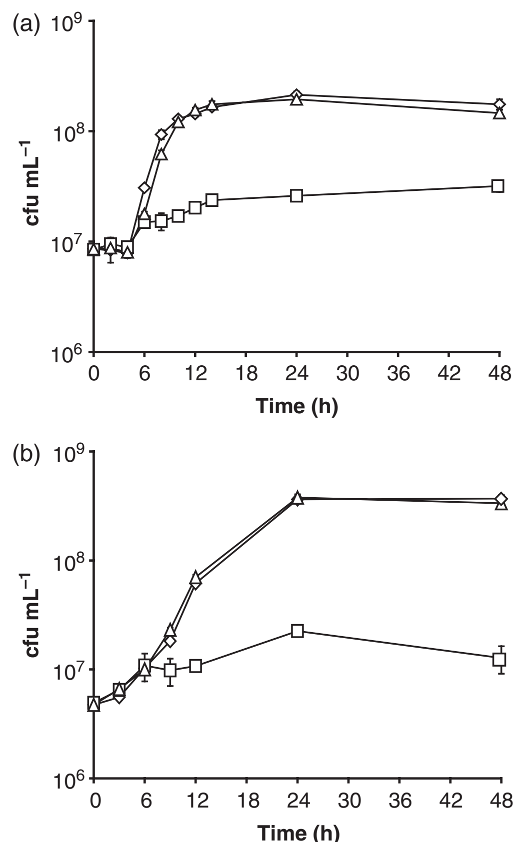

Figure 2. *pinA* enables *Pseudomonas fluorescens* (*Pf*) SBW25 to use β-cyano-L-alanine [Ala(CN)] as a nitrogen source and to tolerate toxic concentrations of Ala(CN).
Pseudomonas fluorescens SBW25 (◇), *Pf*SBW25 Δ*pinA* (□) and *Pf*SBW25 Δ*pinA* (pBBR1MCS5-*pinA*) (Δ) were tested for their ability to use Ala(CN) as a nitrogen source and for their sensitivity to Ala(CN). Each value represents the average of three replicates from one experiment ± standard deviation. Both experiments were carried out at least twice, producing similar results. cfu, colony-forming units.
(a) Bacterial growth in M9 lacking ammonia, supplemented with 2 mM Ala(CN) as the sole nitrogen source.
(b) Bacterial growth in M9 with ammonia, supplemented with 200 mM Ala(CN).

pinA *expression is induced by* A. thaliana *seedlings and by Ala(CN), cysteine and cyanide*

Gal *et al.* (2003) observed that *pinA* expression was induced during colonisation by *Pf*SBW25 of sugar beet seedlings in non-sterile conditions. Experiments were carried out to determine whether *pinA* is induced by sterile Arabidopsis seedlings, which would confirm that *pinA* is induced in response to a plant-derived signal. *pinA* induction studies were performed using the *pinA* IVET strain *Pf*SBW25 IVI93 (Gal *et al.*, 2003). This strain contains a deletion mutation in the *dapB* gene and a chromosomally inserted plasmid containing promoterless copies of *dapB* and *lacZY* downstream of the *pinA* promoter. This strain can only grow if the *pinA* promoter is active, thereby driving expression of *dapB* and *lacZY*, or if growth medium is supplemented with diaminopimelate (DAP) and lysine. *Pf*SBW25 IVI93 can therefore be used to examine *pinA* expression *in vitro* and *in vivo*.

Arabidopsis thaliana seedlings were placed on a lawn of either *Pf*SBW25 IVI93, or the control strain *Pf*SBW25 Δ*dapB*. In the absence of DAP and lysine *Pf*SBW25 IVI93 formed colonies around seedlings, while *Pf*SBW25 Δ*dapB* failed to grow. On plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), colonies of *Pf*SBW25 IVI93 growing in close proximity to seedlings appeared blue (Figure 3a). This blue coloration is the result

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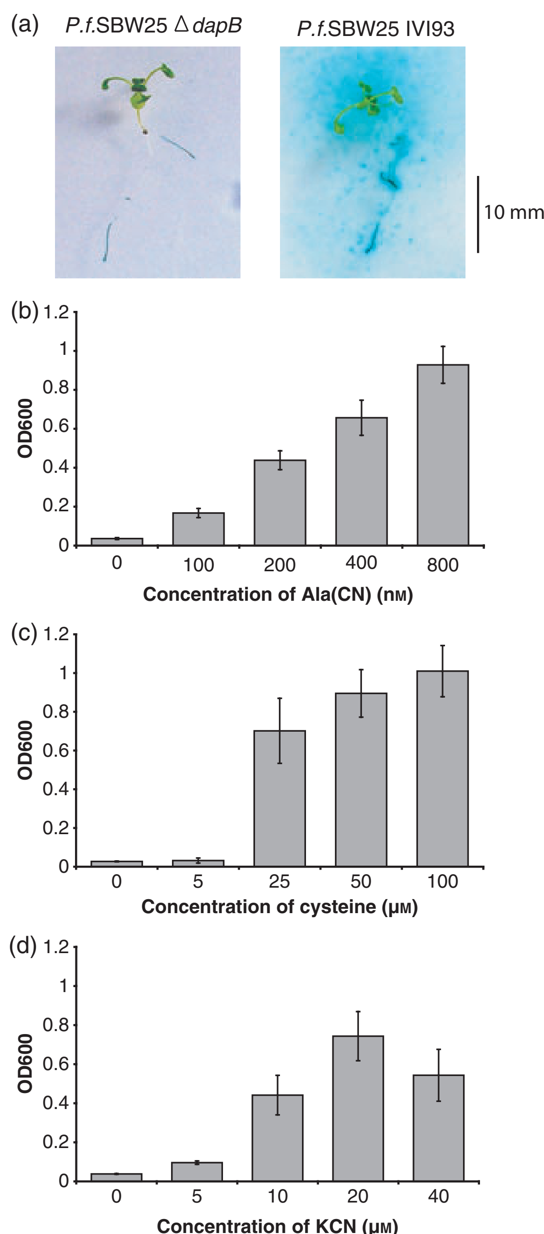

Figure 3. *pinA* expression is induced by *Arabidopsis thaliana* seedlings and by β-cyano-L-alanine [Ala(CN)], cysteine and cyanide.
pinA expression was examined using the *pinA* IVET strain *Pseudomonas fluorescens* (*Pf*) SBW25 IVI93 and the negative control strain *Pf*SBW25 Δ*dapB*. In media lacking diaminopimelate (DAP) and lysine *Pf*SBW25 IVI93 will only grow if *pinA* expression is induced. Each value shown in (b), (c) and (d) represents the average of three replicates from one experiment ± standard deviation, measured 24 h after inoculation. All experiments were carried out three times, producing similar results.
(a) *Arabidopsis thaliana* seedlings were tested for their ability to induce *pinA* by placing seedlings on a lawn of *Pf*SBW25 IVI93 or *Pf*SBW25 Δ*dapB* on M9 agar supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Seedlings were photographed 24 h after transfer to bacterial lawns. Blue coloration around seedlings is the result of bacterial growth and induction of *pinA*. A total of 15 seedlings were screened for each treatment.
(b) Growth of *Pf*SBW25 IVI93 in M9 supplemented with Ala(CN).
(c) Growth of *Pf*SBW25 IVI93 in M9 supplemented with cysteine.
(d) Growth of *Pf*SBW25 IVI93 in M9 supplemented with potassium cyanide (KCN).

of the induced *pinA* promoter driving the expression of the *lacZY* gene. In the presence of DAP and lysine *Pf*SBW25 IVI93 and *Pf*SBW25 Δ*dapB* formed a lawn covering the entire plate, and bacterial growth was not inhibited by the presence of seedlings (data not shown). These results, along with previously published data, support the hypothesis that *pinA* gene expression is induced by a plant-derived signal.

Having shown that *pinA* is induced by plants, experiments were carried out to identify the inducing signal for gene expression. *Pf*SBW25 is exposed to a wide range of chemicals that are secreted from plant roots during rhizosphere colonisation. Root exudates modify the rhizosphere environment and may have important roles in plant–plant and plant–microbe interactions (Flores *et al.*, 1999; Walker *et al.*, 2003). These exudates may include nitrile compounds, which are found in many pathways in plant metabolism (Halkier and Gershenzon, 2006; Legras *et al.*, 1990), or amino acids (Phillips *et al.*, 2004), and may induce nitrilase activity at both a transcriptional and a post-translational level. For example, previous studies have shown nitriles to induce association of nitrilase subunits and subsequent enzyme activation (Hoyle *et al.*, 1998; Layh *et al.*, 1998). Twenty amino acids, nine nitriles and potassium cyanide (KCN) were tested for their ability to induce *pinA* using *Pf*SBW25 IVI93 as a reporter of *pinA* expression. Only Ala(CN), cysteine and KCN were found to induce *pinA* expression (Figure 3b–d). This is of interest since cysteine and cyanide are the substrates for Ala(CN) biosynthesis in the reaction catalysed by Ala(CN) synthase and cysteine synthase (Hatzfeld *et al.*, 2000). *pinA* was found to be induced by Ala(CN) at nanomolar concentrations, with the minimum concentration required for induction being approximately 100 nM. Cysteine and KCN both induced *pinA* at low micromolar concentrations.

PinA *hydrolyses Ala(CN) to release ammonia*

Nitrilase activity can be studied *in vitro* by measuring ammonia synthesis when a nitrilase is incubated with a nitrile compound. PinA and the negative control protein RABE were expressed in *Escherichia coli* as N-terminal histidine-tagged proteins using the expression vector pGAT4. RABE is an *A. thaliana* GTPase and should confer no nitrilase activity. A band corresponding to the predicted size of the PinA protein was visible on a Coomassie stained gel and on a western blot membrane probed with anti-HIS antibody (data not shown). Purified PinA protein was insoluble in solution and thus crude extracts of PinA and RABE were used to examine nitrilase activity *in vitro*. When Ala(CN) was incubated with HIS-tagged PinA the concentration of ammonia rose from 0.17 mM at the start of the experiment to 1.69 mM after 3 h (Figure 4). There was no change in the ammonia concentration in the control reactions: RABE with and without Ala(CN), PinA without

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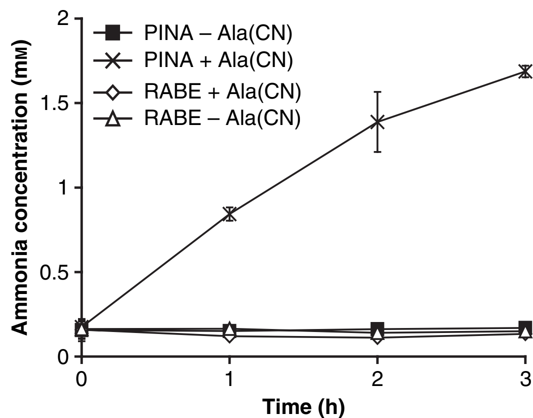

Figure 4. PinA hydrolyses β-cyano-L-alanine (Ala(CN)) to release ammonia. One hundred micrograms of crude protein extract was incubated with and without 3 mM Ala(CN), and ammonia production measured using the Nessler assay. The *Arabidopsis thaliana* GTPase, RABE, was tested as a negative control. The following reactions were examined: PinA with Ala(CN) (x), PinA without Ala(CN) (■), RABE with Ala(CN) (◇) and RABE without Ala(CN) (Δ). The experiment was carried out twice and each value represents the average of three replicates from one experiment ± standard deviation.

Ala(CN) and heat-denatured PinA with and without Ala(CN) (data not shown).

Heterologous expression of pinA *confers Ala(CN) hydrolysing activity and increased tolerance to Ala(CN) in both bacteria and plants*

The observation that PinA can be functionally expressed in *E. coli* provides clear evidence that PinA hydrolyses Ala(CN) to release ammonia, and demonstrates that PinA can function independently of other *P. fluorescens* proteins to confer Ala(CN) hydrolysis. To assess whether *pinA* can be used to create recombinant bacteria that can degrade Ala(CN), *pinA* was expressed in two genome-sequenced strains of the plant pathogen *Pseudomonas syringae*. *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 both contain putative nitrilase genes, as shown in Figure 1 and Table S1, but these nitrilases are quite dissimilar to *pinA*. *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728a were unable to use Ala(CN) as a nitrogen source, confirming that the nitrilase homologues present in the genomes of these bacteria are unlikely to act on Ala(CN). However, bacteria transformed with pBBR1MCS5-*pinA* were able to grow in media in which the sole nitrogen source was Ala(CN) (Figure 5a), and displayed increased tolerance to toxic concentrations of Ala(CN) (Figure 5b, results for *P. syringae* pv. *tomato* DC3000 are not shown).

As *pinA* mutants of *Pf*SBW25 were impaired in Ala(CN) tolerance, and PinA could be shown to be functionally equivalent to the plant nitrilase NIT4, it was logical to hypothesise that NIT4 mutants of *A. thaliana* would also be impaired in Ala(CN) tolerance and to investigate whether PinA can replace NIT4 activity in plants. Wild-type *A. thaliana* and *A. thaliana* plants with a *NIT4* insertional mutation (*A. thaliana* Δ*nit4*, SALK_016289, Joseph R. Ecker) were transformed to constitutively express either *pinA* or *gfp*. *pinA* was found to complement the *NIT4* mutation in *A. thaliana*, allowing plants to grow in concentrations of Ala(CN) that prove fatal to *A. thaliana* Δ*nit4* (Figure 6). In addition, *pinA* improved the level of Ala(CN) tolerance observed in wild-type plants expressing NIT4, enabling these plants to grow in Ala(CN) concentrations that would normally inhibit root growth. In the absence of Ala(CN) the root lengths of wild-type *A. thaliana*, *A. thaliana* Δ*nit4* and *A. thaliana* Δ*nit4* + *pinA* were similar, indicating that Δ*nit4* plants were

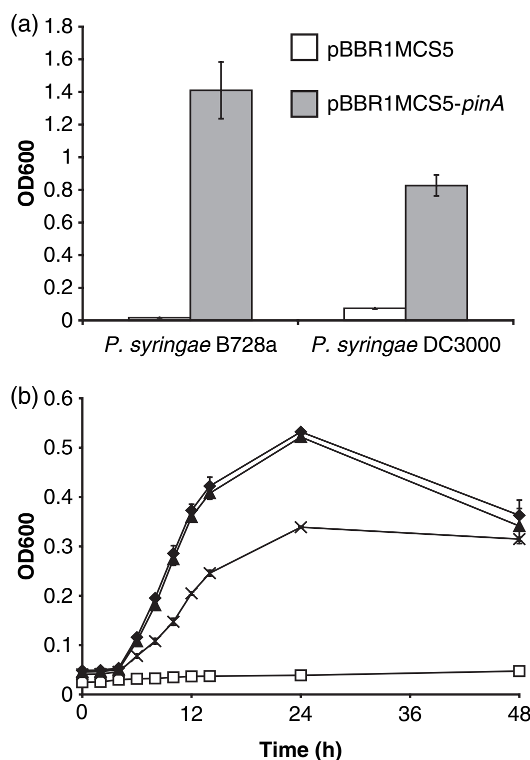

Figure 5. Heterologous expression of *pinA* in *Pseudomonas syringae* confers β-cyano-L-alanine [Ala(CN)]-hydrolysing activity and increased tolerance to Ala(CN).
(a) Bacterial growth after 24 h in M9 with 2 mM Ala(CN) as the sole nitrogen source. *pinA* was expressed in *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 using pBBR1MCS5-*pinA* (solid bars). Bacteria were transformed with pBBR1MCS5 as a control (clear bars). Each value represents the average of three replicates from one experiment ± standard deviation. The experiment was carried out twice, producing similar results.
(b) Bacterial growth in M9 supplemented with 100 mM Ala(CN). *P. syringae* pv. *syringae* B728a (pBBR1MCS5), 0 mM Ala(CN) (▲); *P. syringae* pv. *syringae* B728a (pBBR1MCS5–*pinA*), 0 mM Ala(CN) (♦); *P. syringae* pv. *syringae* B728a (pBBR1MCS5-*pinA*), 100 mM Ala(CN) (x); *P. syringae* pv. *syringae* B728a (pBBR1MCS5), 100 mM Ala(CN) (□). Each value represents the average of three replicates from one experiment ± standard deviation. The experiment was carried out twice, producing similar results.

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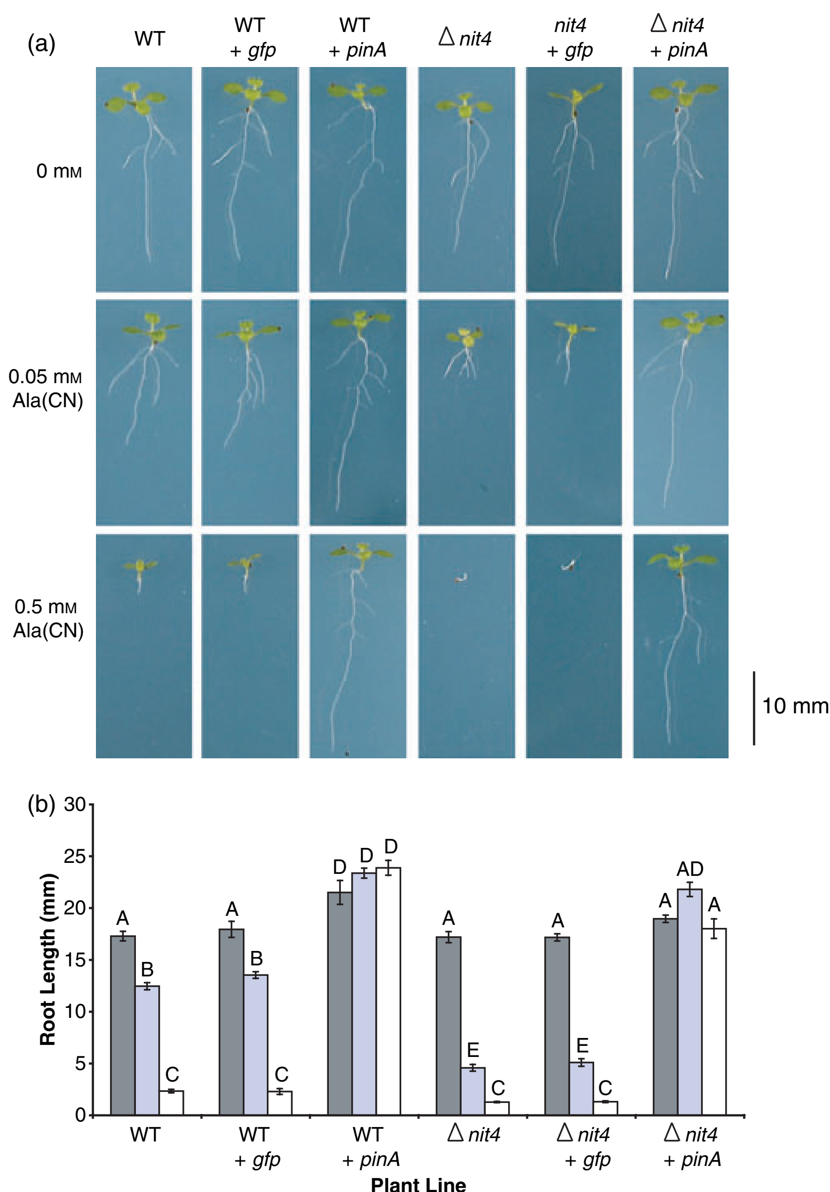

Figure 6. *pinA* can replace NIT4 activity in *Arabidopsis thaliana*.
Arabidopsis thaliana and *A. thaliana* Δnit4 lines transformed with either *pinA* or *gfp* were sown on 1× MS medium and on 1× MS media supplemented with 0.05 mM β-cyano-L-alanine [Ala(CN)] and 0.5 mM Ala(CN).
(a) Images of representative seedlings 10 days after sowing.
(b) Tap root length 10 days after sowing. Each value represents the average of at least 20 seedlings from one individual experiment ± standard error. No Ala(CN) (dark grey bars), 0.05 mM Ala(CN) (light grey bars) and 0.5 mM Ala(CN) (clear bars). Letters indicate significant differences between each line at each Ala(CN) concentration (analysis of variance, *post hoc* least significant difference, $P < 0.05$). The experiment was set up in triplicate for each treatment and each line. Two individual lines were tested for each construct, producing similar results; the graph shows data for one representative line.

not subject to a general fitness effect. Interestingly, wild-type plants expressing *NIT4* and *pinA* showed increased root elongation even in the absence of externally applied Ala(CN), demonstrating that increasing Ala(CN) nitrilase activity can have a significant effect on root development.

Discussion

Nitrilases have been characterised in plants, bacteria and fungi and are thought to be important in nitrile detoxification, utilisation of nitrogen and plant hormone synthesis. While the biological role of plant nitrilases is well understood, very little is known about the importance of these enzymes in bacteria. This study aimed to characterise a plant-induced nitrilase (*pin*A) in the plant growth-promoting rhizobacterium *Pf*SBW25. We have shown that *pinA* encodes a NIT4-type nitrilase that specifically hydrolyses Ala(CN). We have confirmed that *pinA* expression is induced by plant metabolites and have discovered that *pinA* is also induced by Ala(CN) and its precursors, cysteine and cyanide. Deleting *pinA* in *Pf*SBW25 causes enhanced sensitivity to Ala(CN), while expressing this nitrilase in other bacteria and plants confers Ala(CN)-hydrolysing activity and increased tolerance to toxic concentrations of Ala(CN). In this study we have systematically characterised a novel, plant-induced enzyme discovered using IVET technology, identifying both the substrate and the signals that are likely to be involved in plant-specific expression.

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NIT4 enzymes have previously been identified in a number of plant species, including *A. thaliana*, *N. tabacum*, *Lupinus angustifolius* and members of the Poaceae family, and play an important role in cyanide detoxification (Jenrich *et al.*, 2007). Cyanide is produced by all higher plants during ethylene biosynthesis and may also be produced during the synthesis or degradation of glucosinolates and cyanogenic glycosides (Dewick, 1984; Halkier and Gershenzon, 2006; Peiser *et al.*, 1984). Cyanide is converted to Ala(CN) by the activity of β-cyano-L-alanine synthase or cysteine synthase (Blumenthal *et al.*, 1968; Hatzfeld *et al.*, 2000). The Ala(CN) is subsequently detoxified by NIT4 (Piotrowski *et al.*, 2001). Could the same pathway exist for cyanide detoxification in bacteria? Such a pathway would be beneficial to *Pf*SBW25 when colonising plant tissues rich in cyanide or Ala(CN) or when growing in close proximity to cyanogenic microbes, which are common in the plant environment (Laville *et al.*, 1998; Ramette *et al.*, 2003). In plants Ala(CN) is synthesised from cyanide and cysteine by cysteine synthase. *Pf*SBW25 has two cysteine synthase genes, *cysK* and *cysM*. While the activity of these genes has not been confirmed it seems likely that *Pf*SBW25 converts cyanide to Ala(CN) using *cysK* or *cysM*, and that Ala(CN) generated by these enzymes is subsequently hydrolysed by *pinA*. Both cyanide and cysteine promote increased levels of *pinA* expression, probably by promoting increased synthesis of Ala(CN), which was shown to induce expression of *pinA* at nanomolar concentrations.

As already mentioned, NIT4 enzymes are distributed throughout the plant kingdom. This study provides evidence that NIT4 enzymes are also present in a variety of microorganisms. *pinA* homologues were identified in three strains of *P. fluorescens*, *P. entomophila* L48 and in other environmental and plant-associated bacteria such as *Burkholderia* spp. Previously, Ala(CN)-hydrolysing activity was detected in two *Pseudomonas* species (Luque-Almagro *et al.*, 2005; Yanase *et al.*, 1982, 1983). However, sequence data has not been published for these enzymes and they have yet to be cloned and characterised. While further experiments need to be completed to confirm that *pinA* homologues are responsible for this activity, it seems likely that PinA-mediated Ala(CN) hydrolysis is an important and widely distributed trait in *P. fluorescens*, which is conserved beyond the strains examined in the current study. The presence of Ala(CN)-hydrolysing enzymes in bacteria and plants has most likely been achieved by convergent evolution, since these enzymes are not found in a single phylogenetic clade. Interestingly, all of the *P. fluorescens* and *Burkholderia* strains that lie within the *pinA* grouping in the phylogenetic tree (Figure 1) encode a conserved LysR family regulator adjacent to *pinA*, which is absent from *P. entomophila* L48 and other genome sequenced *Pseudomonas*. LysR-type transcriptional regulators are in general positive regulators and have been shown to regulate a diverse range of prokaryote genes (Schell, 1993). It is logical to speculate that this gene encodes a positive regulator of *pinA* expression and that the absence of Ala(CN)-hydrolysing activity in *P. entomophila* is linked to the absence of this gene.

Heterologous expression of *pinA* in plants and bacteria has shown that PinA can function in a wide range of organisms. This raises the possibility of using PinA to develop microorganisms or plants with enhanced Ala(CN) tolerance and Ala(CN)-degrading ability as part of an integrated strategy to degrade cyanide present in the environment. The difference in Ala(CN) tolerance between wild-type and *pinA*-expressing plants was very clear, so *pinA* may also have applications as a novel marker gene for transformation, although researchers would need to take into account any physiological effects that could arise from the release of ammonia and the degradation of endogenous and exogenous Ala(CN). Interestingly, wild-type *A. thaliana* seedlings expressing *pinA* showed moderately increased root length in the absence of Ala(CN) and an even greater increase in root length in the presence of Ala(CN). The latter observation could be attributed to increased ammonia and amino acid levels resulting from the hydrolysis of Ala(CN). However, the observation that PinA expression stimulates root growth even in the absence of Ala(CN) suggests that increasing the activity of this enzyme can affect plant development, either by reducing growth constraints associated with inhibitory levels of cyanide and Ala(CN) or by altering other regulatory or metabolic processes.

The increase in root elongation shown by wild-type plants transformed with *pinA* highlights a further intriguing possibility with respect to the role of PinA in bacterial colonisation of plant roots. Root-colonising bacteria have been shown to use a range of strategies to suppress ethylene signalling and promote root elongation, including the use of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase to degrade and assimilate the ethylene precursor ACC (Glick *et al.*, 1998; Penrose and Glick, 2001). It is possible that PinA directly and indirectly stimulates root growth by removing toxic metabolites and modifying plant physiology to promote root elongation, thereby benefiting both the plant and the bacterium.

In summary, we have shown that *pinA* encodes a plant-induced NIT4-type nitrilase that specifically hydrolyses Ala(CN). However, the role of this enzyme in plant colonisation and in environmental competence remains unclear. PinA enables *Pf*SBW25 to grow in toxic concentrations of Ala(CN) and to use Ala(CN) as a sole nitrogen source, so this enzyme may have roles in both nitrile detoxification and nitrogen acquisition. The ability to hydrolyse Ala(CN) may be particularly useful when colonising plant tissues rich in Ala(CN), when competing with cyanogenic microorganisms or when growing in nutrient-poor conditions. The presence of NIT4 enzymes in a range of plants and plant-associated microorganisms clearly suggests that Ala(CN) hydrolysis is

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an important trait in the plant environment, although interestingly, neither of the plant pathogenic *P. syringae* strains tested in this study could hydrolyse Ala(CN). Experiments examining bacterial fitness in environments in which cyanide and Ala(CN) are present in high concentrations, along with further investigations into the effect of PinA and Ala(CN) on plant gene expression and root development, will advance our understanding of NIT4 enzymes.

Experimental procedures

Bioinformatic and phylogenetic analyses

Nitrilase sequences were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov/) and from the *Pseudomonas* database PseudoDB (http://pseudo.bham.ac.uk/). Amino acid sequences were aligned using Se-Al (Rambaut, 1996) and phylogenetic trees were generated by parsimony analyses using PAUP 4.0 (Sinauer Associates, http://www.sinauer.com/). Statistical support was provided for trees using the bootstrap test. Pairwise amino acid sequence analyses were carried out using the European Bioinfomatics Institute program Emboss Align (Rice *et al.*, 2000). The following settings were used: global analysis, gap open 10.0, gap extend 0.5, matrix Blosum62.

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Table S2. *Escherichia coli* strains were grown at 37°C while *Pseudomonas* strains were grown at 28°C, in Luria–Bertani (LB) broth, M9 minimal medium (M9) or on LB agar plates (Sambrook *et al.*, 1989). Antibiotics and supplements were used at final concentrations of: ampicillin (50 μg ml^{-1}), kanamycin (25 μg ml^{-1}), tetracycline (12.5 μg ml^{-1}), gentamicin (10 μg ml^{-1}) and X-Gal (40 μg ml^{-1}). *Arabidopsis thaliana* seedlings were grown vertically on 1.2% agar plates supplemented with 1× Murashige and Skoog (MS) micro and macro nutrient basal salts (Sigma, http://www.sigmaaldrich.com/). Seedlings were grown at 21°C and a photoperiod of 16 h light and 8 h dark.

Construction and complementation of a pinA *mutant*

pinA was deleted from *Pf*SBW25 using a homologous recombination strategy similar to that described by Jones *et al.* (2007). The region flanking the 5′ end of *pinA* was amplified from genomic *Pf*SBW25 DNA using the primers *pinL-F* (TCAATCTGGCCAACAAGACC) and *pinL-R* (CCGAATAATAGGGTTGCCCA) while the region flanking the 3′ end was amplified using the primers *pinR-F* (ACACGGTCGAAATGTGGGAG) and *pinR-R* (CTGACCGCCAACTCGATGAT). The resulting PCR products were ligated into the cloning vector pCR2.1 using a TA Cloning Kit (Invitrogen, http://www.invitrogen.com/). pCR2.1 containing the 3′ flanking region (pCR2.1-*pinA*3′) was digested with *Spe*I and *Xba*I while pCR2.1 containing the 5′ flanking region (pCR2.1-*pinA*5′) was digested with *Xba*I and treated with calf intestinal phosphatase (CIP) (New England Biolabs, http://www.neb.com/). The 3′ flanking region was ligated into pCR2.1-*pinA5*′ to create pCR2.1-*pinA*5′3′. The combined flanking regions were digested out of pCR2.1-*pinA*5′3′ with *Spe*I and *Xba*I and ligated into the *Spe*I site of the integration vector pUIC3. pUIC3-*pinA*5′3′ was transformed into *Pf*SBW25 by electroporation to achieve a two-step allelic exchange of *pinA*. Double recombinants were selected as described by Jones *et al.* (2007). The *pinA* deletion was confirmed by PCR and by sequencing the nitrilase region from an amplified PCR product.

The *pinA* deletion was complemented using the broad host range expression vector pBBR1MCS5 carrying *pinA*. The *pinA* region (*pinA* with 1.2 kb of 5′ and 3′ flanking sequence) was amplified from *Pf*SBW25 using the primers *comp-F* (AGGCCAAAGCCTGCAGAAATG) and *comp-R* (CTTGATCTACGGCCTGGAAGAC) and cloned into pCR2.1. The *pinA* region was moved from pCR2.1-*pinA* into pBBR1MCS5 by digestion with *Eco*RI and *Bgl*II followed by ligation into the expression vector. The verified clone was electroporated into *Pf*SBW25 and *Pf*SBW25 Δ*pinA*. pBBR1MCS5-*pinA* was also electroporated into *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728a for heterologous expression of *pinA*. Control strains were generated by electroporation with pBBR1MCS5.

Bacterial growth studies

Pseudomonas fluorescens SBW25 was tested for growth in M9 supplemented with different nitrile compounds as the nitrogen source. Nine different nitrile compounds were tested: benzonitrile, phenylpropionitrile, propionitrile, indole acetonitrile (IAN), Ala(CN), acetonitrile, mandelonitrile, *m*-tolunitrile and 2-cyanopyridine. Each nitrile was tested at a concentration of 2 mM (except IAN, which was used at a concentration of 1 mM due to the toxicity of this compound). Bacterial growth was also examined in M9 supplemented with nitrile compounds and with ammonia as an alternative nitrogen source to check that nitrile compounds were not toxic to bacteria. Detailed studies of utilisation and toxicity of Ala(CN) were carried out for *Pf*SBW25, *Pf*SBW25 Δ*pinA* and *Pf*SBW25 Δ*pinA* (pBBR1MCS5-*pinA*) and also for *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728a carrying pBBR1MCS5-*pinA* or pBBR1MCS5. Strains were grown in M9 with or without ammonia and supplemented with varying concentrations of Ala(CN), and bacterial growth was measured over 48 h.

pinA *expression analyses*

Pseudomonas fluorescens SBW25 IVI93 and *Pf*SBW25 Δ*dapB* were grown overnight in M9 minimal medium supplemented with 800 μg ml^{-1} diaminopimelate (DAP) and 80 μg ml^{-1} lysine (tetracycline was added when growing *Pf*SBW25 IVI93). Overnight cultures were washed to remove DAP and lysine and resuspended in M9 lacking DAP and lysine to a final optical density (OD) of 0.02 at 600 nm. For nitrile and amino acid induction assays a single amino acid or nitrile was added to bacterial cultures at a final concentration of 2 mM (except IAN which was used at 1 mM and KCN which was used at 0.4 mM due to the toxicity of these compounds). The OD_{600} of bacterial cultures was measured after 24 h. Those chemicals found to induce *pinA* expression were tested to determine the minimum concentration of chemical required for gene expression. To examine *pinA* expression in the presence of *A. thaliana* seedlings 200 μl of the washed bacterial suspension was plated onto the surface of M9-agar plates with and without DAP and lysine and X-Gal. Twelve-day-old *A. thaliana* seedlings were transferred onto the surface of each plate at a density of five seedlings per 9 cm diameter plate. Plates were incubated overnight in the dark at 28°C.

PinA activity in vitro

pinA was amplified from genomic *Pf*SBW25 DNA using the primers *gate-F* (CACCATGCCTGTTTCTACTGTGG) and *gate-R* (TCAGTCAGTGACAAAGCGCACGC) and cloned into the Gateway entry

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vector pENTR/D-TOPO® using a Directional TOPO Cloning Kit (Invitrogen). *pinA* was transferred from the entry vector into the Gateway destination vector pGAT4, for N-terminal histidine tagging, using Gateway LR Clonase® II enzyme mix (Invitrogen). For the generation of crude protein extracts *E. coli* Rosetta-gami cells expressing pGAT4-*pinA* and pGAT4-*RABE* were grown at 37°C to an OD_{600} of 0.6. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and cultures grown for a further 3 h at 37°C. Cells were harvested by centrifugation and cell pellets were resuspended in 5 ml of lysis buffer [20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) pH 8.0, 150 mM NaCl, 0.01% Tween 20]. Lysozyme (Fluka, http://www.sigmaaldrich.com/Brands/Fluka___Riedel_Home.html) was added to cells at 0.5 mg ml^{-1} and the solution was incubated on ice for 30 min followed by three cycles of freeze–thaw in liquid nitrogen. The suspension was centrifuged for 20 min at 9 500 ***g*** and the supernatant, which constitutes the crude protein extract, was removed for immediate use in enzyme assays. Protein extracts were also analysed by SDS-PAGE and western blotting using penta-HIS antibody (Qiagen, http://www.qiagen.com/).

Nitrilase activity was examined *in vitro* by measuring ammonia production in the presence of Ala(CN). One hundred micrograms of crude protein extract was incubated with 3 mM Ala(CN) at 37°C. Heat denatured protein extracts were used as a negative control and were generated by boiling extracts at 100°C for 10 min. Forty microlitre samples were taken at 0-, 1-, 2- and 3-h time points for the measurement of ammonia using the Nessler assay (Morrison, 1971). Samples were incubated with 140 μl H_2O and 20 μl Nessler reagent (Chemlab, http://www.chem-lab.be/) for 10 min at room temperature and the OD measured at 480 nM. A standard curve was generated using known concentrations of ammonium chloride solution.

Heterologous expression of pinA *in plants*

pinA was transferred from pENTR/D-TOPO® into the Gateway compatible binary T-DNA destination vector, pH2GW7 (Karimi *et al.*, 2002), using Gateway LR Clonase® II enzyme mix (Invitrogen). Verified clones were electroporated into *Agrobacterium tumefaciens* C58 GV3101. Wild-type *A. thaliana* Col-0 plants and *A. thaliana* Δ*nit4* plants (SALK_016289, Joseph R. Ecker) were transformed with either pH2GW7-*pinA* or pH2GW7-*gfp* according to the floral dipping method (Clough and Bent, 1998). Homozygous transformants were generated for each construct and lines were screened for their tolerance to Ala(CN). Seeds were sown on MS agar supplemented with 0.5 mM, 0.05 mM and 0 mM Ala(CN). Seedlings were grown vertically and tap root lengths were measured 10 days after sowing using ImageJ software (National Institutes of Health, http://www.nih.gov/).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Amino acid sequence comparison of *pinA* with other characterised nitrilases.

Table S2. Bacterial strains and plasmids used in this study.

Appendix S1. References for Supplementary Tables.

Figure S1. Alignment file of sequences used to generate the nitrilase phylogenetic tree.

Figure S2. A *pinA* deletion mutant of *Pseudomonas fluorescens* SBW25 is not impaired in growth *in vitro* in the absence of β-cyano-L-alanine [Ala(CN)].

Figure S3. *pinA* enables *Pseudomonas fluorescens* SBW25 to tolerate high concentrations of β-cyano-L-alanine [Ala(CN)].

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