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Breakthrough Technologies
Expression of an Entire Bacterial Operon in Plants

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Multigene expression is required for metabolic engineering, i.e. co-regulated expression of all genes in a metabolic pathway for the production of a desired secondary metabolite. To that end, several transgenic approaches have been attempted with limited success. Better success has been achieved by transforming plastids with operons. IL-60 is a platform of constructs driven from the geminivirus Tomato yellow leaf curl virus (TYLCV). We demonstrate that IL-60 enables non-transgenic expression of an entire bacterial operon in tomato plants without the need for plastid (or any other) transformation. Delivery to the plant is simple and the rate of expressing plants is close to 100%, eliminating the need for selectable markers. Using this platform, we show the expression of an entire metabolic pathway in plants, and delivery of the end-product secondary metabolite (pyrrolnitrin—PRN). Expression of this new secondary metabolite resulted in the appearance of a new plant phenotype—disease resistance. PRN production was already evident 2 days after application of the operon to plants and persisted throughout the plant's life span. Expression of entire metabolic pathways in plants is potentially beneficial for plant improvement, disease resistance and biotechnological advances, such as commercial production of desired metabolites.
Prokaryotic genes are usually clustered in operons under the control of a common promoter, and proteins are translated from a polycistronic mRNA (Jacob and Monod, 1961; Kozak, 1983; Salgado et al., 2000; Ermolaeva et al., 2001). Eukaryotic proteins are translated from their generally monocistronic mRNAs in a 5'-dependent manner (Kozak, 1983). Nevertheless, evidence shows that in some cases, eukaryotic genes associated with functionally related activities are also clustered together and co-expressed (Descombes and Schibler, 1991; Yamanaka et al., 1997; Welm et al., 1999; Ramji and Foka, 2002; Hurst et al., 2004; Amoutzias and Van de Peer, 2008; Field and Osbourn, 2008; Pawel, 2008). Gene clustering may also result in co-regulation of gene expression, and in some cases, genes participating in a certain metabolic pathway are indeed clustered and co-regulated. However, they do not always share a common cis-element, such as a promoter, and they are not always co-regulated by translation from the same polycistronic mRNA (Amoutzias and Van de Peer, 2008). Several cis-controlled clusters have arisen from gene duplication and divergence e.g. the human β-globin gene cluster; (Hardison et al., 1997). However, such clusters represent homologues of the same ancestral gene and not diverse genes requiring coordinated regulation for a particular metabolic pathway. On the other hand, heterologous gene clusters participating in the same metabolic pathway have been found in plants (Gierl and Frey, 2001; Shimura et al., 2007; Field and Osbourn, 2008; Jonczyk et al., 2008). Clustering indicates the possibility of co-segregation (preserving the clusters upon meiosis and recombination). However, despite the "operon-like" clustering and co-expression of pertinent genes (Field and Osbourn, 2008), expression from a single transcription unit, such as the classical operon, has not yet been demonstrated in eukaryotes. The current dogma is that in most cases, co-regulation of eukaryotic gene clusters is due to chromosomal arrangements and chromatin potentiation which affects an entire chromosomal domain (for example, Kingston and Narlikar, 1999; Gierman et al., 2007; Pawel, 2008). Cases in which expression of dicistronic mRNAs is regulated by readthrough of a stop codon have also been reported (e.g. Descombes and Schibler, 1991; García-Ríos et al., 1997).

Operon-like gene organization has been found in nematodes, yeast and some higher eukaryotes (e.g. Ben-Shahar et al., 2007; Shimura et al., 2007; Vázquez-Manrique et al., 2007; Field and Osbourn, 2008; Qian and Zhang, 2008). However, the term "operon-like" may not be accurate in all cases; "clustered organization" better describes the various situations. Nematodes and Drosophila carry gene clusters
which are transcribed to polycistronic pre-mRNAs and on a structural basis, can be defined as "operon-like" (summarized in Blumenthal, 2004). However, unlike in prokaryotes, the polycistronic pre-mRNAs are processed to monocistronic (sometimes dicistronic) mature mRNAs and are co-regulated by virtue of being transcribed from the same promoter. Intergenic regions (IRs) are removed by splicing and open reading frames (ORFs) are condensed to form a number of mRNAs. The resultant monocistronic mRNAs are trans-spliced to SL2 (a species of small nuclear RNA) which carries a cap structure, enabling 5′ initiation of translation from each individual monocistronic structure (Blumenthal et al., 2002).

Several rhizospheric bacteria produce antifungal and antibacterial secondary metabolites and their use as biocontrol agents of soilborne plant pathogens has been attempted (Weller et al., 2002; Spadaro and Gullino, 2005; Lugtenberg and Kamilova, 2009). Arima et al. (Arima, 1964) were the first to report the antibiotic compound 3-chloro-4-(2′-nitro-3′-chlorophenyl)-pyrrole (pyrrolineitrin, PRN) produced by a number of Pseudomonas pyrocinia strains. PRN has been found active against a wide range of pathogens (e.g. Chernin et al., 1996; El Banna and Winkelmann, 1998) and is produced by other bacterial species as well, including Pseudomonas fluorescens, Burkholderia cepacia, and Serratia plymuthica (Yoshihisa et al., 1989; Burkhead et al., 1994; Hill et al., 1994; Kalbe et al., 1996; Hammer et al., 1999; Kamensky et al., 2003; Ovadis et al., 2004). A four-gene operon coding for an enzymatic pathway converting tryptophan to PRN has been identified (Kirner et al., 1998), and the function of every encoded protein determined (Kirner et al., 1998). No significant homology has been found between the first three enzymes (PrnA, PrnB, and PrnC) and any plant protein. However, pheophorbide a deoxygenase (Pao; synonymous with ACD1, LLS1 and Tic55) from several plants shows 42 to 48% similarity to PrnD.

IL-60 is a platform of constructs derived from the geminivirus Tomato yellow leaf curl virus (TYLCV). The IL-60 system has provided universal expression or silencing in all plants tested to date (Peretz et al., 2007). We present a case in which the universal DNA plant vector system, IL-60, mediated the introduction and expression of an entire bacterial operon in tomato plants. The operon was transcribed and translated in the plant in a manner conforming to that of classical bacterial operons. Expression of the entire pathway resulted in the appearance of a new secondary metabolite (PRN), creating a new beneficial plant phenotype.
The biosynthesis of a secondary metabolite involves numerous enzymes, the genes of which constitute a metabolic pathway. Attempts to introduce multiple genes into plants by various techniques (e.g., gene stacking) have been somewhat successful (Halpin, 2005). However, lack of co-regulation remains the main obstacle in this latter technique. The best method to date for metabolic engineering is plastid transformation. Since plastids are of prokaryotic origin, they can express several genes from a single polycistronic mRNA. The ability to express a string of co-regulated genes could potentially result in activation of an entire metabolic pathway and the production of its end product, a non-proteinaceous secondary metabolite (Wang et al., 2009). However, for this to happen, an operon along with its regulatory elements has to be artificially constructed, and concerted regulation and optimization of the stoichiometry of the various components has yet to be achieved.

Here we report on a vector system that introduces an entire operon into plants; its genes are expressed, producing the end-product secondary metabolite. The need for plastid transformation is circumvented, administration to plants is easy and yield of successfully expressing plants is high, making the use of selectable markers unnecessary.

RESULTS

Delivery, Replication, Expression and Spread of the prn Operon in Tomato Plants

The components of the plant universal vector IL-60 employed throughout this study are described in Peretz et al. (2007). Briefly, a plasmid was inserted into the rep gene of TYLCV disabling rolling-circle replication, but maintaining replication from dsDNA to dsDNA which is directed solely by host factors. Any DNA placed downstream of the viral IR which carries the origin of replication and two bidirectional promoters will replicate in the cells into which it has been delivered. However, to spread to other cells it requires a helper virus or IL-60-BS, which promotes movement throughout the plant without causing disease. The various constructs employed in this study are illustrated in Fig. S1. All constructs were administered to the plants by root uptake. The root tips of young seedlings were slightly trimmed and immersed in an aqueous solution containing 1 μg of each plasmid per plant. Tap water was added when the solution was fully absorbed by the plants and the plants were immersed for 3 days, after which they were planted in soil.
The entire prn operon of Pseudomonas fluorescens strain Pf-5 (GenBank accession no. CP000076.1; bases 4157074 to 4162815) was cloned in front of the IR in the plasmid pD-IR as described in the Supplemental Experimental Procedures and was designated IR-PRN. The plasmid construct consisted of the IR, a 160-bp stretch of the 5' end of gene V2 (pre-coat) of TYLCV carrying a postulated plant ribosomal binding site (RBS), followed by the prn operon starting at the first ATG of prnA. IR-PRN was administered to plants along with the IL-60-BS "driver" as described in Materials and Methods. IR-PRN replication and spread in the plant was determined by PCR of DNA extracted from leaves and other plant organs. Primers used for amplification of the various segments of the operon are shown in Table S1 and Fig. S2. Two weeks after administration to plants, prn DNA was found in all tested tissues, including flowers and fruits at flowering and fruit set (Fig. 1).

Transcription from IR-PRN was determined by reverse transcription (RT)-PCR (Fig. 2), by analyzing ribosome-bound RNA (as cDNA) and by northern blot analysis (described further on).

Plants Harboring the prn Operon Produce Biologically Active PRN

In bacteria, the end product of the prn operon is PRN. We demonstrated that plants treated with the IR-PRN vector system produce PRN. Extracts of various plant organs were analyzed by HPLC as described in Materials and Methods. HPLC samples were adjusted for equal protein content. PRN was found in extracts of roots and leaves of plants carrying IR-PRN (Fig. 3A) but not in fruits (Fig. 5 IV. LC-MS analysis (Fig. 3C) identified a peak in IR-PRN-treated plants which was absent in control plants, corresponding in mass (254.97 Da) to PRN.

PRN is an antifungal, antibacterial compound that inhibits the growth of a wide spectrum of plant pathogens. One of the affected pathogens is the fungus Rhizoctonia solani. Therefore, we tested the various HPLC-eluted fractions for their capacity to inhibit the growth of R. solani. Biological tests showed that the plant-extracted, HPLC-purified PRN was indeed inhibitory to R. solani (Fig. 3B). It was concluded that the plants produced PRN identical to that produced in bacteria by the same operon.
Further on, prn expression in plants is shown to be polycistronic. To determine whether, in addition to prnA (which carries a viral RBS in front of it), the other three ORFs are also required for PRN production and are not replaced by functionally equivalent plant proteins, we mutated those genes. We first tested whether translation of the most 3’-distal ORF (prnD) originates from the IR-directed construct and not from a gene directed by a plant promoter. We fused GFP (5’UTR was deleted to remove predicted RBSs and the first ATG was omitted to prevent possible translation initiation at the start of the GFP coding region) to prnD to produce IR-PRN-GFP (described in Materials and Methods and illustrated in Fig. S1), and then administered this construct to plants. GFP fluorescence in these plants indicated that prnD was translated from IR-directed polycistronic mRNA and was not of plant origin. The protein prnD-GFP appeared to be secreted into the vacuole and to aggregate (Fig. 4).

A series of controlled experiments indicated that all four genes of the PRN operon are required for PRN production in plants. Plants carrying IR-PRN with deletions in prnB (Fig. 5 VII) or prnC (data not shown) did not produce PRN. Plants expressing IR-PRN-GFP did not produce PRN (probably due to inactivation of the GFP-fused protein PrnD). Plants in which PRN had been replaced by an irrelevant gene (GUS), or TYLCV-infected plants, did not produce PRN (Fig. 5). Only the positive controls exhibited PRN (elution time 27.9 min). PRN could not be detected by HPLC in fruits (Fig. 5 IV) even though the fruit harbored IR-PRN-DNA (Fig. 1B), probably due to interference in expression.

**Generation of a New Phenotype: PRN-Expressing Plants Are Resistant to Damping-Off Disease Caused by R. solani**

Since PRN is inhibitory to R. solani and was produced in all plant tissues except fruits, the PRN-expressing plants were tested for resistance to damping-off disease of tomato caused by R. solani.

Young untreated and PRN-expressing tomato plants were planted in pots containing soil mixed with R. solani mycelium. The control plants, which did not express PRN, wilted within 2 weeks whereas the PRN-expressing plants remained healthy (Fig. 6). Various PRN-producing bacteria have been tested by others for biological control of phytopathogens, but despite their potential antifungal activity,
results have been inconsistent (e.g. Compan et al., 2005). In comparison, 80 to 100% of the PRN-expressing plants were protected from infection.

**PRN-Expressing Plants Produce Operon-Long Transcripts**

A true polycistronic mode of expression has never been demonstrated for eukaryotes. To determine whether the bacterial operon is correctly expressed in plants, we performed northern blot analyses. Figure 7 (lanes 1 and 2) shows that the prn operon was transcribed into two long transcripts (the size of the major transcript is 5.5 to 6 kb and the shorter one is approximately 4 to 5 kb, probably due to different termination signals).

In addition, we isolated polyribosomes from PRN-expressing plants, prepared cDNA from the ribosomal-bound RNA (as described in Materials and methods) amplified long cDNA (5 to 6 kb and p) by PCR with a PRN-specific primer (Fig. 7, lane 3). Two major cDNA species were observed: one was 5 to 6 kb long and the other was longer (possibly due to incomplete 3’ trimming of the nascent transcript). Nevertheless, smaller-size prn transcripts were not detected, indicating lack of further processing to monocistronic mRNAs. Hence, the polycistronic long transcript itself, rather than processed mature RNAs, served as the template for translation, as is the case in prokaryotes.

**DISCUSSION**

The IL-60 system provides a universal expression or silencing tool for all plants tested to date (Peretz et al., 2007). In this study, we report on IL-60-derived expression of a complete bacterial operon in tomato. We chose the prn operon (around 6 kb) of the rhizospheric biocontrol strain Pf-5 of *P. fluorescens* which encodes the broad-range antifungal and antibacterial PRN (Loper *et al.*, 2007; Gross and Loper, 2009) to investigate whether: (i) the IL-60 system can provide delivery and expression of this rather large bacterial operon in plants, (ii) expression of the prn operon in plants leads to the appearance of a major new metabolic trait, manifested in the ability to produce a functionally active secondary metabolite (PRN), and (iii) the ability to produce PRN increases resistance of the treated plants to root rot disease caused by the fungus *R. solani* used as a model plant pathogen.
Secondary metabolites are non-proteinaceous compounds that contribute to the molecular programs required for normal growth and development in plants (Pierre, 2005). They serve as mediators in many metabolic pathways and contribute to plant interactions with the environment, some of which are modulated by plant hormones (Creelman and Mullet, 1997; Wasternack and Parthier, 1997; Loreti et al., 2008). Pigments and fragrance attract pollinators (Raguso, 2004; Grotewold, 2006) and are therefore essential for plant reproduction. At the same time, plant volatiles serve as repellents to herbivores (Gibson and Pickett, 1983; Francis et al., 2004) and following infestation by a pest, alarm pheromones are emitted which attract natural enemies of that pest (Francis et al., 2004). Plant secondary metabolites also participate in the elicitation of induced resistance to pathogens and pests (Fawcett and Spencer, 1966; Baily, 1982; Walling, 2009). Plants produce a plethora of secondary metabolites which are major ingredients in a wealth of potentially economically valuable substances such as pharmaceuticals, food additives, fragrances, natural pesticides and more (Morant et al., 2007). However, the production of these metabolites from plants and plant cultures has not yet seen the transition from economic potential to commercial success (Hadacek, 2002; Zhang et al., 2004). A number of approaches have been attempted to improve the yield of a desired metabolite to a commercially relevant level: metabolic engineering employs genetic engineering techniques to increase production by enhancing gene expression, manipulating a gene's regulatory system (usually transcription factors), preventing branching off to another pathway by downregulating the competing enzyme, and minimizing catabolism (Verpoorte et al., 1999; Verpoorte and Memelink, 2002).

Here, plants expressing the native bacterial prn operon cloned to the IL-60 platform were shown to produce physiological amounts of the secondary metabolite PRN, sufficient to engender a new phenotype, without any additional manipulation. PRN production following administration of the prn operon to tomato was at the pmol/mg fresh tissue level (Fig. 3), a biologically active level which indeed induced the appearance of a new phenotype. The obtained IL-60-PRN construct is transcribed into a polycistrionic mRNA which serves as a template for translation; it can therefore be defined as an operon. An entire metabolic pathway is expressed, producing the secondary metabolite PRN. Operon-type expression was demonstrated by the appearance of operon-long transcripts, an apparent lack of further processing of those transcripts, the ribosome-bound PRN transcripts being of a size compatible with a
full-length polycistronic RNA, and the last gene of the operon indeed being transcribed and translated from an IR-directed transcript. Translation into a polyprotein that would be further processed to maturity and biological activity is inconceivable because of the presence of stop codons and intergenic spaces, and due to the fact that not all ORFs are at the same translation frame.

A plant RBS is present on the IR-PRN construct upstream of \textit{prnA}. However, the issue of plant ribosome recognition of the other RBSs, in front of each of the downstream ORFs, is currently under study. Mutational analyses clearly demonstrated that expression of all of the operon's genes is required for production of the end product PRN. Taken together, these results indicate that under the control of the IL-60 platform, a plant can express an entire metabolic pathway from an operon in a polycistronic manner. To our knowledge, this is the first report indicating that polycistronic mRNA in a eukaryotic system is a template for translation, rather than a pre-mRNA that is further processed to smaller mRNAs.

Operon transformation mediated by the IL-60 system presents several advantages over plastid transformation as described by other authors (Elghabi et al., 2011; Sanz-Barrio et al., 2011; Wei et al., 2011). A comparison between the two operon-expression systems in plants indicates that: (i) the IL-60 system is not transgenic while plastid transformation produces transgenic plants; (ii) preparation and handling of the IL-60 system is much simpler than plastid transformation protocols, and (iii) IL-60 delivery into plants circumvents the need to use selectable markers. It is conceivable that further manipulation of any of the pathway's signals and genes may elevate the level of PRN production to match that of other manipulated native secondary metabolites.

The obtained resistance to root rot disease presents an obvious potential advantage of PRN-producing crops over various PRN-producing bacteria, which have been tested by others for biological control of phytopathogens: despite their potential antifungal activity, results have been inconsistent, probably due to the diversity of environmental niches and sensitivity of PRN to environmental factors such as UV light (Compant et al., 2005). In comparison, 80 to 100% of the PRN-expressing plants were protected from infection. It seems that when expressed within plant tissues, PRN is protected from the external environment, it is continuously produced and it does not need to diffuse into the infected tissue to inhibit the invading pathogen. This may explain the appearance of a new efficiently resistant phenotype in our plant system. It
is worth noting that PRN was found in different parts of the treated plants but not in fruits. Since the operon itself is present in fruits, it is assumed that some conditions/compounds in tomato fruits modify, destroy or are antagonist to PRN activity. This needs to be further investigated.

CONCLUSION

The ability to express an entire metabolic pathway in plants opens a potentially new way of manipulating secondary metabolite levels and production. Once the regulatory elements enabling a polycistronic mode of expression are further elucidated, non-plant metabolites of commercial importance can potentially be biobarmed.

MATERIALS AND METHODS

Vectors, their Administration to Plants and Molecular Procedures

Molecular procedures were carried out according to standard protocols (Sambrook, 2001). The RiboRuler high-range RNA ladder (Fermentas) was used to determine the size of RNA bands in northern blot analyses. IL-60-BS is described in Peretz et al. (2007). The IR segment of TYLCV and the following 166 bp of ORF V2 were PCR-amplified with added KpnI and PstI restriction sites and cloned into the plasmid pDrive (Qiagen). The PRN Operon (GenBank accession # CP000076.1; bases 4157074 to 4162815) was inserted into the same plasmid downstream of IR-V2 between the BamHI and XbaI sites. The construct was designated IR-PRN. In another construct, the gene for GFP was fused to prnD of IR-PRN. A clone of tobacco mosaic virus with GFP of improved fluorescence (30B-GFP3; Shivprasad et al., 1999) was obtained from Dr. William O. Dawson (University of Florida, Lake Alfred). The coding region of GFP was amplified with primers 1334 and 1335 (Table S1) carrying restriction sites for BglII and NdeI. Inverse PCR of IR-PRN, starting at both ends of the stop codon of prnD, was performed with primers 1332 and 1333 carrying restriction sites matching those of the GFP primers. Following ligation, the obtained construct carried IR, part of V2, PRN (without the prnD stop codon) and the coding region of GFP (initiation ATG was deleted). This enabled the translation of a fused,
continuous, prnD-GFP protein. The various constructs are illustrated in Fig. S1. Deletion in prnB was done by cleaving with AjiI and re-ligating. Deletion in prnC was done by cleaving with ScaI and re-ligating.

Polyribosomes were prepared as described by Nur et al. (1995). A cDNA library was prepared from polyribosomal RNA using Smart™ cDNA construction kit (Clontech) according to the manufacturer's instructions. The primer for reverse transcription was 5' GCCAGATAGTCATGAATACCTCGCAAAGAG 3'.

**Extraction of PRN from Plant Tissue, HPLC and LC-MS Analyses**

Plant tissue (4 g) was taken for each extraction. The protein content in each extract was determined separately. The plant tissue was homogenized in chloroform and the homogenate was kept at 4°C for at least 24 h. The mixture was filtered through Miracloth followed by 10 min centrifugation at 3000 g. The supernatant fluid was mixed with an equal volume of 0.1 M K2HPO4 and following phase separation, the aqueous fraction was discarded. The chloroform fraction was rota-evaporated and the resultant dry material was dissolved in 100 µl acetonitrile. Approximately 30 µl of the extract (equivalent to 70 µg protein in the initial plant extract) was subjected to HPLC separation. HPLC was performed on a 100 RP-18 column (Merck, Lichrospher, 15 µm, 250 x 4.6 mm). Elution was done in 45% H2O:30% acetonitrile (GC grade) and 25% methanol or in 65% H2O:35% acetonitril. Flow rate was 1 ml/min and detection was at 225 nm.

In addition, LC-MS analyses were performed on plant extracts. HPLC analysis was performed on an Accela High Speed LC system (Thermo Fisher Scientific Inc) which consists of an Accela pump, autosampler and PDA detector. The Accela LC system was coupled with the LTQ Orbitrap Discovery hybrid FT mass spectrometer (Thermo Fisher Scientific Inc.) equipped with an electrospray ionization ion source. Mass spectrometer was operated in negative ionization mode, with the following ion source parameters: spray voltage 3.5 kV, capillary temperature 250°C, capillary voltage -35 V, source fragmentation was disabled, sheath gas rate (arb) 30, and auxiliary gas rate (arb) 10. Mass spectra were acquired in the m/z 150-2000 Da range. The LC-MS system was controlled and data were analyzed using Xcalibur software (Thermo Fisher Scientific Inc.). The presence of PRN in the sample was
confirmed by high resolution LC-MS analysis (measured mass 254.97284, calculated atomic composition of deprotonated pseudomolecular ion $C_{10}H_5O_2N_2^{35}Cl_2$, error -2.0 ppm).

**Bioassay for Inhibition of *R. solani* in Vitro**

*R. solani* was cultured on potato dextrose agar (PDA; medium # M127 of Bacteriological Analytical Manual, 2001, Food and Drug Administration) at 28°C. A disk (30 mm in diameter) of *R. solani*-containing agar was transferred to another Petri dish containing PDA and 10 µl of HPLC elution buffer (negative control), 10 µl of various plant extracts, or 10 µl containing 0.2 µg synthetic PRN in elution buffer (Sigma, positive control). Inhibition of fungal growth was determined following incubation at 28°C for 3 days.

**Assay for *R. solani* Resistance**

Roots of tomato seedlings were immersed in a water solution of IL-60-BS and IR-PRN or IR-PRN-GFP (1 µg per plantlet), until the entire solution was sucked up by the plants. The plants were then potted and kept in the greenhouse at 24°C. Tomato seedlings, 2 weeks after application of IL-60-BS and IR-PRN, were transferred to pots with 0.5 kg soil mixed with 0.5 g *R. solani* mycelium. Control plants (not carrying PRN) were similarly treated. Plants were kept for 2 to 3 weeks at 24°C until symptom appearance.

**Confocal Microscopy**

Tomato leaf sections, after peeling away the epidermis, were observed under the confocal microscope (Zeiss 100M). Excitation was at 488 nm. GFP emission was detected at 505 to 550 nm. Autofluorescence of chlorophyll was detected at wavelengths greater than 560 nm. Data were processed by the built-in program LSM 51.
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FIGURE LEGENDS

Figure 1. Analysis for the presence of prn genes in treated plants. A. DNA was extracted from systemic leaves and subjected to PCR using different sets of primers (Table S1). M: Size markers. Amplification of: a prnA fragment (lane 1), TYLCV-IR (lane 2), a prnB and prnC-spanning fragment (lane 3), a prnD fragment (lane 4). B. Presence of prnA in various tomato organs. Amplification with prnA primers of DNA extracted from roots (lane 1), leaves (lane 2), fruits (lane 3), leaves of an untreated plant (lane 5), and without template (lane 6). Lanes 4 and 7: Empty. M: Size markers.

Figure 2. RT-PCR analysis for transcription of the prn operon. The amplified sequence is a segment of prnD. RT-PCR of the same plant RNAs amplifying a fragment of tomato actin is depicted in the bottom panel. M: Size markers. Lanes 1-8: Template RNA extracted from various prn-carrying plants. Lane 9: Control; RNA was extracted from untreated plant.

Figure 3. HPLC and LC-MS analyses of the metabolites produced in PRN-expressing tomato plants. A. HPLC elution profiles. I: Synthetic PRN (positive control). II: Root extract of PRN-expressing plants. III: Leaf extract of PRN-expressing plants. IV: Negative control. Root extract of untreated plants. Positions of PRN elution are indicated by arrows. B. Biological activity of the various fractions of panel AII. Each plate contains a disk of agar with R. solani mycelium and addition of (left to right) fractions eluted between 1 and 3 min, fractions eluted between 3 and 5 min, fractions eluted between 5 and 25 min, fractions eluted between 30 and 32 min, and synthetic PRN (0.2 µg/plate). C. LC-MS analysis of tomato extracts. The two upper panels present extracts from plants not carrying IR-PRN. The two bottom panels present extracts from IR-PRN-harboring plants. LC patterns [TIC: total (negative) ion chromatogram] are shown in the first and third panels from the top. Searches for PRN by mass (EIC: extracted ion chromatogram) are shown in the second and fourth panels.

Figure 4. Expression of GFP in tomato plants harboring IR-PRN-GFP. Plant samples were taken 2 weeks after administration of the construct and examined under a confocal microscope (see Materials and methods). A. Picture taken following excitation for GFP fluorescence. B. Picture taken with a filter that masks GFP fluorescence (chlorophyll autofluorescence is in red). C. Picture taken without excitation. D. Superposition of the two upper panels.

Figure 5. HPLC analysis of PRN in control tomato plants. I: Positive control (synthetic PRN). II: Positive control. Extract of roots from plants carrying IR-PRN (same as in Fig. 3AII). III: Extract of roots carrying IR-PRN-GFP. IV: Extract of plants carrying IR-GUS (GUS replacing PRN). V: Extract of plants carrying IR-PRN with a deletion in prnB. Solid arrows mark the position of PRN elution. Dashed arrow mark the expected positions of the eluted PRN.

Figure 6. PRN-expressing plants are resistant to damping-off disease. Left frame: IR-PRN was introduced into tomato seedlings which were then planted in non-infested soil. The plants were transferred to R. solani-infested soil a week after administration of IR-PRN. The picture was taken 2 weeks after R. solani infestation. A PRN-treated plant is shown on the left and a non-treated plant on the right. Right frame: IR-PRN was administered to tomato seedlings which were immediately planted in R. solani-infested soil. The group of plants on the right was PRN-treated and the group on the left consists of untreated plants. The picture was taken 5 days after planting.

Figure 7. IR-PRN is expressed as a long transcript. Lanes 1 and 2: Northern blot analyses of RNA from IR-PRN-harboring plant (lane 1) and untreated plant (lane 2). A segment of prnA served as a probe. Lane 3: Long-distance PCR of cDNA reverse-transcribed from ribosome-bound RNA. Primers for reverse transcription and PCR were from both ends of the prn operon (Supplemental Data). M: Size markers.
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