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ORIGINAL PAPER

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Organization of phenylalanine ammonia lyase (*PAL*), acidic *PR-5* and osmotin-like (*OSM*) defence-response gene families in the potato genome

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Abstract Defence-response (DR) genes are candidates for the genetic functions underlying quantitative resistance to plant pathogens. The organization of three DR gene families encoding phenylalanine ammonia lyase (PAL), acidic PR-(pathogenesis-related) protein 5, and basic PR-5, or osmotin-like (OSM), proteins was studied in the potato genome. A bacterial artificial chromosome (BAC) library containing ~50,000 clones was constructed from high-molecular weight genomic DNA of the diploid potato clone PD59, a hybrid between *Solanum tuberosum* and *S. phureja*. BAC clones carrying one or more copies of the DR genes were identified and characterized by Southern hybridization, sequence analysis and genetic mapping. *PAL*, acidic *PR-5* and *OSM* (basic *PR-5*) genes were all organized into gene families of varying complexity. The *PAL* gene family consisted of at least 16 members, several of which were physically linked. Four acidic *PR-5* homologous were localized to a 45-kb segment on potato chromosome XII. One of these, *PR-5*/*319*, codes for the acidic thaumatin-like protein C found in intercellular fluids of potato. Nine *OSM* genes were organized at two loci: eight form a 90-kb cluster on chromosome VIII, and a single gene was found on chromosome XI. The topology of a phylogenetic tree based on PR-5 and OSM protein sequences from Solanaceae suggests a mode of evolution for these gene families. The results will form the basis for further studies on the potential role of these defence-related loci in quantitative resistance to pathogens.

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Introduction

Natural genetic variation for plant resistance to plant pathogens such as viruses, bacteria and fungi appears in two forms: as a qualitative resistance response to a specific pathogen triggered by a single *R* gene, or as quantitative or partial resistance, which is controlled by an unknown number of genes. The map positions of genes controlling quantitative resistance can be delimited by quantitative trait locus (QTL) analysis using molecular markers. However, genes underlying quantitative resistance have not yet been identified at the molecular level, whereas *R* genes and genes controlling the various defence responses triggered by them have been studied extensively (reviewed in Kombrink and Somssich 1995; McDowell and Woffenden 2003). In potato, a number of *R* genes and QTLs for pathogen resistance have been localized on molecular genetic maps. Some *R* loci co-localize with resistance QTLs (Gebhardt and Valkonen 2001; Simko 2002; Bonierbale et al. 2003), suggesting that quantitative resistance may result from allelic series of *R* genes or from clustered families of *R* gene homologues (Leonards-Schippers et al. 1994). The potato molecular map also contains a number of defence-response (DR) loci, which have been identified by restriction fragment length polymorphism (RFLP) mapping, using cloned DR genes from potato and tobacco as probes (Leonards-Schippers et al. 1994; Gebhardt et al. 2001). Few of these DR loci are located in the same map segments as resistance QTL.

Quantitative trait loci for resistance to late blight caused by the oomycete *Phytophthora infestans* have been mapped to six potato chromosomes in a diploid interspecific hybrid population derived from a cross between the diploid *Solanum phureja* and dihaploid

S. tuberosum ssp. *tuberosum* (the 'PD family'; Ghislain et al. 2001). Markers were developed for a series of DR genes and were found to be linked to QTLs for late blight resistance in the PD family (Trognitz et al. 2002). Based on their functional role in resistance to pathogens, and the observed genetic correlations with resistance QTLs, both *R* gene homologues and defence-response genes are candidates for the genes that underly quantitative resistance (Leister et al. 1996; Faris et al. 1999; Li et al. 1999; Wang et al. 2001; Pflieger et al. 2001).

The gene(s) underlying a given QTL are expected to be present as molecularly and functionally diverse alleles in the genetic material used for QTL mapping. The causal role of a candidate gene in the trait governed by the QTL has to be confirmed by cloning the alleles and using them as transgenes for quantitative complementation analysis in suitable genetic backgrounds. A candidate locus may be complex, however, containing candidate gene families rather than single genes (Trognitz et al. 2002; Paal et al. 2004). The use of large DNA segments for functional complementation, such as the plasmid with a 90-kb insertion used by Ercolano et al. (2004), helps to simplify this task. However, a specific large-insert library must be constructed for the genotype that is heterozygous for the QTL and candidate-gene alleles.

As a prerequisite for the further evaluation of the role of DR genes in quantitative resistance to late blight in the PD family, we have constructed a bacterial artificial chromosome (BAC) library from high-molecular-weight DNA obtained from one hybrid clone of the PD family. Using this BAC library, we determined the structure and genomic organization of three families of defence-response genes encoding phenylalanine ammonium lyase (PAL; a key enzyme in the phenylpropanoid pathway leading to the accumulation of phytoalexins), osmotin-like (OSM) proteins, and acidic pathogenesis related protein 5 (PR-5). These three gene families were selected from the large number of potential DR candidate genes based on their putative linkage to QTL for late blight resistance that have been mapped in the PD population.

Materials and methods

Plant material

The diploid potato clone PD59 was used to construct the BAC library. PD59 is one of 246 hybrids (the PD population) derived from an inter-specific cross between *S. phureja* (clone CHS-625) and dihaploid *S. tuberosum* ssp. *tuberosum* (line PS-3) (Ghislain et al. 2001). PD59 was selected for its resistance to late blight and, based on linked marker alleles, for combining positive effects at QTLs on chromosomes III, V, VII, VIII, XI and XII. PD59 was obtained as an in vitro culture from the International Potato Center (CIP, Lima, Peru). Plants were propagated from stem cuttings in MS medium (Murashige and Skoog 1962). Rooted plantlets were transferred to the greenhouse and grown under normal daylight conditions. Before harvesting leaves for protoplast isolation, plants were kept in the dark for 3 days to decrease leaf starch content. For mapping purposes, two diploid populations were used. The PD population, in which QTLs for late blight resistance have been mapped (Ghislain et al. 2001) and population F1840, which consists of 92 F1 individuals obtained from the cross P18 (H82.337/49) × P40 (H80.696/4) (Gebhardt et al. 1991, 2003; Leister et al. 1996).

Preparation of high-molecular-weight (HMW) DNA

High-molecular-weight DNA was prepared from PD59 protoplasts. Leaves were cut into thin slices with a sterile razor blade and incubated in 100 ml in Protoplast Enzyme Solution [containing per 100 ml: 450 mg MS salts, 13.7 g sucrose, 10 mg inositol, 25 mg xylose, 0,1 ml MS vitamins, 100 mg cellulase R10 (Sigma-Aldrich, St. Louis, USA), 500 mg macerozym R10 (Serva, Heidelberg, Germany), pH 5.6] overnight at 28°C in the dark. Leaf debris was removed, first by filtering the protoplast suspension through a stainless steel sieve (50-µm mesh) and then by centrifugation at 800 rpm for 5 min at 4°C. The cells floating on top were transferred to a new tube filled with W5 buffer (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, pH 5.6–6.0) and centrifuged at 500 rpm for 5 min at 4°C. The protoplasts were then checked under the microscope and resuspended in a suitable volume of MaMg buffer (450 mM mannitol, 15 mM $MgCl_2$, 5 mM MES, pH 5.5–6.7) to a concentration of 2×10^7 protoplasts/ml. The cells were mixed with an equal volume of 1% low-melting-point agarose (Epicentre) dissolved in MaMg buffer, and aliquoted into plug molds (Bio-Rad). Plugs were washed according to Choi and Wing (http://www.genome.clemson.edu/protocols) and stored in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) at 4°C.

Plasmid vector preparation

pCLD04541 (Jones et al. 1992) was isolated using a Plasmid Maxi Kit (Qiagen) and further purified by two rounds of cesium chloride gradient centrifugation (Sambrook et al. 1989). After complete digestion with *Hin*dIII, the plasmid was extracted with phenol/chloroform, resuspended in TE and stored at −20°C. Prior to ligation, the vector was dephosphorylated with shrimp phosphatase (Roche Diagnostics) by incubating 500 ng of plasmid with 1 U of the enzyme at 37°C for 1 h. The enzyme was then inactivated by incubation at 65°C for 30 min.

Library construction and characterization

Individual plugs were sliced into eight pieces and incubated for 1 h on ice in a total volume of 150 µl of restriction enzyme buffer (10 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, pH 8.0) containing 0.1 mg/ml BSA and 1.6 mM spermidine. After adding 6 U of *Hin*dIII, the reaction was further incubated on ice for 10 min. After digestion at 37°C for 20 min, the reaction was placed on ice, and a 1/10th volume of 0.5 M EDTA (pH 8.0) was added to inhibit the enzyme. The agarose pieces were loaded onto a 1% low melting point agarose gel in 0.5×TBE. Lambda ladder PFGE (New England Biolabs) was used as a size marker. Pulsed field gel electrophoresis (PFGE) was performed in a CHEF-DR III (Bio-Rad) using the following settings: initial switching time 60 s, final switching time 90 s, voltage gradient 6 V/cm, angle 120°, running time 19 h at 14°C. After electrophoresis, the gel piece containing the size ladder and 0.5 cm of digested HMW DNA was stained with ethidium bromide and visualized under UV light. Stained and unstained gel pieces were aligned side by side and fragments in the 100–500 kb range were cut from the unstained gel. Gel slices containing different DNA size fractions (fraction 1: 250–300 kb, fraction 2: 150–250 kb, fraction 3: 100–150 kb) were collected separately. The gel slices were incubated with gelase (Epicentre) following the supplier's instructions, except that the concentration of NaCl in 1× gelase buffer was increased to 100 mM to improve the recovery of DNA fragments larger than 100 kb. Aliquots (100 ng) of partially restricted, size-selected DNA were ligated to the same amount of *Hin*dIII-restricted and dephosphorylated pCLD04541 vector in 110 µl of 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000, containing 6 U of T4 DNA ligase (Life Technologies). Ligations were incubated at 16°C for at least 24 h. After ligation, salts were removed by dialysis against 0.1 × TE using 30,000 NW filters (Millipore). For transformation, 1 µl of ligation reaction was mixed with 25 µl of transformation-competent DH10B *Escherichia coli* cells (Life Technologies) and transformed by electroporation using a Gene Pulser (Bio-Rad). The electroporator settings used were: capacitance 25 µF, voltage 1.8 kV, resistance 100 Ω. Transformed cells were recovered in 600 µl of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4/MgCl_2$ and 20 mM glucose), incubated at 37°C for 50 min and plated on LB medium containing tetracycline (15 mg/l), IPTG (14 mg/l) and X-gal (60 mg/l). After incubation for 24 h, recombinant clones were picked and checked for insert size (see below). Ligation mixtures with acceptable insert size and transformation efficiency were used to construct the library. Multiple transformations were carried out and plated on 22×22 cm plates. Recombinant clones were picked using a Q-Pix robot (Genetix) and inoculated into 384-well plates prefilled with LB freezing medium (Sambrook et al. 1989). Replicas were made from each 384-well plate using a BioGrid robot (BioRobotics) and stored at −70°C. Plasmid DNA was isolated from 90 randomly picked BAC clones by alkaline lysis (Sambrook et al. 1989). The inserts were released by digestion with *Not*I, and their sizes were determined by PFGE on 1% agarose (initial switching time 5 s, final switching time 15 s, potential gradient 6 V/cm angle 120°, running time 16 h at 14°C).

Library screens

High-density colony filters were prepared from the BAC library using the BioGrid robot. In all, 27,648 independent clones were double-spotted onto Pall membranes (Biodyne) in a 5×5 pattern. The filters were processed according to Sambrook et al. (1989). The probes were labeled with [$\alpha-^{32}$P]dCTP using the random priming method (Feinberg and Vogelstein 1984). Filter hybridization and determination of BAC position was performed as described previously (Gebhardt et al. 1989; Ballvora et al. 2002). The library was screened with labeled probes for the following defence-response genes: a cDNA (NtPR-5) from tobacco encoding the pathogenesis related gene PR-5, provided by John Ryals (Payne et al. 1988); a cDNA (PAL) from potato encoding phenylalanine ammonia-lyase, provided by Erich Kombrink (Fritzemeier et al. 1987), and potato osmotin-like genes. Primers for osmotin-like genes (Table 1) were designed on the basis of the consensus sequence of the GenBank accessions X65701, S40046, X61679, AF093743, X72927, X72928, X67244 and M21346. A 700-bp PCR product was amplified and used as the probe.

Construction of BAC subclones

DNA fragments obtained by digestion of BACs (1 µg of BAC plasmid DNA) with *Hin*dIII, *Xho*I, *Xho*I/*Not*I or *Eco*RV which showed homology to genes for osmotin-like or acidic PR-5 proteins were subcloned into pBluescript (Stratagene) after purification on Quiaquick columns (Qiagen), and transformed into DH10B cells using standard procedures (Sambrook et al. 1989). Osmotin subclones were identified with the osmotin primers (Table 1). Subclones harboring acidic PR-5 sequences were identified by PCR screening with E22 primers (Table 1). The E22 primers were designed based on sequence motifs conserved between the T7 end of BAC clone BB43d14 and GenBank accessions X15224 and × 15223.

Sequence analysis

Plasmid DNA was isolated using the Plasmid Midi Kit (Qiagen). The BACs and subclones were end-sequenced

Table 1 PCR-based markers and hybridization probes

Probe and marker names	Forward (f) and reverse (r) primers (5′ → 3′)	TA[a] (°C)	Size (bp)	Marker type and enzyme[b]
PR-5, E22	GATGGTAGTGGCCGAGGCAAATG (f) TCCAGCAGGACATGTAAACAAACT (r)	56	400 + 500	SCAR
PR-5, 319	GGACACGAACCTACAATTGCAGATG (f) GGTAATGTCAAAAGTGGCAGCATGAGT (r)	56	400	CAPS; *Ava*II
PR-5, 321c	CACACAACAATTACGATACTCCTTC (f) GAGATGTCCTTAAACAGAGAGAGTAG (r)	56	500	CAPS; *Apo*I
Osmotin	GAGGTACGCAACACCTGTCCATAC (f) AGGGGAAATTTGGGCTAGTAACAC (r)	60	700	–
Contig A	GCTTCACCATGAAACGGGTCTACA (f) CCTTTCGACTTACATGACCATTACG (r)	58	800	CAPS; *Alu*I, *Mbo*I
Contig B	TGATGGTGCTGGTAGAGGTTCTTG (f) AAGACCGATTGCCTGAAGCATTAG (r)	58	700	CAPS; *Alu*I
S. phu pA35	GAGAAAATGAGTCACTTGACAA (f) TCAGATCTTAGCCACTTCAAGC (r)	60	844	CAPS; *Mbo*I

[a]TA annealing temperature
[b]*CAPS* Cleaved amplified polymorphic sequence; *SCAR* sequence-characterized amplified region

using T3 and T7 primers. Subclones were sequenced by primer walking. Custom sequencing was performed by the ADIS unit at the Max Planck Institute for Plant Breeding Research, using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and an AB377 automated DNA Sequencer (PE Biosystems). DNA sequence analysis was performed using the Wisconsin Package, version 10.0 (Genetics Computer Group). The programs BLASTN and BLASTX from NCBI were used to search for sequence homology. Sequences of the subclones were assembled and analyzed using Jellyfish 1.3 (Bioware). SignalP software (http://www.cbs.dtu.dk/services/SignalP) was used to predict signal sequences. The sequence alignment for the phylogenetic tree was performed with the Wisconsin Package, and the phylogram was constructed with the program TreeTop-Phylogenetic Tree Prediction, provided by Genebee (http://www.genebee.msu.su/services/phtree_reduced.html).

Determination of the parental origin of BACs

Amplicons were generated with the same primers from 'haploid' BAC plasmid DNA and from genomic DNAs of the *S. phureja* and *S. tuberosum* parents of PD59, based on BAC end or internal sequence information. The amplicons were sequenced and the sequences were aligned. Single nucleotide polymorphisms (SNPs) that discriminated between the parents were used to determine the parental origin of BACs, based on the SNP allele present in the BAC sequence.

PCR-based markers and linkage mapping

Genomic DNA templates (25 ng) were amplified in 25 µl of PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 200 µM dNTPs, 2.5 mM $MgCl_2$, containing 0.3 pmol of each primer (Table 1) and 2 U of Taq polymerase (Life Technologies). Cycle parameters were as follows: 96°C for 5 min, followed by 30 cycles of 94°C for 20 s, 56–60°C (see Table 1 for precise annealing temperatures) for 30 s and 72°C for 1 min, and a final extension cycle at 72°C for 6 min. Segregating PCR fragments were scored as present or absent in the diploid mapping populations F1840 (Gebhardt et al. 2003) or PD (Ghislain et al. 2001). The fragments were mapped relative to the existing marker framework databases using the software package MAPRF (Ritter et al. 1990) or MAPMAKER/EXP v3.0b.

Results

BAC library construction and characterization

A BAC library of about 50,000 clones was constructed from HMW DNA of the diploid, interspecific hybrid clone PD59. After partial digestion with *Hin*dIII and size-fractionation by PFGE, ligations of three DNA size fractions (250–300, 150–250, and 100–150 kb) were used for transformation, and the average insert size of random clones was assessed by PFGE after digestion with *Not*I. The largest average insert size of around 80 kb was obtained with fraction 3, which contained DNA fragments in the size range from 100 kb to 150 kb. Slight differences in DNA concentration influence mobility during PFGE electrophoresis, such that the DNA size marker does not always provide an exact indication of fragment size. Similar results have been obtained by others (Vinatzer et al. 1998; Allouis et al. 2001; Ming et al. 2001). Cloning of the larger sized fractions resulted in smaller sized inserts, most probably, due to the preferential transformation of smaller DNA fragments that were trapped in these fractions during PFGE (Woo et al. 1994; Zhang et al. 1995;

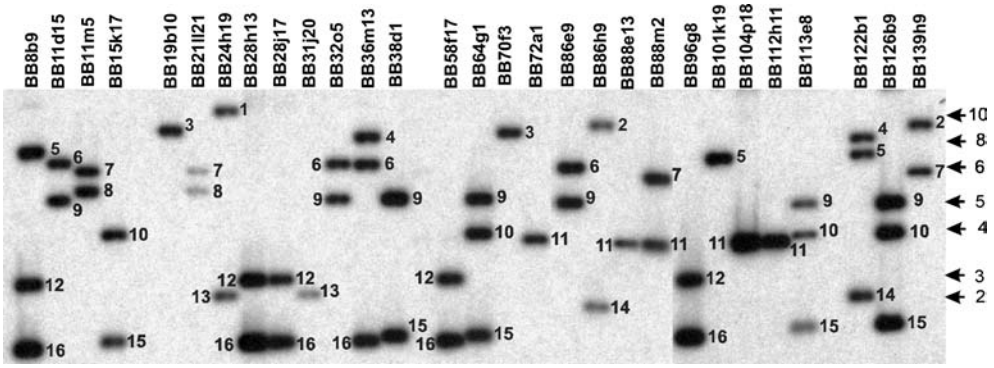

Fig. 1 Southern analysis of 29 BACs with a potato PAL cDNA probe. Plasmid DNA was digested with *Hin*dIII. Positions of size markers (sizes in kb) are indicated by the *arrows* on the *right*. The different *Hin*dIII fragments are numbered from *1 to 16*. The

Fritjers et al. 1997). The library constructed with DNA from fraction 3, with an average insert size of 80 kb, was used for all subsequent analyses. Based on a potato haploid genome size of ~850 Mb (Arumuganathan and Earle 1991) and an average insert size of 80 kb, the 50,000 clones in this library represent 4.7 genome equivalents. Due to the interspecific nature of PD59, the BAC library actually represented around 2.3 genome equivalents each of the *S. tuberosum* and *S. phureja* genomes.

Genomic organization of *PAL* genes

Screening of high-density colony filters of the entire BAC library with a potato phenylalanine ammonia-lyase (*PAL*) probe identified 29 cross-hybridizing clones. Southern analysis of the *Hin*dIII restricted BAC plasmids revealed at least 16 different cross-hybridizing restriction fragments in the size range between 1 kb and 10 kb (Fig. 1). Assuming that each restriction fragment corresponds to at least one gene copy, 16 *PAL* genes, including orthologous genes from *S. tuberosum* and *S. phureja*, are represented in the BAC library. The true gene number might be lower due to the presence of internal *Hin*dIII restriction sites or higher if more than one gene copy is present per restriction fragment. The frequency of individual restriction fragments in the 29 BACs was between one and six, with an average of 3.4 (1.7 for each parent), indicating a lower redundancy (genome coverage) of the library than was estimated based on genome size, average insert size and clone number (see above). Seventeen different, partially overlapping fragment patterns were distinguished among the 29 BACs, of which fourteen were composed of two or three restriction fragments (Fig. 1). This shows that potato PAL is encoded by a multigene family, and that members of the family are often closely linked in the genome.

Genomic organization of acidic *PR-5*-homologous genes

Screening of the entire BAC library with the tobacco cDNA probe NtPR-5 (Accession No. X12739; Payne et al. 1988; Ward et al. 1991) identified a single, 45-kb BAC clone (BB43d14). A Southern gel blot bearing *Hin*dIII-restricted DNA of PD59 and BB43d14 was probed with NtPR-5 (Fig. 2). The three major cross-hybridizing fragments detected in PD59 were all present in BB43d14, suggesting that most, if not all, potato *PR-5* homologues were included in this BAC. Sequencing of both ends of the BB43d14 insertion revealed one end with 89 and 81% sequence identity to the tobacco genes E22 (X15224) and E2 (X15223), respectively. E22 and E2 encode the major and minor forms, respectively, of PR-S (= PR-R = NtPR-5) from *Nicotiana tabacum* (Van Kan et al. 1989). *Eco*RV, *Xho*I and *Xho*I-*Not*I restriction fragments of BAC BB43d14 containing *PR-5* homologous sequences were subcloned and partially sequenced. Four different PR-5-homologous ORFs were found in subclones 319 (7 kb), 321 (7 kb) and 21 (4 kb). ORFs PR-5/319 (227 amino acids; GenBank Accession No.

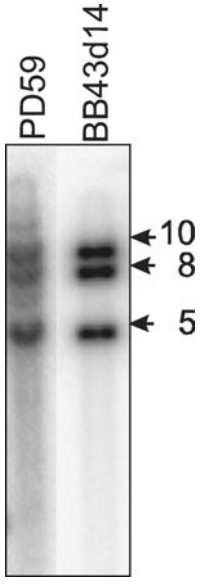

Fig. 2 Southern analysis of plant PD59 and BAC clone BB43d14 with the NtPR-5 cDNA probe. DNA was restricted with *Hin*dIII. The positions of size markers are shown on the *right*

AY737315) and PR-5/21 (229 amino acids; AY737316) were 3 kb apart, whereas, ORFs PR-5/321c (246 amino acids; AY737317) and PR-5/321d (221 amino acids; AY737317) were tandemly duplicated without any intervening sequence. ORF PR-5/321d was shorter due to the apparent lack of the expected N-terminal sequence (Fig. 3). BB43d14, and therefore, all PR-5 ORFs, originated from the *tuberosum* parent of PD59. Like other *PR-5* genes (Velzhahan et al. 1999), the four ORFs did not have introns. The three apparently complete ORFs included a putative signal peptide of 21–25 amino acids at the N-terminus. The physical distance of PR-5/321c and PR-5/321d from ORFs PR-5/319 and PR-5/21 was not determined. The alignment of the deduced amino acid sequences of the four ORFs (Fig. 3) shows that the conserved regions of the proteins include 16 cysteine residues.

The position of the acidic *PR-5*-homologous genes on potato chromosome XII (Fig. 4; Gebhardt et al. 2001) was confirmed by mapping a 500-bp PCR product obtained with E22 primers (Table 1), which segregated in the F1840 population. To map the *PR-5* locus in the PD population, two CAPS markers (PR-5/319, PR-5/321c, Table 1) were developed based on single nucleotide polymorphisms (SNPs) between the alleles present in the *S. phureja* parent of the PD population, which was heterozygous in the putative promoter region of genes *PR-5/319* and *PR-5/321c* (not shown). Both markers mapped to linkage group XII of the *S. phureja* parent (not shown). This map position does not overlap with that of the late blight QTL on linkage group XII of the *S. phureja* parent (Ghislain et al. 2001). Due to apparently high homozygosity at the *PR-5* locus in the *S. tuberosum* parent, we could not assess whether the *PR-5* locus overlaps with the late blight QTL on linkage group XII of the *S. tuberosum* parent.

Genomic organization of basic *PR-5* (osmotin-like)-homologous genes

Screening of the BAC library with an osmotin probe identified six positive BACs: BB18h19 (85 kb), BB41d14 (65 kb), BB48h15 (90 kb), BB82a11 (70 kb), BB86e10 (100 kb) and BB99n12 (35 kb). Comparison of *Hin*dIII restricted DNAs of PD59 with the BACs on the Southern blots hybridized to the osmotin probe showed that all gene copies present in PD59, with the exception of two weakly cross-hybridizing genomic fragments, were accounted for in the six BACs, (Fig. 5a). Sequencing of the BAC ends revealed 94% sequence identity between the T3 end of BB41d14 and an osmotin-like gene of *S. commersonii* (X72927). PCR primers were designed from the BAC insertion ends in order to detect overlaps and orient overlapping BACs relative to each other. This resulted in the assembly of two contigs of approximately 100 kb (A) and 120 kb (B), respectively (Fig. 5b). Comparative sequence analysis of the amplicons from BACs and parental lines confirmed the overlaps and determined the origin of each BAC from one or other of the parents of the PD population. Eight genomic *Hin*dIII fragments were distinguished and

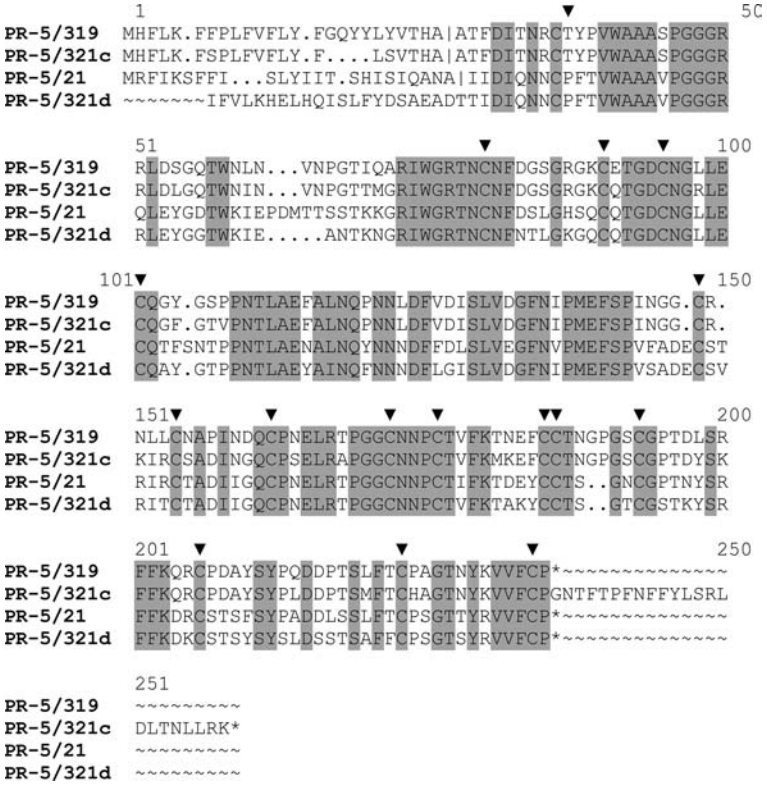

Fig. 3 Alignment of amino acid sequences deduced from *PR-5* homologous genes located on BB43d14. ORFs PR-5/21, PR-5/319 and PR-5/321c are complete. ORF PR-5/321d lacks the N-terminal region when compared to the other genes, and is fused to the stop codon of ORF PR-5/321c. Amino acids conserved in all genes are *shaded*. The 16 cysteine residues conserved in all PR-5 proteins are indicated by *inverted filled triangle*, and stop codons by *asterisks*. The most likely site of cleavage of the putative signal peptide is indicated by a *vertical line*

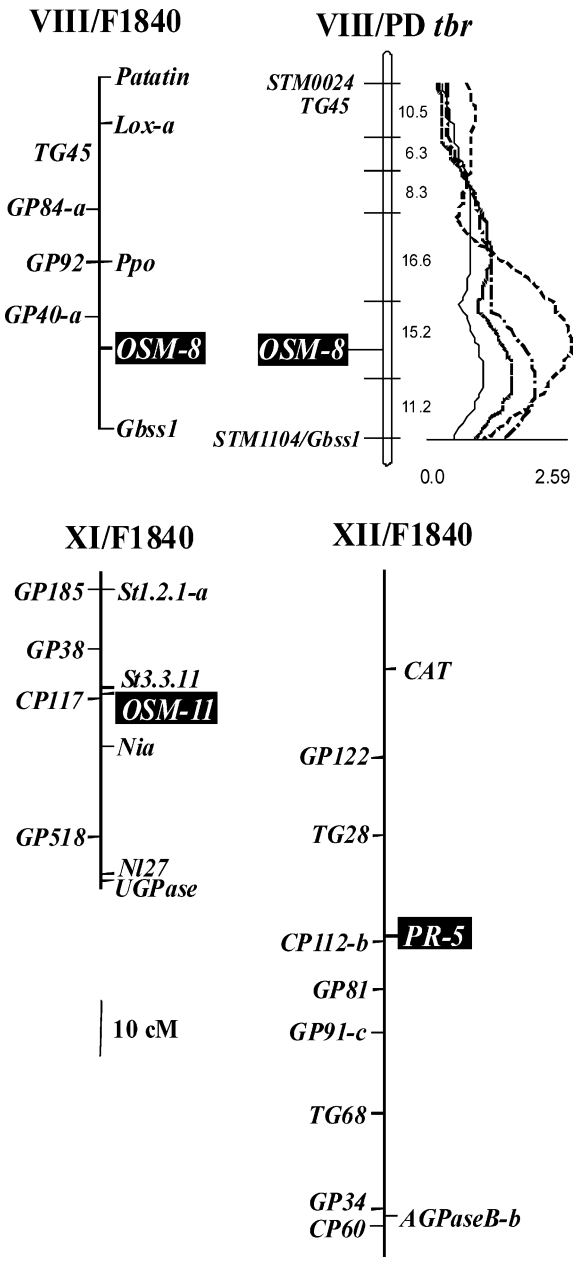

Fig. 4 Positions of *PR-5* and *OSM* loci on potato molecular maps. Linkage groups VIII, XI and XII of the F1840 mapping population are shown, together with a subset of the RFLP loci that have been mapped to each (http://gabi.rzpd.de/PoMaMo.html). RFLP loci identified by functional gene probes are shown to the *right* of the linkage groups. The suffixes *-a*, *-b* and *-c* indicate that more than one RFLP locus was detected with the same marker probe. *Lox* lipoxygenase; *Ppo* polyphenol oxidase; *Gbss1* granule-bound starch synthase 1: *St1.2.1*, *St3.3.11* resistance-gene-like sequences; *Nia* nitrate reductase; *Nl27* N-resistance-gene-like sequence; *UGPase* UDP-glucose pyrophosphorylase; *CAT* catalase; *AGPaseB* ADP-glucose pyrophosphorylase B. Linkage group VIII of the *S. tuberosum* parent of mapping population PD was adapted from Ghislain et al. (2001) and is based on AFLP (names not shown) and microsatellite (STM****) markers. STM1104 is a microsatellite within the noncoding region of the *Gbss1* gene (Milbourne et al. 1998). Marker loci *TG45*, *OSM-8* and *Gbss1* are shared between the two molecular maps of potato chromosome VIII. LOD curves for a late blight QTL on linkage group VIII of the *S. tuberosum* parent of population PD (Ghislain et al. 2001) are shown to the *right* of the linkage group

numbered from one to eight (Fig. 5a). Based on the presence or absence in the overlapping BACs, the approximate positions of the eight *Hin*dIII fragments in the contigs were deduced (Fig. 5b). *Hin*dIII fragments 1 (7.2 kb, GenBank Accession No. AY737312) and 2 (5.7 kb, AY737313) of BB18h19, *Hin*dIII fragments 3 (8 kb), 4 (5.8 kb, AY737311) and 5 (3.4 kb) of BB48h15 and *Hin*dIII fragment 8 (6.8 kb, AY737314) of BB82a11 were subcloned and sequenced. *Hin*dIII fragments 5 and 3 were adjacent to each other (Accession No. AY737310). This was confirmed by sequencing an amplicon from BB48h15, which spanned the adjacent ends of the subclones bearing *Hin*dIII fragments 3 and 5 (Fig. 5b). Comparison of the sequences of the subclones with the databases revealed nine osmotin-like ORFs without apparent introns: OSM-5a, OSM-3b, OSM-3f, OSM-3c, OSM-4d, OSM-1g, OSM-1h and OSM-2d in contig A, and OSM-8e in contig B (Fig. 5b). The ORFs OSM-2d and OSM-4d (derived from *S. tuberosum* and *S. phureja*, respectively) were most similar to each other (97.7% at the nucleotide and 99.2% at the amino acid sequence level). Upstream and downstream sequences were also highly similar (97.2 and 94.8% identity, respectively). This suggested that *OSM-2d* (*S. tuberosum*) and *OSM-4d* (*S. phureja*) are orthologous genes (Fig. 5b). The alignment of the deduced amino acid sequences is shown in Fig. 6. The eight osmotin-like genes in contig A encoded proteins of between 226 and 250 amino acids (OSM-2d, OSM-4d and OSM-3c: 250 amino acids, OSM-1h and OSM-1g: 226 amino acids, OSM-3f: 246 amino acids, OSM- 3b and OSM-5a: 247 amino acids), which all had the 16 cysteine residues that are highly conserved in the PR-5 family, and shared more than 90% similarity with other osmotin-like genes. The single osmotin-like gene present in contig B encodes a shorter polypeptide of 220 amino acids (OSM-8e). An internal 10-amino acid deletion included cysteine residues 11 and 12. Signal peptides of 21–26 amino acids length were predicted for the osmotin-like proteins in contigs A and B.

The CAPS markers specifically developed for contigs A and B (Table 1, Fig. 5b) segregated in the F1840 mapping population and identified two loci, *OSM-8* and *OSM-11*, on linkage groups VIII and XI, respectively (Fig. 4). A further PCR marker was designed for the osmotin-like gene pA35 of *S. phureja* (Accession No. AY743928) (Table 1), which is most closely related to OSM-2d, OSM-3c and OSM-4d (Fig. 7, see below) located within contig A on chromosome VIII. This marker segregated in population PD (Ghislain et al. 2001) and identified the same *OSM-8* locus (Fig. 4). *OSM-8* on chromosome VIII of the *S. tuberosum* parent of the PD population was linked to a QTL for late blight resistance (Fig. 4).

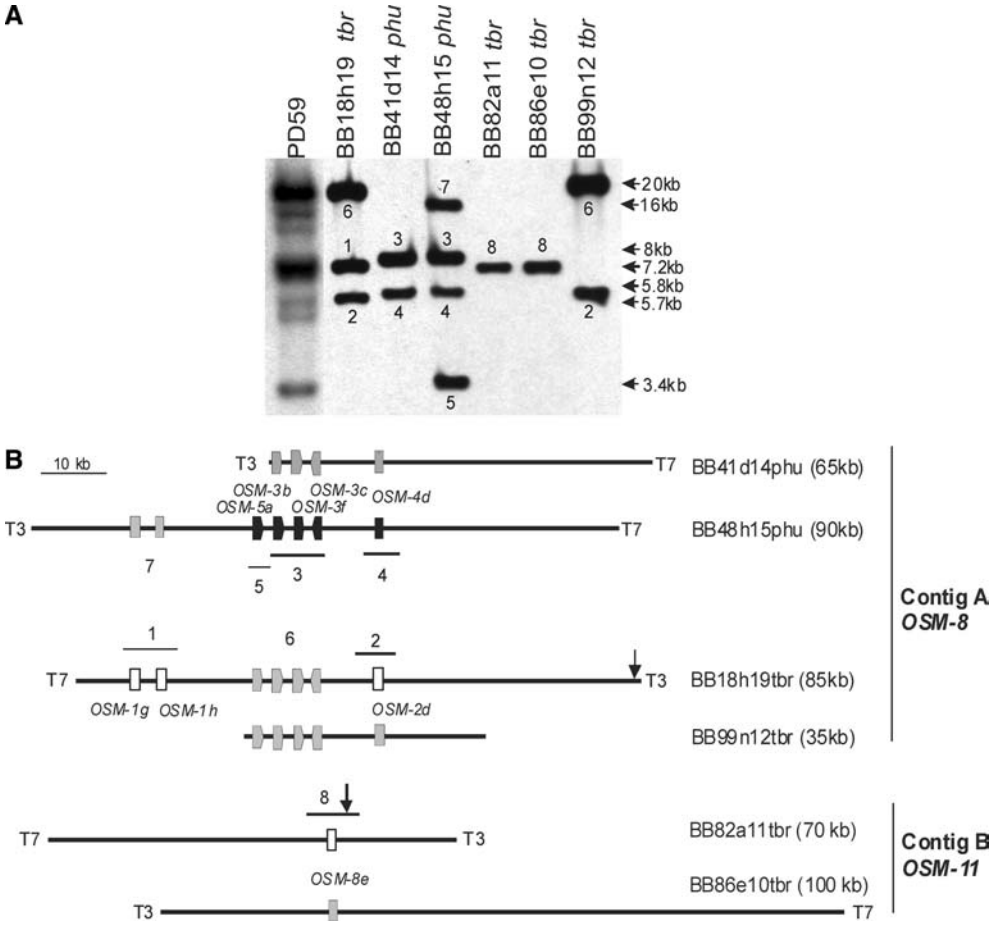

Fig. 5 a, b Genomic organization of osmotin-like genes. **a** Southern analysis of plant PD59 and six BACs selected with the osmotin-like probe. DNA was digested with *Hind*III. The different *Hind*III restriction fragments are numbered from one to eight and their sizes (kb) are indicated on the *right*. **b** Contig A and Contig B based on four and two overlapping BACs, respectively. The relative and approximate positions of eight *Hind*III restriction fragments in contigs A and B are shown, and the sequenced subclones are indicated by the corresponding fragment numbers. Nine osmotin-like genes (*OSM*) were identified by sequencing the subcloned *Hind*III fragments 1, 2, 3, 4, 5 and 8. *OSM* genes of *S. phureja* and *S. tuberosum* are indicated as *black* and *white boxes*, respectively. Inferred *OSM* genes that were not sequenced are shown as *grey boxes*. The *arrows* indicate the positions of the CAPS markers (Table 1) used to map contigs A and B

Phylogenetic tree of acidic and basic PR-5 polypeptides in solanaceous species

A phylogenetic tree was constructed based on the deduced, full-length sequences of acidic PR-5 and basic PR-5-like (osmotin-like) polypeptides from *S. phureja* and *S. tuberosum*, homologues from other solanaceous species, and the two most closely related acidic PR-5 homologues from *Arabidopsis thaliana* (Fig. 7). The polypeptides from solanaceous species were clearly separated from those of Arabidopsis, and formed four major clusters. Cluster I included acidic PR-5 proteins from *S. tuberosum* and *Nicotiana tabacum*. Cluster II included osmotin-like proteins OSM-3c, OSM-2d, OSM-4d and homologues from *S. lycopersicum* (tomato), *S. commersonii, S. phureja* and *N. sylvestris*. Cluster III included OSM-3b, OSM-3f, OSM-5a, homologues from other *Solanum* species (*S. commersonii, S. lycopersicum, S. nigrum*, *S. dulcamara*) and from *Capsicum annum, Petunia hybrida* and *N. tabacum*. Cluster IV was new and consisted of osmotin-like proteins OSM-1h, OSM-1g (both encoded on chromosome VIII) and OSM-8e (encoded on chromosome XI). The protein encoded by *PR-5/21* was separated from all four clusters and formed a unique branch.

Discussion

Large insert libraries of tuber-bearing *Solanum* species

We have constructed a BAC library from an interspecific hybrid between *S. tuberosum* and *S. phureja*, which are both cultivated potato species. Other BAC libraries have been constructed for different *S. tuberosum* genotypes (Kanyuka et al. 1999; Ballvora et al. 2002) and for the wild potato species *S. bulbocastanum* (Song et al. 2000; Vander Vossen et al. 2003). Together with large-insert libraries for the highly colinear genome of tomato, *Solanum lycopersicum* (Martin et al. 1992; Bonnema et al. 1996; Hamilton et al. 1999; Budiman et al. 2000), these represent a unique resource for comparative genome analysis at the intra- and interspecific level in the Solanaceae family. High-density colony filters of the library can be screened by hybridization with homologous probes to isolate full-length genomic clones in a single step. With an average insertion size of 80 kb, the library is also suitable for investigating the organization of gene families in physically linked clusters. The analysis of the *OSM-8* locus showed that orthologous genomic regions from *S. tuberosum* and *S. phureja* can be identified, which may then be tested for quantitative

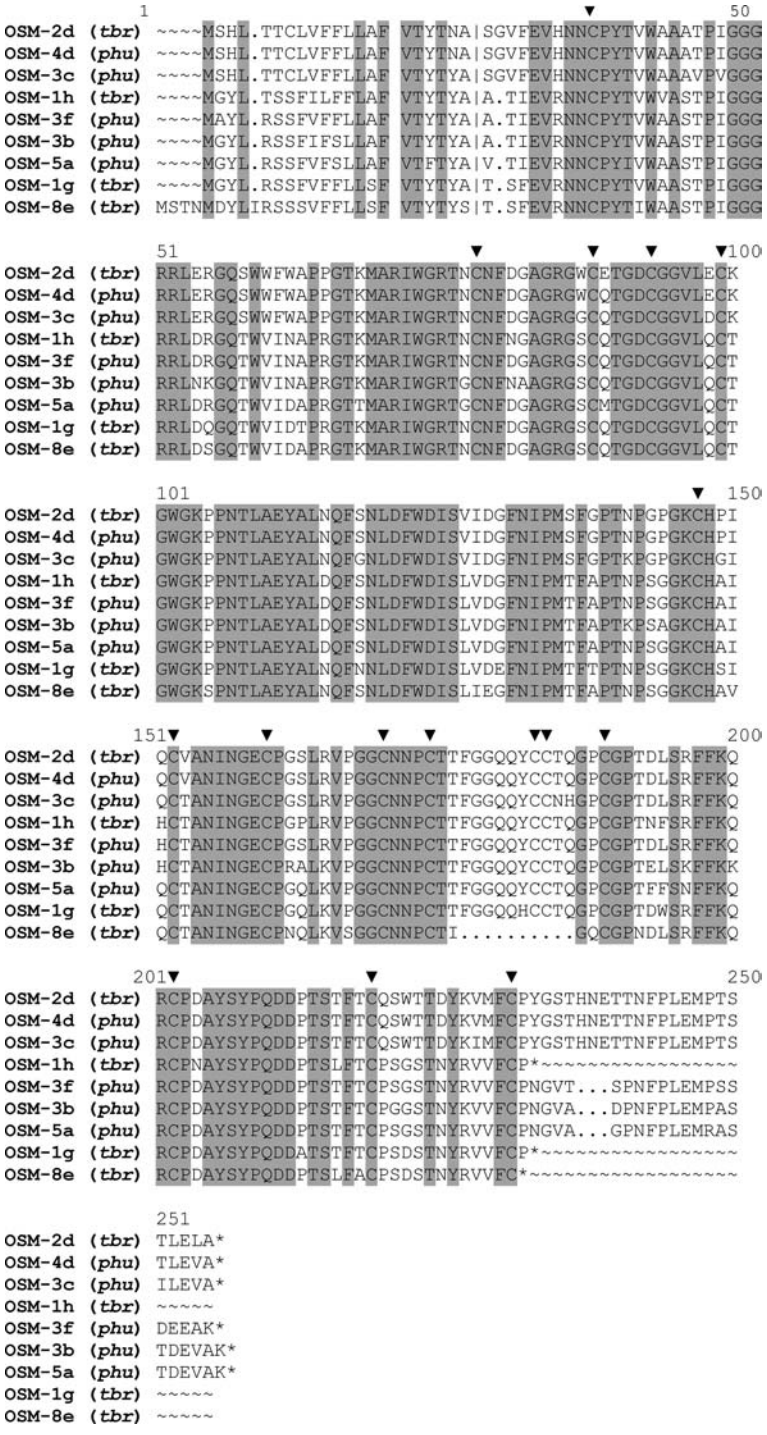

Fig. 6 Alignment of amino acid sequences deduced from nine osmotin-like genes located on BB48h15 (*S. phureja*), BB18h19 (*S. tuberosum*) and BB82a11 (*S. tuberosum*). Amino acids conserved in all genes are *shaded*. The 16 cysteine residues conserved in all OSM proteins are indicated by *inverted filled triangle*, and stop codons are indicated by *asterisks*. The most likely site for cleavage of the putative signal peptide is indicated by the *vertical line*

complementation by ballistic transformation of whole BAC DNA (Ercolano et al. 2004).

Genomic organization of defence-response genes

Three defence-response genes were studied by Southern analysis of selected BACs, sequencing and genetic mapping. All three genes are members of multigene families, which are clustered at one or more loci in the potato genome. The *PAL* family was the most complex. The dihaploid potato genome was estimated to contain 40–50 *PAL* genes, based on copy number titration (Joos and Hahlbrock 1992), which corresponds to 20–25 gene copies per haploid genome. Our estimate of around 16 copies (based on the number of different *Hin*dIII restriction fragments present in 29 BACs) is somewhat lower. This difference may reflect the imprecision of both methods for estimating gene copy number, or it may be due to incomplete representation of *PAL* genes

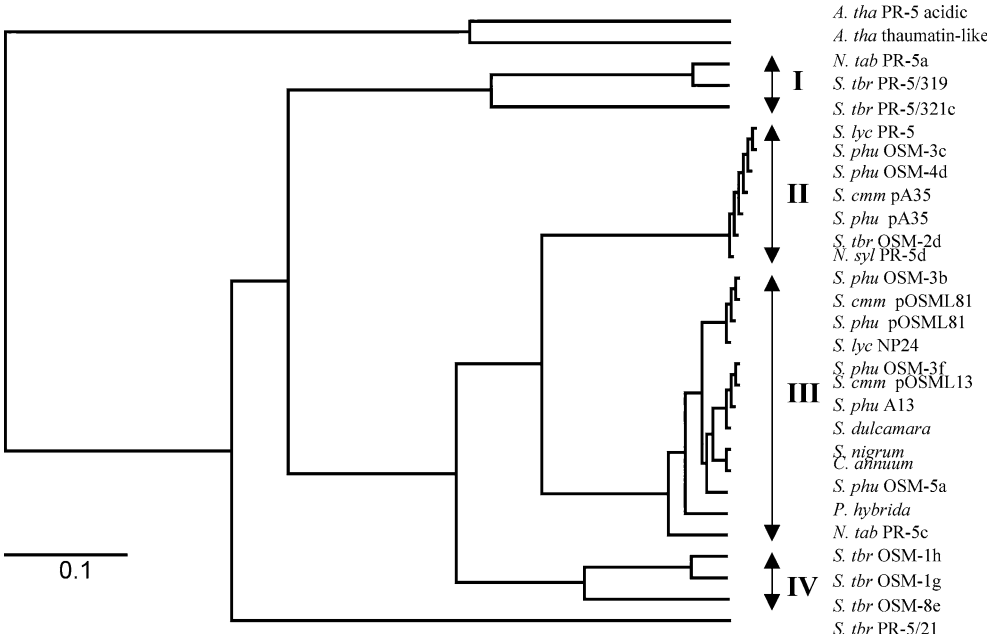

Fig. 7 Phylogenetic tree (GeneBee) constructed on the basis of the amino acid sequences deduced from three full-length *PR-5* homologous genes of *Solanum tuberosum* and nine osmotin-like genes of *S. tuberosum* or *S. phureja* described in this paper, and homologues from other plants. *A. tha* PR-5 acidic and *A. tha* thaumatin-like: *Arabidopsis thaliana* GenBank Accessions M90510 and U83490. *N. tab* PR-5a and *N. tab* PR-5c: *Nicotiana tabacum* GenBank accessions X12739 and S30157. *N. syl* PR-5d: *Nicotiana sylvestris* GenBank accession D76437. *S. lyc* PR-5 and *S. lyc* NP24 : *Solanum lycopersicum* GenBank accessions AJ277064 and AF093743. *S. cmm* pA35, *S. cmm* pOSML81 and *S. cmm* pOSML13: *Solanum commersonii* GenBank accessions X67244, X72927 and X72928. *S. phu* A13, *S. phu* pA35 and *S. phu* pOSML81: *Solanum phureja* GenBank accessions AY743928, AY743929 and AY 743930. *Solanum dulcamara* GenBank accession AY007309, *Solanum nigrum* GenBank accession AAL87640, *Capsicum annuum* GenBank accession AF297646, *Petunia hybrida* GenBank accession AF376058

in the BAC library. RFLP mapping in various populations has identified six *PAL* loci on potato chromosomes III, IX, X and XII (Gebhardt et al. 2001, https://gabi.rzpd.de/PoMaMo.html; Trognitz et al. 2002), some of which may contain clusters of two or more *PAL* genes. This is indicated by the finding that most PAL BACs contained more than one genomic *Hin*dIII fragment. *PAL* homologous loci on chromosomes III, IX and XII have been linked to QTLs for late blight resistance in potato (Leonards-Schippers et al. 1994; Trognitz et al. 2002). In *Phaseolus vulgaris*, a QTL for resistance to *Colletotrichum lindemuthianum* was also linked to *PAL* genes (Geffroy et al. 2000). Anchoring of the PAL, BACs to the potato genetic map, sequencing and analysis of molecular and functional variability will give further insight into the structure of *PAL* loci in the potato genome and their potential role in quantitative resistance.

Four homologues of genes for acidic PR-5 proteins were identified, all of which were located within a 45-kb stretch on potato chromosome XII. The BAC clone carrying the four genes harbors all acidic *PR-5* genes of this homology group in the potato genome. However, we cannot exclude the possibility that additional members of this gene family are present on this same BAC. Three of the sequenced genes were complete, whereas, the fourth gene (*PR-5*/*321d*) was truncated at the 5′ end and fused to the 3′ end of *PR-5*/*321c*, which makes it unlikely that it is expressed. The amino acid sequence deduced from the complete gene *PR-5*/*319* was 100% identical to the sequenced portion (58 N-terminal amino acids) of the acidic thaumatin-like protein C that has been identified in intercellular fluids of potato plants during aging and after salicylate treatment (Pierpoint et al. 1990). Sequences of RT-PCR products generated from PD59 leaves all corresponded to *PR-5*/*319* (R. Castillo-Ruiz, unpublished results). Thus *PR-5*/*319* most probably encodes protein C and is probably secreted into the intercellular space in potato leaves. To the extent that it has been sequenced in PD59 and its parents, the *PR-5* locus showed little polymorphism between the alleles. It was also not polymorphic in two tetraploid populations of *S. tuberosum* that were analyzed for late-blight QTLs (Bormann et al. 2004). Acidic *PR-5* homologues also seem to be highly conserved in a range of solanaceous species (Castillo-Ruiz 2002). This limited genetic variation points to strong functional constraints on this gene family in the Solanaceae. Nine osmotin-like genes were found to be organized at two loci, eight genes in a 90-kb cluster on chromosome VIII and one single gene on chromosome XI. However, it cannot be excluded that additional copies are present on *Hin*dIII fragments 6 and 7, which were not sequenced (Fig. 5). These nine genes do not represent all osmotin-like genes of this homology group in the potato genome, as indicated by additional cross-hybridizing bands on genomic Southern blots of clone PD59. Three osmotin-like proteins and their corresponding genes have been

characterized in the tuber-bearing wild species *S. commersonnii* (Zhu et al. 1995a, b). The *S. commersonnii* genes *OSML13* and *OSML81* were present in tandem orientation on the same Lambda genomic clone, like the *S. phureja* genes *OSM-3b* and *OSM-3f*, which were located within a segment of 5 kb at the *OSM-8* locus (Fig. 5). *OSM-3b* is most closely related to *OSML81*, whereas *OSM-3f* is most similar to *OSML13* (Fig. 7). Thus, the *S. commersonnii* locus containing genes *OSML13* and *OSML81* is orthologous to the *OSM-8* locus of *S. phureja* and *S. tuberosum*.

The branching topology of the phylogenetic tree suggests the following mode of evolution for the osmotin-like gene family. An ancestral gene appears to have undergone two rounds of duplication with subsequent divergence before speciation took place within the Solanaceae family. One of the four ancestral genes was then translocated to chromosome XI and evolved into *OSM-8e*, whereas, the other three genes remained in a cluster on chromosome VIII, and each of them has more recently undergone at least one more round of duplication.

Osmotin-like genes are functional candidates for quantitative resistance to late blight. Accumulation of osmotin-like proteins upon infection with *P. infestans* has been demonstrated in *S. commersonnii* (Zhu et al. 1995a, b). Over expression of tobacco osmotin and OSML13 from *S. commersonnii* in potato resulted in a delay in the development of symptoms (Liu et al. 1994; Zhu et al. 1996). Osmotin-like genes are also positional candidates for such loci, based on co-localization with late blight QTLs. PCR-based markers for the potato locus *OSM-8* have been linked with a late blight QTL in the PD population, and markers for both the *OSM-8* and *OSM-11* loci tagged a QTL for late-blight resistance in the tetraploid progeny of a cross between the varieties Nikita and Leyla (Bormann et al. 2004). However, osmotin-like genes are not the only genes present in these genomic regions, and the discovery of other candidates may have to await the sequencing of the complete potato genome. In any case, the putative role of osmotin-like genes in the natural variation in resistance to late blight and other pathogens will require further validation. This can be approached by association mapping (Gebhardt et al. 2004; Simko et al. 2004) and ultimately by quantitative complementation analysis using different alleles.

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