ARTIFICIAL EVOLUTION CORRECTS A REPULSIVE AMINO ACID IN POLYGALACTURONASE INHIBITING PROTEINS (PGIPs)

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SUMMARY

Polygalacturonase-inhibiting proteins (PGIPs), extracellular proteins that specifically inhibit fungal endopolygalacturonases (PGs), play a critical role in plant protection by favouring the accumulation of oligogalacturonides (OGs), which are elicitors of plant defence responses. The genes encoding PGIP2 of P. vulgaris and the variant PGIP2.Q224K were subjected to error prone PCR (epPCR) to generate mutated inhibitors with novel and improved recognition capabilities. Using a Pichia pastoris expression library and a high-throughput screening method, two mutated PvPGIP2.Q224K-derived inhibitors active against the PG produced by the phytopathogenic fungus F. phyllophilum (FpPG) were isolated. Both variants were better inhibitors than PGIP2.Q224K and were characterized by the replacement of the lysine in position 224, supporting the view that the absence of this positively charged amino acid at position 224 is a primary requirement for gaining the inhibition capability against FpPG.

Key words: artificial evolution, PGIPs, LRR, polygalacturonase, Pichia pastoris, Error prone PCR.

INTRODUCTION

Molecular recognition events play a key role in plant defence against pathogens. For example, microbe-associated molecular patterns (MAMPs) are recognized by specific plant pattern recognition receptors (PRR) to trigger defence responses. On the other hand microbial effectors, secreted to help the pathogenicity process, may act as avirulence products upon recognition by specific receptors (resistance proteins) and elicit resistance responses (Boller and Felix, 2009). Furthermore, several enzymes produced by microbes or insects are specifically recognized by plant proteins for inhibition of their enzymatic activity (De Lorenzo and Ferrari, 2002) or as non-self molecules for activation of the immune response (Boller and Felix, 2009), in order to limit colonization and disease.

The majority of plant recognition proteins share domains characterized by leucine-rich repeats (LRR). This structure is also found in many receptors involved in hormone response and development (Becraft, 2002; Szekeres, 2003; Boller and Felix, 2009). A prototypical plant LRR protein involved in defence is polygalacturonase-inhibiting protein (PGIP). PGIP exhibits 10 imperfect LRRs of the extracellular type [consensus motif: xxLxLxxNxLt/sGxIPxxLxxLxx] and acts as a specific inhibitor of endopolygalacturonases [poly (1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15; PGs] from fungi and insects, but not of plant-derived PGs (De Lorenzo et al., 2001). The degradation of the mechanical barrier represented by the plant cell wall is a critical event during pathogenesis caused by phytopathogenic fungi that produce a variety of cell wall degrading enzymes both to facilitate the invasion of plant tissues and to release nutrients to be used as carbon source. PGs are among the first enzymes produced during infections and degrade homogalacturonan, a component of pectin that acts as a cohesive element for the entire cell wall structure (Lionetti et al., 2010). To accommodate pathogenesis in a variety of conditions and on various hosts, many PG isoenzymes, which often exhibit polymorphism in different isolates or races, are produced by pathogens (Caprari et al., 1993; De Lorenzo et al., 2001; Daroda et al., 2001; Poinssot et al., 2003; Mariotti et al., 2009). Against these PGs, plants have evolved many PGIPs, often encoded by small gene families of clustered paralogs, with different specificities and regulation (Frediani et al., 1993; D’Ovidio et al., 2004). For instance, all four members of the Phaseolus vulgaris PGIP family inhibit, with different efficiencies, PGs from Botrytis cinerea and Colletotrichum lupini, but only PvPGIP2 inhibits PG from Fusarium moniliforme FC-1 strain [now reclassified as F. phyllophilum strain FC912 (Mariotti et al., 2008)] (Leckie et al., 1999; D’Ovidio et al., 2004). By interacting with PGs, PGIPs favour the formation of oligogalacturonides (OGs) capable of inducing plant defense responses (De Lorenzo et al., 2004).
and Ferrari, 2002; Brutus et al., 2010). PGIPs hamper the invasion process by limiting host tissue colonization and can therefore be exploited to obtain transgenic plants with increased resistance (Powell et al., 2000; Ferrari et al., 2003; Aguero et al., 2005; Manfredini et al., 2005; Joubert et al., 2007; Janni et al., 2008).

Computational analysis of the PvPGIP2 structure predicts that the xxLxLxx region within each LRR has a strong propensity to be involved in protein-protein interactions (Sicilia et al., 2005; Casasoli et al., 2009) and a biochemical analysis has shown that this region contains residues influencing the specificity of PvPGIP2 (Leckie et al., 1999; D’Ovidio et al., 2004; Sicilia et al., 2005). The highly variable xxLxLxx motifs in the LRR are responsible for ligand binding. In the crystal structure of PGIP, which is so far the only available structure of a plant LRR protein, these motifs form a concave beta-sheet surface. Single residues located in this concave surface differentially affect the inhibition of fungal PGs. For example, the glutamine residue 224 is crucial for the inhibition of PG of F. phyllophilum, hereon indicated as FpPG (Leckie et al., 1999). The combination of evolutionary analyses with structural, biochemical, and physiological data allowed the identification of residues in the third LRR (H104A, Y105A, and Y107A) and in the seventh LRR module (F201) of PvPGIP2 as “hotspots” for the interaction, i.e., essential residues that if mutated impede or severely affect protein-protein interactions (Casasoli et al., 2009).

In this work, an in vitro evolution approach has been used in the attempt to obtain inhibitors with novel and improved recognition capabilities against a fungal PG that is not inhibited by any known PGIP such as that from Fusarium verticillioides (Raiola et al., 2008). Approaches of artificial evolution based on random mutagenesis by error-prone PCR (epPCR) have been already applied to tobacco LRR resistance protein Rx to generate variations in disease resistance specificities (Farnham and Baulcombe, 2006), indicating directed evolution as a common laboratory tool for altering and optimizing the functions of proteins characterized by a high evolvability (Romero and Arnold, 2009). Random mutagenesis is a suitable method to generate in vitro variability of LRR proteins, because leucine, the key aminoacid for the maintenance of the stability of the protein scaffold, is coded by six different codons and characterized by the lowest mutation frequency. Here we present the results obtained by screening two different libraries of PGIP variants expressed in Pichia pastoris. The first library was generated using, as a starting protein, PGIP2Q224K, which is impaired in recognition of FpPG and was mainly aimed at recovering gain-of-function variants and demonstrating the feasibility of the approach. The second library was generated using the wild-type PvPGIP2 and aimed at isolating variants with novel and improved recognition specificities.

**MATERIALS AND METHODS**

**Construction of the expression vector containing the PvPGIP2 gene.** The wild-type PvPGIP2 gene was cloned in pGAPZαA (Invitrogen BV, The Netherlands) using the EcoRI and XbaI restriction sites introduced by the following primers: EcoFw 5’-ATCGATGAAATTCGAGCTATGCAACCCAA-3’ and XbaRvtag 5’-CTTGTTCTGAGAAGTGCAGGCAGG-3’ (underlined sequence indicates the restriction site introduced), to generate a fusion protein where the signal sequence of PvPGIP2 was replaced by the yeast alpha factor signal sequence for secretion and a 6 histidine tag was added at the C-terminus. pGAPZαA allows the constitutive expression of heterologous proteins under the control of the GAP promoter. The construct was amplified by transforming E. coli DH5α competent cells [genotype: F-Φ80lacZAM15 ΔlacZYA-argF]U169 deoR recA1 endA1 bsdR17(ter-, mcr +) pboA supE44 thi1 gyrA96 relA1- provided by Invitrogen BV, The Netherlands]. Transformants were selected on low-salt Luria-Bertani (LB) plates containing 25 μg/ml zeocin (Duchefa Biochemie, Italy) and analysed by direct PCR amplification using specific primers for the alpha-factor signal peptide and the 3’-AOX terminator sequences according to the manufacturer's instruction. One PCR-positive colony was picked with a sterile tip and used to inoculate 5 ml of low-salt LB medium [1% tryptone (Duchefa Biochemie, Italy), 0.5% yeast extract (Duchefa Biochemie, Italy), 0.5% NaCl (Carlo Erba Reagents, Italy), pH 7.4] containing 25 μg/ml zeocin. The culture was grown overnight at 37°C at 250 rpm. Plasmid DNA was extracted from the cells using a plasmid mini prep kit (Macherey and Nagel, Germany) and analyzed by digestion with EcoRI and XbaI restriction enzymes, followed by 1% agarose gel analysis.

**Site-directed mutagenesis of PvPGIP2.Q224K and PvPGIP2.Q224E.** Site-directed mutagenesis of the PvPGIP2 gene was performed using the Quick Change site-directed mutagenesis kit (Stratagene, Canada). PCR was carried out directly on pGAPZαA/ PGIP2 construct. The internal overlap primers that hybridize at the site of the desired mutation, containing the relevant mismatched bases (underlined bases), were the following: PGIP2.Q224K Fw: 5’-CGGATCGATAAGAACACG AAGAGATACATCTGGCAGAAG-3’; PGIP2.Q224K Rw 5’-CTTCGCCAGA TGTATCTTTCGTTCTT TT ATCTGATCCG-3’; PGIP2.Q224E Fw 5’-CGGA TGAGATAAGAACCGGAGAGAGATACCTCGGCC GAAG-3’; PGIP2.Q224E Rw 5’-CTTCGCCAGA TG TATCTTTCGTTCTT TT ATCTGATCCG-3’.

Transformation and growth of E. coli as well as selection and analysis of transformants were performed as reported in the previous paragraph. The mutations were checked by sequencing analysis (PRIMM, Italy).
Error-prone PCR. The conditions for random mutagenesis were optimized on the basis of the method described by Leung et al. (1989). The reaction mixture (50 µl final volume) contained: 10 mM Tris-HCl pH 8.3 (Carlo Erba Reagenti, Italy), 50 mM KCl (Carlo Erba Reagenti, Italy), 7 mM MgCl₂ (Carlo Erba Reagenti, Italy), 0.35 mM MnCl₂ (Carlo Erba Reagenti, Italy), 25 pmol of each primers, unbalanced dNTPs mix (1mM dCTP and dTTP, 0.2 mM dATP and dGTP) (Bioline, England) and 2.5 U Taq polymerase (Bioline, England). The primers used were EcoFw and XbaRvtag described above. We used 22 ng of pBS23-Pvpgip2Q224K (Leckie et al., 1999) for each reaction tube for a total of 50 tubes. This was the program utilized: 1x2 min 94°C, 26-30 x (1 min 94°C, 1 min 56°C, 1 min 72°C), 1 x 7 min 72°C.

Electroporation. The bacterial and yeast libraries were obtained by electroporation using the Gene-Pulser Xcell Electroporation System (Bio-Rad Laboratories, USA). Preparation and electroporation of DH5αw was carried out according with the Gene-Pulser Xcell Electroporation System manual. Preparation and electroporation of Pichia pastoris X33 (genotype: wild type, pro tease Δ) was prepared as described (Bonivento et al., 2008). A homogeneous PG of Aspergillus niger (strain N400) was prepared according to D’Ovidio et al. (2004). Botrytis cinerea (strain BO5-10), Fusarium phyllophilum (strain FC912), Fusarium graminearum (strain XM91184) and Fusarium verticillioides (strain PD; (Raiola et al., 2008)) were grown for 20 days on potato dextrose agar (Oxoid, Italy) at 22°C under constant light. Mycelium of Botrytis cinerea was grown for 20 days on potato dextrose agar (Oxoid, England) and 2.5 U Taq polymerase (Bioline, England). The primers used were EcoFw and XbaRvtag described above. We used 22 ng of pBS23-Pvpgip2Q224K (Leckie et al., 1999) for each reaction tube for a total of 50 tubes. This was the program utilized: 1x2 min 94°C, 26-30 x (1 min 94°C, 1 min 56°C, 1 min 72°C), 1 x 7 min 72°C.

Preparation of PGs. A homogeneous PG of Colletotrichum luvini (strain SHK788 var setosum) was prepared as described (Bonivento et al., 2008). A homogeneous PGII of Aspergillus niger (strain N400) was prepared according to D’Ovidio et al. (2004). Botrytis cinerea (strain BO5-10), Fusarium phyllophilum (strain FC912), Fusarium graminearum (strain XM991184) and Fusarium verticillioides (strain PD; (Raiola et al., 2008)) were grown for 20 days on potato dextrose agar (Oxoid, Italy) at 22°C under constant light. Mycelium of Botrytis cinerea, F. phyllophilum, F. graminearum and F. verticillioides (1 cm²) were harvested, used to inoculate Czapek-Dox medium (2 g l⁻¹ NaNO₃, 1 g l⁻¹ K₂HPO₄, 0.5 g l⁻¹ MgSO₄, 0.5 g l⁻¹ KCl, 10 mg l⁻¹ FeSO₄, pH 7.0; all chemicals were from Carlo Erba Reagenti, Italy), and supplemented with 1% citrus pectin (Sigma Aldrich, Italy). Cultures were incubated in a rotary shaker at 180 rpm and 21°C for 5 days, and filtrates were used for the PG activity assay.

Screening of the libraries. P. pastoris colonies obtained by electroporation were replicated by replica plating using a sterile filter paper and transferred to a plate containing 40 ml of YTGA medium [25 mM citrate buffer pH 4.5, 1.3% glycerol, 0.5% yeast extract (Duchefa Biochemie, Italy), 1% tryptone (Duchefa Biochemie, Italy), 0.8% polygalacturonic acid from citrus fruit (Sigma Aldrich, Italy), 1.2% agarose (Jena Bioscience, Germany), 100 µg/ml zeocin (Duchefa Biochemie, Italy)]. After replica plating, plates were incubated at 28°C for 4 days. Plates were covered by a solution containing the polygalacturonase of Fusarium phyllophilum (1.5 ng/ml of FpPG in 25 mM citrate buffer pH 4.5) and incubated overnight at 28°C. After incubation, the development of the assay was acted adding 6 M HCl for 1 min.

PG-PGIP assay. PG-PGIP assay was performed as previously published (Taylor and Secor, 1988; Ferrari et al., 2003). The sample containing purified PGIP and purified PG (for AnPGII and ClPG) or the fungal crude preparation (for FpPG, FgPG, FvPG and BcPG) was added to 0.9 cm wells on plates containing 100 mM sodium acetate (Carlo Erba Reagenti, Italy), pH 4.6, 0.5% PGA from citrus fruit (Sigma Aldrich, Italy) and 0.8% agarose (Jena Bioscience, Germany). Plates were incubated for 16 h at 30°C, and the halo caused by enzyme activity was visualized after 1 min of treatment with 6 M HCl. PG activity was expressed as agarose diffusion units, with 1 agarose diffusion unit defined as the amount of enzyme that produced a halo of 0.5 cm radius (external to the inoculation well) after 16 h at 30°C.

Production and purification of PGIP. The medium used for production of PGIP2, PGIPA and PGIPB contained 1% yeast extract (Duchefa Biochemie, Italy), 1% tryptone (Duchefa Biochemie, Italy) and 2% glucose (Carlo Erba Reagenti, Italy). Purification included the following steps: cultural filtrates were concentrated using a Vivaflow 200 (Sartorius Stedim, France) and dialyzed against 20 mM Na acetate pH 4.6 (Carlo Erba Reagenti, Italy). Dialyzed proteins were mixed with a suspension of diethylaminoethyl (DEAE) cellulose (DE52, Whatman, UK) pre-equilibrated with 20 mM Na acetate pH 4.6. The not-absorbed proteins were passed on a column HiTrap SP-Sepharose (GE Healthcare, USA) pre-equilibrated with 20 mM Na acetate pH 4.6 (Carlo Erba Reagenti, Italy). Elution were acted using a linear gradient of NaCl (0 to 1 M in 10 min) (Carlo Erba Reagenti, Italy). Fractions that showed the highest inhibitory activity were assayed by SDS gel 10% acrylamide (Sigma Aldrich, Italy), quantified by Blue Coomassie staining and immunoblotting.

SDS-PAGE and Western blotting. Purified proteins were analyzed by SDS-PAGE acrylamide gel 10% (Sigma Aldrich, Italy) and stained using a solution of Coomassie brilliant blue R-250 (Sigma Aldrich, Italy). Western blotting was done utilizing the antibody His probe (Santa Cruz Biotechnology, USA) at the concentration suggested by the manual. Protein bands were detected by chemiluminescence by ECL reagent (Amer sham, USA) and quantified using the program Quantity One (Bio-Rad Laboratories, USA).
Statistical analysis. Each experiment (PG- PGIP assay) was performed at least five times. Data are reported as means. Statistical significance between samples was calculated using the Student’s t-test. P<0.003 was considered significant.

RESULTS AND DISCUSSION

In a previous paper, *P. vulgaris* PvPGIP2, in which glutamine 224 had been substituted by a lysine (Q224K), was reported to be affected in its inhibitory activity against FpPG (Leckie *et al.*, 1999). In order to assess whether PGIP is amenable to random mutagenesis to obtain improved variants, the PvPGIP2.Q224K mutated gene was chosen as a starting point for epPCR to generate a library of PGIP variants for the isolation of function-gaining inhibitors of FpPG. The signal peptide sequence and the stop codon of the *Pvpgip2* gene were removed and the ORF was fused in frame between the sequence encoding the yeast α-factor sequence for translocation in the ER and the sequence encoding the histidine tag (6His) present in the expression vector pGAPZαA. The resulting plasmid was subjected to site-directed mutagenesis to obtain the plasmid pGAPpgip2Q224K. This was introduced into *P. pastoris* and the inhibitory activity of the expressed protein was tested by the agar diffusion assay against FpPG as well as against PGs from *Aspergillus niger* (AnPGII), *Colletotrichum lupini* (CLPG), *Botrytis cinerea* (BcPG) *Fusarium graminearum* (FgPG) and *Fusarium verticillioides* (FvPG), in parallel with the wild type PGIP2 (PGIP2wt) (Table 1).

The gene pgip2Q224K was then used as a template for epPCR and the amplified DNA was used to obtain an *E. coli* library (about 2×10^6 independent clones) in pGAPZαA. The average mutation frequency of the pgip2Q224K gene was 5.4±3.6 mutations/kb of DNA as determined by sequencing 40 independent transformants. Substitutions were randomly distributed along the gene without apparent preferential sites; in some cases (8%) single-base deletions were observed. It was decided to introduce a low number of mutations because preliminary experiments had shown that a high number of mutations causes a large proportion of transformed *P. pastoris* colonies with no expression of PvPGIP2Q224K, as determined by Western blot analysis using an anti-His antibody (data not shown). This is likely due to the instability and degradation of highly mutated PvPGIP2 and is in agreement with the notion that the mutation rate of *in vitro* evolutionary approaches should be decided on the basis of the evolvability degree of the specific protein, i.e. the ability of a protein to adapt in response to mutation and to selective pressure (Romero and Arnold, 2009).

![Tridimensional structure of PGIP](image)

Fig. 1. Tridimensional structure of PGIP. Green and purple circles indicate the mutated sites found in PGIP.A and PGIP.B, respectively.
The plasmid DNA purified from the bacterial library was linearized with 
AvrII and used to transform 
P. pastoris by electroporation (Wu and Letchworth, 2004). An 
in vitro high-throughput screening method was de- 
veloped, based on the detection of the inhibitory activity 
secreted by single transformed 
P. pastoris colonies. The 
colonies were grown for 4 days on YTGA medium con-
taining polygalacturonic acid. A PG solution was 
poured into the YTGA Petri dish and the enzyme incu-
bated overnight at 28°C. HCl 6 M was used as a detec-
tor of enzyme activity; a typical dark spot, due to the 
absence of substrate degradation, appeared around the 
colonies producing an active PGIP. Upon screening 
3x10^3 clones, two colonies showing FpPG inhibition 
were isolated. Sequencing of the insert showed that the 
proteins secreted by both colonies had a missense muta-
tion on residue 224, with two different amino acids in-
troduced, i.e. glutamic acid and methionine. The mu-
tant proteins, named PGIP .A and PGIP .B, had two 
(K99N and N289I) and one (S70T) additional substitu-
tions. PGIP .A showed activity against FpPG, it was less active 
whether the amino acid substitution at site 224 (Q224E) 
results in nearly wild type activity, including the ability to 
In other PG-PGIP combinations this complexity is 
not sufficient for PGIP2 to acquire the capability to in-
hbit FvPG; probably due the complexity of the inter-
action. A second library of variants was generated starting 
from the wild-type 

| Table 1. Inhibitory activities* of native and mutated PGIP2 variants. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                             | PGIP2wt | PGIP2.Q224K | PGIP.A | PGIP.B | PGIP2.Q224E |
| AnPG                        | 0.4     | 15*         | 0.4   | 0.9*   | 0.4           |
| FpPG                        | 1.3*    |             | 1.5   | 7.5*   | 1.5           |
| CIPG                        | 2.1*    |             | 1.3*  | 3*     | 1.1*          |
| BcPG                        | 1.2*    |             | 4.0*  | 2.5*   | 1.0           |
| FpPG                        | 1.2     |             | 0.9   | 3*     | 0.8           |
| FvPG                        |     |             |      |       |               |

*Values indicate the amount (in ng) of PGIP that determines 50% inhibition of 1 agarose plate unit of PG. The symbol ∞ indicates >600 ng. The asterisk indicates cases where inhibitory activities of PvPGIP2 and the mutated variant are significantly different (P < 0.003).

Te apparent discrepancy between these values and those reported in previous articles (D’Ovidio et al., 2004) is due to the different enzyme preparations used in this work (for details see Materials and Methods).
vidio et al., 2006) or members belonging to different gene families may be mixed, fragmented into random size pieces and reassembled into full-length genes via self-priming PCR and extension. This process may yield crossovers carrying different PGIP contact points that can be analyzed in the P. pastoris expression system.

REFERENCES


Farnham G., Baulcombe D.C., 2006. Artificial evolution extends the spectrum of viruses that are targeted by a disease-resistance gene from potato. Proceedings of the National Academy of Sciences USA 103: 18828-18833.


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