Theor Appl Genet (1993) 87:369–373

THEORETICAL AND APPLIED GENETICS

Cytological localization of the *PGIP* genes in the embryo suspensor cells of *Phaseolus vulgaris* L

M. Frediani[1], R. Cremonini[1], G. Salvi[2], C. Caprari[2], A. Desiderio[2], R. D'Ovidio[3], F. Cervone[2], G. De Lorenzo[2,4]

[1] Dipartimento di Scienze Botaniche della Università, via L. Ghini 5, I-56100 Pisa, Italy
[2] Dipartimento di Biologia Vegetale, Università La Sapienza, piazzale A. Moro, I-00185 Roma, Italy
[3] Dipartimento di Agrobiologia e Agrochimica, Università della Tuscia, via S. Camillo DeLellis, I-01100, Viterbo, Italy
[4] Istituto di Botanica, Università di Bari, via G. Amendola 175, I-70126 Bari, Italy

Received: 2 April 1993 / Accepted: 20 April 1993

Abstract. Polygalacturonase-inhibiting protein (*PGIP*) is a cell wall protein which inhibits fungal *endo*polygalacturonases. A small gene family encodes *PGIP* in the genome of common bean, as indicated by Southern-blot experiments performed at high-stringency conditions. Southern-blot analysis of DNA extracted from different cultivars of *Phaseolus vulgaris* and from *Phaseolus coccineus* showed length polymorphism of the hybridizing restriction fragments. The cytological localization of the *PGIP* genes was determined in polytene chromosomes of the *P. vulgaris* embryo suspensor cells. In-situ hybridization experiments using the cloned *PGIP* gene revealed labelling over a single region of the pericentromeric heterochromatin of chromosome pair X, next to the euchromatin, suggesting that *PGIP* gene family may be clustered in one chromosomal region.

Key words: Polygalacturonase-inhibiting protein – *Phaseolus vulgaris* – Polytene chromosomes – Cytological localization – Copy number

Introduction

Fungal endopolygalacturonases (EC 2.3.1.15) have important functions during the early stages of plant pathogenesis. These enzymes hydrolyze the homogalacturonan of the plant cell wall, thereby assisting in the colonization of plant tissues and providing nourishment for the fungus (reviewed by Hahn et al. 1989). All dicots thus far examined contain a cell-wall-associated protein (*PGIP*) which specifically inhibits fungal endopolygalacturonases (Cervone et al. 1986; Salvi et al. 1990). In-vitro experiments have shown that, in the presence of *PGIP*, the ability of fungal endopolygalacturonase to hydrolyze polygalacturonic acid is reduced while the existence in the reaction mixture of oligogalacturonides with a degree of polymerization between 10 and 14 is strongly prolonged (Cervone et al. 1989). These oligogalacturonides are able to elicit plant defence responses, including phytoalexin accumulation: (Jin and West 1984; Nothnagel et al. 1983; Hahn et al. 1989) and lignification (Nothnagel et al. 1983). Thus, it appears that plants have evolved the production of *PGIP* as a mechanism to regulate the activity of pathogen-produced enzymes, thereby increase the production of elicitor-active oligogalacturonides. Recently, the gene encoding *PGIP* of *Phaseolus vulgaris* has been cloned and characterized (Toubart et al. 1992). Evidence has been obtained that the expression of *PGIP* is induced by elicitor treatment, wounding, and infection, indicating that *PGIP* can be an important factor for plant resistance to phytopatho-genic fungi (Bergmann et al., manuscript in preparation).

This paper reports on the genomic organization of the *PGIP* genes in the *P. vulgaris* genome and their cytological localization in the polytene chromosomes of embryo suspensor cells.

Materials and methods

DNA extraction

Total DNA preparations were obtained by grinding 40–50 g of leaves of different cultivars of *P. vulgaris* and of *P. coccineus* to a fine powder, with liquid nitrogen in a mortar. The powder was

Communicated by F. Mechelke
Correspondence to: R. Cremonini

transferred to a beaker containing 100 ml of extraction buffer (50 mM Tris HCl, pH 7.8, containing 8 M urea, 20 mM EDTA, 100 mM NaCl and 2% sarkosyl) and shaken gently for 10 min at room temperature. The slurry was added to an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v), swirling gently for 5 min before centrifuging at 9,000 g at room temperature. The aqueous phase was added to 60 ml of isopropanol, gently mixed and kept at room temperature for 10 min. The precipitate was then recovered by centrifugation, washed with 70% ethanol, and air dried. The residue was dissolved in 10 ml of 10 mM Tris HCl, pH 8.0, containing 1 mM EDTA and 0.1% sarkosyl. The resulting DNA was further purified by two cycles of ultracentrifugation at 33,000 g for 15 h in a CsCl-EtBr gradient. The DNA in 1.2 ml of CsCl-EtBr was extracted six times with an equal volume of water-saturated n-butanol, diluted 1:3 with water, brought to 0.3 M Na Acetate, pH 5.2, and precipitated with 2 vol of 100% ethanol. The yield of high-molecular-weight DNA was approximately 500 µg per g fresh tissue.

Southern blotting, hybridization and determination of the gene copy number

DNA was transferred after gel electrophoresis onto Hybond-N filters (Amersham), using the capillary blotting procedure of Southern (1975).

High-stringency hybridization conditions were as described (D'Ovidio et al. 1990) using 100 µg/ml of calf thymus DNA as a carrier. Blots were washed twice in 6 × SSPE and 0.1% SDS at room temperature for 5 min, once in 1 × SSPE and 0.1% SDS at 65 °C for 30 min. The final wash was in 0.1 × SSPE and 0.1% SDS at 65 °C for 20 min.

Hybridization probes were prepared by random oligonucleotide priming (Feinberg and Vogelstein 1983; Hodgson and Fisk 1987) of a PCR-amplified 1026-bp fragment including the region from nt 1 through nt 1026 of the *PGIP* gene (Toubart et al. 1992).

As a standard, a recombinant plasmid (pPT-2) containing the *PGIP* gene (Toubart et al. 1992) was used. The amount of pPT-2 DNA that corresponded to a single-copy gene in a *Phaseolus* haploid genome [C = 1.85 pg, as determined by Ayonoadu (1974)] was calculated. Relative to the applied amount of total DNA of *Phaseolus* (20 µg), the samples of the recombinant pPT-2 DNA loaded on the gel corresponded to 1, 5 and 10 copies of *PGIP* genes per haploid genome. pPT-2 DNA was added to sheared calf thymus DNA, electrophoresed, blotted and probed along with the *Phaseolus* genomic DNA, previously digested with different restriction enzymes (*Eco*RI, *Hin*dIII and *Ava*II).

In-situ hybridization

A PCR-amplified 758-bp fragment, corresponding to the region from nt 126 to nt 884 of the *PGIP* gene was labelled by the nick-translation method (Rigby et al. 1977), using ^{3}H-dATP and ^{3}H-dCTP (Amersham). The specific activity was 9.1×10^6 cpm/µg.

Embryo suspensor cells from *P. vulgaris* seeds, fixed in ethanol/acetic acid 3:1 (v/v), were squashed in a drop of 45% acetic acid after treatment with a 5% aqueous solution of pectinase (Sigma) for 30 min at 37 °C and in-situ hybridization was performed according to Macgregor and Mizuno (1976). Chromosomal DNA was denatured in 0.07 N NaOH for 3 min at room temperature and the ^{3}H-labelled DNA probe was hybridized at a concentration of 1 µg/ml. After incubation, unbound DNA was removed by stringent washings including hot SSC and trichloroacetic acid (Hennen et al. 1975). The slides were then covered with Ilford L4 emulsion and developed after exposure times ranging from 1 to 3 months.

Results

The organization of the *PGIP* sequences in the bean genome was analyzed by Southern-blot analysis. Genomic DNA extracted from *P. vulgaris* L. cv Pinto was cut with the *Eco*RI restriction enzyme, which does not cut within the *PGIP* gene, and *Hin*dIII and *Ava*II enzymes, which cut within the 1026-bp gene fragment used as a probe.

The probe hybridized at high stringency with five *Eco*RI fragments (8.4 kb, 6.8 kb, 5.7 kb, 5.5 kb and 3.6 kb). By comparing the intensity of each hybridization signal with those of copy standards, it was estimated that 4–5 copies per haploid genome corresponded to two of the *Eco*RI fragments (6.8 and 5.7 kb), 2–3 copies corresponded to the 3.6-kb *Eco*RI fragment, and one copy to the other fragments. Digestion with *Hin*dIII and *Ava*II still produced multiple hybridizing restriction fragments, some of which were present in more

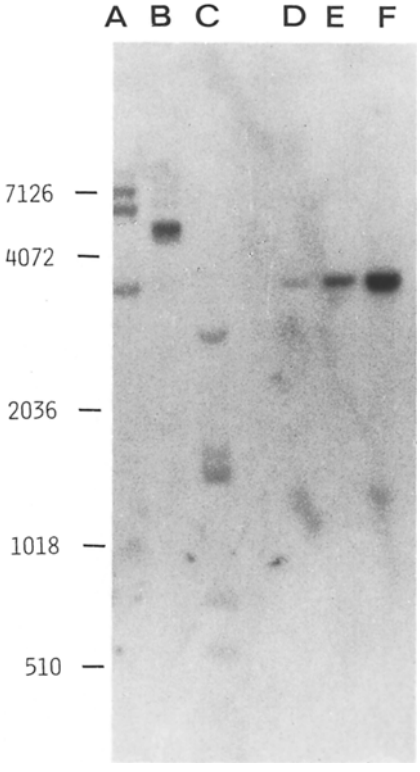

Fig. 1. Southern-blot analysis of genomic DNA (20 µg/lane) from *Phaseolus vulgaris* cv Pinto digested with different restriction enzymes and probed with a labeled 1026-bp fragment corresponding to the coding region of the bean *PGIP* gene. Appropriate amounts of pPT-2 DNA (see Material and methods) were electrophoresed as copy standards. *A*, *Eco*RI; *B*, *Hin*dIII; *C*, *Ava*II; *D*, one copy per haploid genome; *E*, five copies per haploid genome; *F*, ten copies per haploid genome. Marker sizes are in bp

than one copy, confirming the existence of multiple copies of the *PGIP* genes in the bean genome (Fig. 1).

The genomic organization of the *PGIP* genes in several cultivars of *P. vulgaris* and in *P. coccineus* was also analyzed. Multiple hybridizing restriction fragments were present in all cultivars of *P. vulgaris*, while a single fragment was observed in *P. coccineus*. Length polymorphism of the hybridizing restriction fragments was also observed (Fig. 2).

In-situ hybridization of *PGIP* on polytene chromosomes of *P. vulgaris* embryo suspensors showed labelling over one single region of the pericentromeric heterochromatin of a metacentric chromosome, at the boundary between condensed and decondensed chromatin (Fig. 3).

The idiogram of *P. vulgaris* polytene chromosomes that resembles the *P. coccineus* set (our analysis and personal communication from Nagl) and the distribution of heterochromatin according to Nagl (1967) are reported in Fig. 4. On the basis of the absence of any intercalary bands and the occurrence of smaller blocks

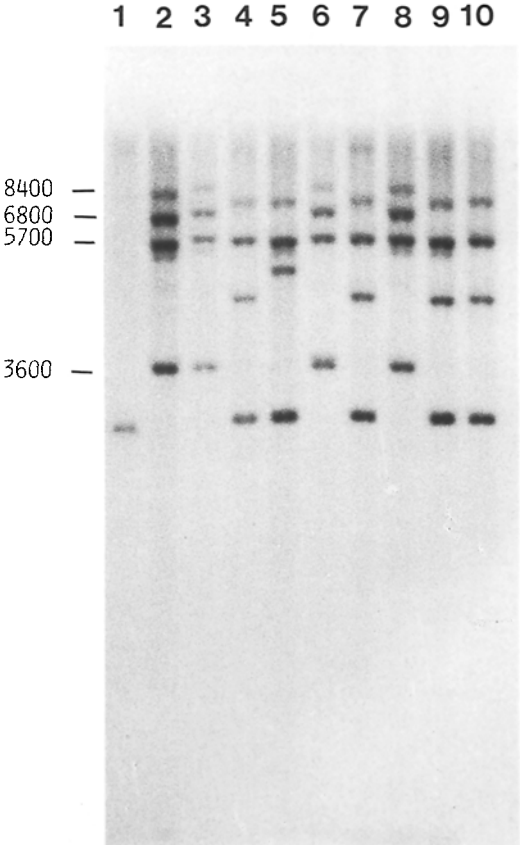

Fig. 2. Southern-blot analysis of genomic DNA (20 µg/lane) from *Phaseolus coccineus* and from different cultivars of *P. vulgaris* digested with *Eco*RI and probed as described in Fig. 1. *1*, *P. coccineus*; *2–10*, *P. vulgaris*; *2*, Great Northern; *3*, Pinto; *4*, Cannellino; *5*, Brown Bean; *6*, Borlotto; *7*, Cannelloni; *8*, St. Fiacre; *9*, Marconi; *10*, Red Kidney

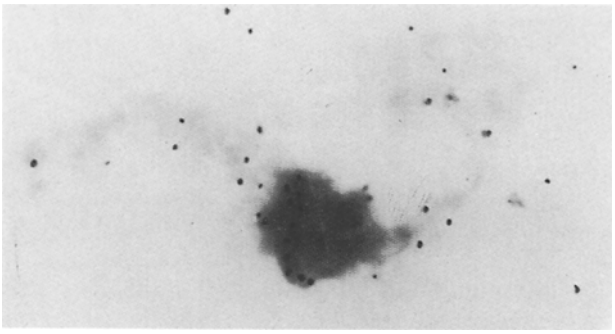

Fig. 3. In-situ hybridization of *PGIP* on an embryo suspensor cell of *P. vulgaris*. Chromosome X. Giemsa, × 900

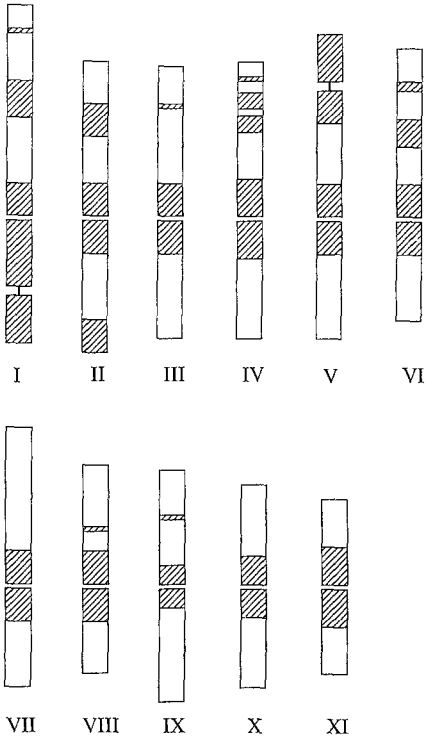

Fig. 4. Idiogram of polytene chromosomes of *Phaseolus* embryo suspensor cells. *Dashed segments*, distribution of heterochromatin (Nagl 1967)

of heterochromatin in the pericentromeric regions, in comparison with the metacentric chromosome pair XI, it is possible to state that the *PGIP* genes are localized on chromosome pair X.

Discussion

A small family of *PGIP* genes is present in the genome of *P. vulgaris*, as indicated by Southern-blot hybridization carried out at high-stringency conditions. It cannot be decided whether the sequences hybridizing less

strongly represented genes that were highly homologous to the isolated *PGIP* gene and present at a smaller copy number or whether they were genes that had diverged. Western-blot analysis of proteins extracted from bean hypocotyls, probed with an antibody specific to *PGIP*, revealed the presence of at least three different reacting polypeptides (C. Bergmann, personal communication). The detection of only one species of mRNA by Northern-blotting analysis (Toubart et al. 1992) may indicate that only one gene is expressed under the conditions examined or that different *PGIP* genes have the same-size transcript. It remains to be investigated whether different species of mRNA coding for *PGIP* are developmentally and differentially expressed in different tissues of *P. vulgaris* and whether undetected isoforms of *PGIP* are present in this species. The *PGIP* purification procedure utilized to obtain the protein so far characterized employs a Sepharose-*Aspergillus niger* polygalacturonase affinity column that may not be able to bind all *PGIP* isoforms (Cervone et al. 1987).

In *Phaseolus* the occurrence of an embryo suspensor with highly polytene chromosomes (Nagl 1974) represents a suitable system for determining the cytological localization of sequences present at low copy number in the chromosome complement of a mitotic cell. The cytological hybridization of the *PGIP*-specific probe demonstrated only one labelling site over a single region of the pericentromeric heterochromatin of chromosome pair X, at the boundary between condensed and decondensed chromatin (Fig. 3). Consequently, we conclude that either the *PGIP* gene family is clustered in that region or else that *PGIP* genes localized in other chromosomal regions are not detectable under our experimental conditions. At present, it is impossible to relate the *PGIP* genes to other markers on chromosome X, because the correspondence between the linkage groups of the available molecular marker-based map (Vallejos et al. 1992) and chromosome localization has not yet been determined. We have shown that polymorphism in restriction fragment length exists for the *PGIP* genes among different cultivars of *P. vulgaris*. Thus, it should now be possible, using a segregation analysis, to associate the *PGIP* genes to one of the characterized linkage groups.

To our knowledge, this paper is the first report on the cytological localization of a plant gene coding for a protein in a heterochromatic region and it is worth noting that this protein is involved in plant response to stress. The localization of a functional gene in the heterochromatin is not surprising (Devlin et al. 1990; Pardue and Henning 1990; and references therein). Some data in the literature demonstrate that transcriptionally active genes reside preferentially at the border of euchromatin and heterochromatin (Hutchinson and Weintraub 1985; Kost et al. 1991). Moreover, Forino et al. (1992) recently demonstrated that in *Phaseolus* polytene chromosomes, no difference exists between euchromatic and heterochromatic regions as regards their transcriptional activity. These authors suggested that many functional genes are present in the heterochromatin of *Phaseolus* and that RNA synthesis is independent of chromatin condensation, in agreement with the observations of Nagl (1979). In *Drosophila* salivary gland nuclei, polytene chromosomes have been shown to be anchored to the nuclear envelope's inner surface through telomeres and centromeres and several unique heterochromatic regions within each chromosome (Newport and Forbes 1987). Hadwiger (1988) speculated that "dominant Mendelian traits with a potential for disease resistance could be .... located near the nuclear circumference and .... can be influenced directly or indirectly by elicitors released by the pathogen", thus generating in a short time the pleiotropic gene response observed in the defence reaction. Other genes, in addition to *PGIP*, may reside in chromosomal regions potentially able to interact with the nuclear envelope. Indeed, in wheat, traits for disease resistance have, on the basis of their linkage in segregation ratios of progeny exhibiting the resistant phenotype, been mapped to loci scattered over different chromosomes, with some loci mapping indistinguishably from the centromere (McIntosh 1983). Further investigations on the chromosomal localization of defence genes other than *PGIP* and on changes of plant nuclear structure following challenge by a fungal pathogen are required to evaluate the influence of chromosomal and nuclear structural features on the regulation of these genes.

Acknowledgements. The research was supported by the National Research Council of Italy, Special Project R.A.I.S.A., subproject no. 2 Paper no. 842, by EC Grant BIOT-CT90-0163 and by a grant of M.U.R.S.T., Rome.

References

Ayonoadu UWU (1974) Nuclear DNA variation in *Phaseolus*. Chromosoma 48:41–49

Cervone F, De Lorenzo G, Degra L, Salvi G (1986) Interaction of fungal polygalacturonase with plant proteins in relation to specificity and regulation of plant defence response. In: Lugtenberg B (ed) Recognition in microbe-plant symbiotic and pathogenic interactions. NATO-ASI series, vol H4. Springer-Verlag, Berlin, pp 253–258

Cervone F, De Lorenzo G, Degra L, Salvi G, Bergami M (1987) Purification and characterization of a polygalacturonase-inhibiting protein from *Phaseolus vulgaris* L. Plant Physiol 85:631–637

Cervone F, Hahn MG, De Lorenzo G, Darvill A, Albersheim P (1989) Host-pathogen interactions. XXXIII. A plant protein converts a fungal pathogenicity factor into an elicitor of plant defence responses. Plant Physiol 90:542–548

Devlin RH, Bingham B, Wakimoto BT (1990) The organization

and expression of the *light* gene; a heterochromatic gene of *Drosophila melanogaster*. Genetics 125:129–140
D'Ovidio R, De Lorenzo G, Cervone F (1990) Isolation and characterization of pectin-inducible DNA clones from the phytopathogenic fungus *Fusarium moniliforme*. Mycol Res 94:635–640
Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction fragments to high specific activity. Anal Biochem 132:6–13
Forino LMC, Tagliasacchi AM, Cavallini A, Cionini G, Giraldi E, Cionini PG (1992) RNA synthesis in the embryo suspensor of *Phaseolus coccineus* at two stages of embryogenesis, and the effect of supplied gibberellic acid. Protoplasma 167:152–158
Hadwiger LA (1988) Possible role of nuclear structure in disease resistance of plants. Phytopathology 78:1009–1014
Hahn MG, Bucheli P, Cervone F, Doares H, O'Neill RA, Darvill A, Albersheim P (1989) The roles of cell wall constituents in plant-pathogen interactions In: Nester E, Kosuge T (eds) Plant microbe interactions, vol 3. Macmillan Press, New York, pp 131–181
Hennen S, Mizuno S, Macgregor HC (1975) In-situ hybridization of ribosomal DNA labelled with ^{125}iodine to metaphase and lampbrush chromosomes from newts. Chromosoma 50:349–369
Hodgson CP, Fisk RZ (1987) Hybridization probe size control: optimized "oligolabelling". Nucleic Acids Res 15: 62–65
Hutchinson N, Weintraub H (1985) Localization of DNAse I-sensitive sequences to specific regions of interphase nuclei. Cell 43:471–482
Jin DF, West CA (1984) Characteristics of galacturonic acid oligomers as elicitors of casbene synthetase activity in castor bean seedlings. Plant Physiol 74:989–992
Kost MV, Bolsheva NL, Badaev NS, Zelenin AV (1991) Regularities of in-situ nick translation labelling pattern of cereal metaphase chromosomes. Genome 34:131–138
Macgregor HC, Mizuno S (1976) In-situ hybridization of "nick-translated" ^{3}H-ribosomal DNA to chromosomes from salamanders. Chromosoma 54:15–25
McIntosh RA (1983) A catalogue of gene symbols for wheat (1983 edition). Proc 6th Intl Wheat Genet Symp. Kyoto, Japan. pp 1197–1254
Nagl W (1967) Riesenchromosomen von *Phaseolus coccineus* L.: Baueigentumlichkeiten, Strukturmodifikationen, zusätzliche Nukleolen und Vergleich mit dem mitotischen Chromosomen. Österr Bot Z 114:171–182
Nagl W (1974) The *Phaseolus* suspensor and its polytene chromosomes. Z Pflanzenphysiol 73:1–44
Nagl W (1979) Condensed interphase chromatin in plant and animal cell nuclei: fundamental differences. Plant Syst Evol suppl 2:247–260
Newport JW, Forbes DJ (1987) The nucleus: structure, function and dynamics. Annu Rev Biochem 56: 535–565
Nothnagel EA, McNeil M, Albersheim P, Dell A (1983) Host-pathogen interaction. XII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. Plant Physiol 71:916–926
Pardue ML, Hennig W (1990) Heterochromatin: junk or collectors item? Chromosoma 100:3–7
Rigby WJ, Dieckmann M, Rhodes C, Berg P (1977) Labelling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase I. J Mol Biol 113:237–251
Salvi G, Giarrizzo F, De Lorenzo G, Cervone F (1990) A polygalacturonase-inhibiting protein in the flowers of *Phaseolus vulgaris*. J Plant Physiol 136:513–518
Southern E (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98: 503–517
Toubart P, Daroda L, Desiderio A, Salvi G, Cervone F, De Lorenzo G, Bergmann C, Darvill G, Albersheim P (1992) Cloning and characterization of the gene encoding the endopolygalacturonase-inhibiting protein (PGIP) of *Phaseolus vulgaris* L. Plant Jour 2:367–373
Vallejos CE, Sakiyama NS, Chase CD (1992) A molecular marker-based linkage map of *Phaseolus vulgaris* L. Genetics 131:733–740