Acid phosphatase-1^1, a molecular marker tightly linked to root-knot nematode resistance in tomato

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Proefschrift

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in de landbouw- en milieuwetenschappen
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Stellingen

1. De term isozym wordt vaak ten onrechte gebruikt waar allozym wordt bedoeld.

   R. Messeguer *et al.* (1991). Theor. Appl. Genet. 82: 529-536.
   H. Tanaka *et al.* (1990). Agric. Biol. Chem. 54: 1947-1952.

2. De uitkomst van een PCR-reactie is niet alleen afhankelijk van de aanwezigheid van de "target" nucleotidensequentie op de gebruikte DNA-matrijs, maar wordt mede bepaald door de concurrentie van eventuele nevenprodukten om polymerasemolekulen.

3. De conclusie van Kaneko *et al.* dat celwanden van tabak een zure fosfatase bevatten dat uit acht verschillende subunits is opgebouwd, is niet gerechtvaardigd. Veeleer tonen hun resultaten aan, dat het verkregen fosfatasepreparaat nog onzuiver is.

   Kaneko *et al.* (1990). Phytochemistry 29: 2883-2887.

4. Het is onwaarschijnlijk, dat de eiwitband met pI = 4.37, beschreven door Günther *et al.*, zure fosfatase-1^1 (APS-1^1) van tomaat representeert.

   Günther *et al.* (1988). Electrophoresis 9: 618-620.

5. Voor de betrokkenheid van de aryl hydrocarbon (Ah) receptor bij het carcinogene effect van dioxinen en polyhalogeenbifenylen bestaat onvoldoende bewijs.

   A. Poland en J.C. Knutson (1982). Ann. Rev. Pharmacol. Toxicol. 22: 517-554.
   S. Safe (1990). Crit. Rev. Toxicol. 21: 51-88.
   R.E. Keenan *et al.* (1991). J. Toxicol. Environ. Health 34: 279-296.

6. Het blinde vertrouwen van veel consumenten in de heilzame eigenschappen van natuurlijke voedingsmiddelen is ongenuanceerd, en gaat voorbij aan de gevaren verbonden aan geregelde consumptie van toxische componenten, die van nature in deze produkten aanwezig kunnen zijn.

7. Uit het oogpunt van verantwoord milieubeleid verdient het aanbeveling polychloorbifenylen (PCBs) voor toxicologisch onderzoek uit afgewerkte transformatorvloeistof te winnen.

8. Het afbreken van de ontwikkeling van de menselijke persoonlijkheid naar zijn eigen, unieke voltooiing is in elk levensstadium, zowel prenataal als postnataal, een onherstelbare misdaad.

9. De afbeelding op de jubileumpostzegel, uitgegeven bij het 75-jarig bestaan van de Landbouwuniversiteit, geeft een verkeerde indruk van de biologische bestrijding van witte vlieg met parasitaire sluipwespen.

10. De zogenaamde post-promotionele depressie kan voorkomen worden door aansluitend aan de verdediging van het proefschrift met een nieuw promotie-onderzoek te beginnen.

Stellingen behorende bij het proefschrift
"Acid phosphatase-1[1],
a molecular marker tightly linked to root-knot nematode resistance in tomato"
door Jac M.M.J.G. Aarts, te verdedigen op vrijdag 14 mei 1993 te Wageningen.

Aan mijn ouders en zus

Contents

Summary

SUMMARY

Root knot nematode resistance in tomato is a genetic trait which is determined by a single dominant gene (*Mi*) on chromosome 6 of tomato. Information about the mRNA or protein product is completely lacking, which precludes the cloning of *Mi* by conventional strategies based on gene expression. However, an acid phosphatase-1 allozyme marker (*Aps-1*^{1}) is known, which shows tight genetic linkage to the root knot nematode resistance trait. With a view to isolating *Mi* nucleotide sequences by a positional cloning approach, we have developed a molecular probe for the *Aps-1*^{1} gene, using the polymerase chain reaction (PCR) and a pair of primers, corresponding to amino acid sequence information from the protein encoded by the *Aps-1*^{1} gene.

The *Aps-1*^{1} gene product (APS-1^1) was purified from hydroponic tomato roots and tomato suspension cells using conventional low pressure column chromatographic techniques. The most striking purification was achieved by concanavalin A (Con A)-Sepharose 4B affinity column chromatography, which constituted the fourth step in our purification procedure and produced virtually pure APS-1^1. Unfortunately, however, this step caused contamination of the APS-1^1 preparation with Con A released form the column. Therefore, a final Mono Q-FPLC purification step was added to remove the Con A-contamination. The resulting APS-1^1 preparation was homogeneous in denaturing and non-denaturing polyacrylamide gel electrophoresis. In addition, the purified protein showed co-electrophoresis and co-elution with the APS-1^1 enzymatic activity in non-denaturing PAGE and gel filtration column chromatography, respectively, thereby demonstrating its APS-1^1 identity. The yield of our purification protocol was a few µg of APS-1^1 protein per kg of tomato suspension cells or roots. No major loss of APS-1^1 activity was observed in any of the purification steps, indicating that the low yield attained is truly reflecting the very low expression level of the *Aps-1*^{1} gene.

The purified APS-1^1 preparation was treated with CNBr and trypsin to produce APS-1^1 peptides. Following purification by HPLC, amino acid sequence analysis of two CNBr and seven trypsin cleavage products revealed 61 residues of APS-1^1 amino acid sequence.

The amino acid sequence information from two APS-1^1 peptides consisting of 8 and 14 amino acids respectively, allowed the synthesis of PCR primer pools to direct the amplification of a 2.4 kb *Aps-1*^{1} fragment using a genomic DNA template. Crucial for the effectivity of these pools was the limitation of the number of different primers used to account for codon degeneracy. Restriction of the complexity of the primer pools was achieved by incorporating deoxyinosine or the most probable nucleotide(s) at ambiguous codon positions in the 5' part of the primers. On the other hand, efficient primer elongation was assured by including in the primer pool every possible combination of the three 3'-terminal codons. The 2.4 kb fragment

was only amplified from a genomic template carrying the *Aps-1*^{1} allele and was not found in using a template that carried the *Aps-1*^{3} or *Aps-1*$^+$ allele. Moreover, the 2.4 kb amplification product was found to reveal RFLPs between a pair of nematode resistant and sensitive nearly isogenic lines, which only differed for the *Aps-1*^{1}*/Mi* region. The specific amplification under the direction of the particular *Aps-1* allele from which the primers had been derived and its genetic map position provide evidence showing, that the 2.4 kb PCR product represents the *Aps-1*^{1} target fragment. Using cDNA as a template and the same primers that directed the synthesis of the 2.4 kb genomic PCR product, a 490 bp *Aps-1*^{1} fragment was obtained. An overlapping *Aps-1*^{1} cDNA sequence of 550 bp was amplified using the same upstream primers but a different pool of downstream primers corresponding to a peptide that turned out to represent the C-terminus of APS-1^1. The amount of these cDNA-directed amplification products synthesized in 30 cycles of PCR was so low, that their production was only detectable by Southern blot hybridization using the 2.4 kb genomic PCR product as a probe, which provides another demonstration of the very low expression level of the *Aps-1*^{1} gene.

In addition to the 2.4 kb genomic *Aps-1*^{1} sequence, another PCR product of about 115 bp was obtained using a different pair of primers and either a genomic or a cDNA template. This product was found to comprise several related nucleotide sequences of similar size. Because of the poor performance of the cloned products as a probe, it was not possible to establish their genetic map position by RFLP analysis.

Screening of a cDNA library with the 2.4 kb putative *Aps-1*^{1} sequence identified two clones of related nucleotide sequences, one of which apparently represented an *Aps-1*^{1} cDNA clone, as it encoded three APS-1^1 peptides (together 30 amino acids) in the orientation predicted by the PCR results.

The nucleotide sequence of this *Aps-1*^{1} cDNA clone revealed a stretch of 69 residues of tomato APS-1^1 amino acid sequence starting from the C-terminus, and showed that peptide VII is the C-terminal tryptic peptide. Another stretch of putative APS-1^1 amino acid sequence was deduced from the nucleotide sequence adjacent to the upstream primer in the 2.4 kb genomic PCR product, and comprised 56 amino acids sequence information starting with peptide IX at the N-terminal end. The amino acid sequence of tomato APS-1^1 elucidated so far, did not present major sequence homology with the sequences of any other (acid) phosphatase in the GenBank or EMBL data bases. A striking sequence homology was found, however, with a vegetative storage protein from soybean, VSP-β, that accumulates to very high levels in leaves after depodding of the plants.

The detection of an *Aps-1*^{1}-related cDNA clone using the 2.4 kb genomic *Aps-1*^{1} sequence as a probe, and furthermore, the sequence heterogeneity among the 115 bp PCR product points to the existence of a family of *Aps-1*^{1}-related nucleotide sequences within the tomato genome. Upon comparison of four different nucleotide sequences present in the 115 bp PCR product with the *Aps-1*^{1} cDNA sequence, it was found, that one of them corresponded

with the *Aps-1*^{1} sequence. Neither of the other three, nor the *Aps-1*^{1}-related cDNA clone showed a higher homology at the amino acid level with soybean VSP-β than with tomato APS-1^{1}, which argues against one of these sequences representing the tomato homologue of soybean VSP-β.

In the isolation of an *Aps-1*^{1} cDNA clone, we have shown that it is possible to design highly specific degenerate PCR primer pools. Therefore, whenever going from protein to gene, we recommend to try PCR first in order to obtain a highly selective probe, before turning to library screening using degenerate oligonucleotides. Furthermore, since an *Aps-1*^{1} cDNA clone is available now, a possible starting point or a useful landmark has been provided for a chromosomal walk towards the nematode resistance gene *Mi*.

Chapter 1

General introduction

ROOT-KNOT NEMATODES

History and significance

Nematodes (*Nematoda*) or round-worms constitute a class of vermiform animal species which include a number of major plant pathogens. Among those, the root-knot nematodes (*Meloidogyne* spp.) are regarded as particularly harmful to agriculture.

The first description of a plant-parasitic nematode dates from 1743 when Needham reported his observations of nematodes in blighted wheat kernels [Needham, 1743; Dowler and Van Gundy, 1984]. Root-knot nematodes were mentioned explicitly in 1855 by Berkeley in England as 'vibrios forming excrescences on cucumber roots' [Berkeley, 1855]. Initially, root-knot nematodes were named *Anguillula marioni* by Cornu [1879]. Later they were independently referred to as *Heterodera radicicola* Müller [Müller, 1884], *H. javanica* Treub, *Meloidogyne exigua* Göldi [Göldi, 1887], *A. arenaria* Neal [Neal, 1889], *Tylenchus arenarius* Cobb, *A. vialae* Lavergne, *Heterodera marioni* (Cornu) Marcinowski [Marcinowski, 1909], *Oxyurus incognita* Kofoid and White, and *Caconema radicicola* Cobb [Cobb, 1924]. Finally, Chitwood [1949] revised the genus *Meloidogyne* Göldi 1887 to include all root-knot nematode species. Nowadays, the four major root-knot nematode species are referred to as *M. incognita* (Kofoid and White) Chitwood or southern root-knot nematode, *M. hapla* Chitwood or northern root-knot nematode, *M. javanica* (Treub) Chitwood or Javanese root-knot nematode, and *M. arenaria* (Neal) Chitwood or peanut root-knot nematode.

The four most prevalent root-knot nematode species, *M. incognita*, *M. hapla*, *M. javanica*, and *M. arenaria*, all together show a world-wide distribution and a very broad host range [Sasser, 1977; 1980; Sasser *et al.*, 1983; Sasser and Carter, 1985], including a diversity of economically important crop species such as potato, wheat, rice, peanut, cotton, tomato, beans and other leguminous vegetables [Nickle, 1984; Fassuliotis, 1985]. Together they are responsible for extensive crop losses, estimated as high as 5% of total agricultural production world-wide [Sasser *et al.*, 1983; Sasser and Carter, 1985]. However, the estimations made thus far may still be too low, since they mostly refer to the losses directly attributable to root-knot nematodes, and they do not take into account the collateral damage and the unnoticed crop losses [Sasser *et al.*, 1983; Mai, 1985].

Interaction with the host plant

Root-knot nematodes owe their name to the nodule-like structures formed on the surface of infected roots. These so-called root-knots or galls contain the adult females, which are

sedentary and deposit eggs enclosed in a gelatinous egg sac at their posterior end, the side of the body that is usually placed at the surface of the root.

Following embryogenesis, the juvenile molts once within the egg. Subsequent egg hatching occurs spontaneously without any stimulus from plant roots [Hussey, 1985; Perry, 1987; Idowu and Fawole, 1990]. The resulting second-stage juveniles are mobile and constitute the infective stage. They migrate in the soil and are generally attracted by plant roots. As to what extent roots attract root-knot nematodes, little or no difference was found between host and non-host plants [Lownsbery and Viglierchio, 1961; Hussey, 1985], or between resistant and susceptible cultivars of a particular host plant species [Riggs and Winstead, 1959; Fassuliotis *et al.*, 1970; Reynolds *et al.*, 1970; Griffin and Waite, 1971; McClure *et al.*, 1974; Griffin and Elgin, 1977; Hadisoeganda and Sasser, 1982; Herman *et al.*, 1991; Schneider, 1991]. When the juvenile has contacted a suitable plant root, the cortex is penetrated either directly behind the root cap, or at the branching points of lateral roots, at the penetration sites of other juveniles, or at wounded surfaces. Subsequently, the juveniles migrate intercellularly towards the region where the vascular tissue differentiates, and then start feeding, preferably on cells of the primary phloem or adjacent parenchyma. From this point on, the course of the development is dependent on the compatibility of the interaction between the root-knot nematode and the plant genotype.

Compatible interaction

In a susceptible host, a few cells adjacent to the head of the feeding nematode enlarge to become so-called 'giant cells'. These putative feeding cells develop characteristic ingrowths of the cell wall by which they increase the surface of the plasmalemma to facilitate the transport of solutes to the nematode [Jones and Northcote, 1972; C.S. Huang, 1985]. Giant cells are multinucleate and most probably formed by repeated endomitosis without subsequent cytokinesis as shown by scanning electron microscopy [Jones and Payne, 1978; C.S. Huang, 1985]. Concurrent with giant cell formation, hyperplasia and hypertrophy occur in the surrounding tissues, giving rise to the root gall characteristic of *Meloidogyne* infection.

After having established a parasitic relationship within the plant root and feeding for 3 - 8 weeks, the second molt takes place and the stylet and median bulb of the esophagus disappear. Sufficient metabolic reserves have been stored at this stage to allow two additional molts without any further feeding. This yields the adult (fifth) stage, in which the stylet and the median bulb are regenerated. Soon after the second-stage juvenile has established a parasitic relationship, the genital primordium starts growing, resulting in the differentiation of males and females. Females first become flask-shaped during their development, but after the fourth molt, the adult females restart feeding and assume a pear shape. The females remain sedentary within

the root, where they may live and reproduce for an extended period of three months or more [Heald and Orr, 1984]. The males, on the other hand, evolve a vermiform body shape by molting within the cuticle of the second stage. After the last molt, the adult males (fifth stage) burst out of the cuticle. Unlike the adult females, the adult males do not resume feeding and retain their motility so they can leave the root in search of a female.

Although the ability to generate male individuals has been preserved in the four common root-knot nematode species, males are not necessary for reproduction. *M. incognita*, *M. arenaria* and *M. javanica* reproduce exclusively by mitotic parthenogenesis, whereas in *M. hapla*, facultative meiotic parthenogenesis occurs in some races and obligatory mitotic parthenogenesis in others. Egg masses containing 300 - 1000 single-celled eggs are deposited surrounded by a gelatinous matrix. This gelatinous egg sac is secreted by the rectal glands of the female and sticks to the posterior body end, which is located at the root surface. Depending on the climate and host species, the life cycle can be completed within four to six weeks, resulting in the development of three to five generations in a single growing season.

The activity of the nematode causes a general dysfunction of the root system with poor mineral and water uptake as a main effect. This may result in mineral deficiency symptoms and wilting of the plant during hot periods [Hussey, 1985]. In case of a severe infestation, death of the infected plants can be the ultimate consequence [Johnson and Fassuliotis, 1984; Minton, 1984].

For the sake of a prolonged parasitic relationship, the nematodes probably benefit from maintaining a certain balance between their reproduction rate and the exploitation of the host plant, rather than from killing their host. This might be the reason why the nematode population shows adaptation of its sex ratio in response to high population density [Davide and Triantaphyllou, 1967a] and other stress conditions such as unfavourable temperature [Laughlin *et al.*, 1969], poor plant nutrition [McClure and Viglierchio, 1966; Davide and Triantaphyllou, 1967b] and retarded plant growth [Davide and Triantaphyllou, 1968; Orion and Minz, 1971]. Such conditions were found to increase the proportion of males. From an agricultural point of view, the self-restraint of the nematode population is favourable, since the female nematodes cause most of the crop damage and determine the reproduction rate.

Incompatible interaction

In a resistant plant, several defensive mechanisms, acting at different stages of the host-parasite interaction, may be implicated in the resistance response [Graham and Graham, 1991; Keen, 1992].

As a first defence, root exudates of some plant species contain compounds which are repellant or lethal to root-knot nematodes. In cucumber, for example, certain varieties contain

the bitter (*Bi*) gene, a dominant gene involved in the production of cucurbitacins, triterpenoid compounds, which are toxic to various insects and repel nematodes. Excised roots from bitter cucumber lines were found to attract significantly fewer *M. incognita* nematodes than roots from non-bitter isogenic lines [Haynes and Jones, 1976]. Besides, there is the well-known example of the nematicidal root exudates of marigold (*Tagetes erecta* L.) [Alam *et al.*, 1975], which contain a high concentration of α-terthienyl [Uhlenbroek en Bijloo, 1958]. Although aborted giant cells and dead juveniles and females have frequently been observed in the roots of marigold, the role of α-terthienyl as a nematicide in soil remains unclear. No inhibition of the nematode infestation has been observed during co-cultivation of susceptible tomato plants with marigold [Hackney and Dickerson, 1975; J.-s. Huang, 1985].

If the nematode has succeeded in entering the plant root, post-infectional defence mechanisms are activated. In a localized area adjacent to the nematode hypersensitive necrosis occurs [Dropkin, 1969; Kaplan *et al.*, 1979; Nelson *et al.*, 1990; Ho *et al.*, 1992]. This so-called hypersensitive reaction is considered an important defence response of essentially unknown mechanism that is associated with an incompatible host-pathogen interaction. Cells surrounding the necrotic region often show an increased deposition of callose in their cell walls [Bleve-Zacheo *et al.*, 1982]. The hypersensitive reaction is accompanied by a range of other inducible responses discussed below.

There is a considerable amount of evidence suggesting that nematode resistance involves elevated levels of phenolics [Hung and Rohde, 1973; Singh and Choudhury, 1973], sometimes associated with higher activities of enzymes such as phenylalanine ammonia lyase (PAL) [Brueske, 1980] and β-glycosidase, which are known to play a role in the accumulation of these toxic compounds [Creasy and Zucker, 1974; J.-s. Huang, 1985]. In contrast to the phenolic compounds, which are constitutively formed, phytoalexins [Paxton, 1980], another class of inhibitory compounds, are only produced upon infection. It has been suggested that accumulation of phytoalexins also contributes to the resistance against root-knot nematodes [Veech and McClure, 1977; Veech, 1978; Kaplan *et al.*, 1980].

Another defence reaction often associated with hypersensitive necrosis was investigated by Zacheo *et al.*, [1982]. They showed that peroxidase activity increased and superoxide dismutase activity decreased in nematode-resistant tomato plants upon infection with *M. incognita*, whereas the opposite occurred in susceptible plants. This suggests, that resistant tomatoes generate superoxide and singlet O_2 to inhibit pathogen development. Other data, however, are in contradiction with these results [Ganguly and Dasgupta, 1979].

Increased synthesis of hydroxyproline-containing proteins has also been implicated in nematode resistance through the reduced plasticity of the cell wall, possibly resulting in the suppression of hypertrophy [Giebel and Stobiecka, 1974].

The effect of the defence responses discussed above may be that the invaded nematodes feel urged to migrate out of the root [Reynolds *et al.*, 1970; Griffin and Elgin, 1977; Herman *et*

al., 1991] or even that they are killed within the root before a parasitic relationship has been established [J.-S. Huang, 1985; Schneider, 1991].

The actual importance of the defensive factors suggested remains yet unclear. Molecular cloning of the genes controlling the resistance to root-knot nematodes would provide a first and essential step towards an understanding of the mechanism of the resistance.

BREEDING FOR RESISTANCE AGAINST ROOT-KNOT NEMATODES

Introduction

Transfer of hereditary resistance against root-knot nematodes from resistant germ-plasm of closely or distantly related wild species into adapted crop cultivars would offer an effective and environmentally harmless means to control the root-knot nematode problem [Sasser *et al.*, 1983]. Therefore, several crop plant species and related wild species have been screened for inherited resistance against root-knot nematodes [Fassuliotis, 1985]. To date, useful resistant germ-plasm has been described for a number of crops including tomato (*Lycopersicon esculentum* Mill.) [Bailey, 1941], potato (*Solanum tuberosum* L.) [Mendoza and Jatala, 1985], sweet potato (*Ipomoea batatas* (L.) Lam.) [Jones *et al.*, 1980], wheat (*Triticum aestivum* L.) [Roberts *et al.*, 1982], soybean (*Glycine max* (L.) Merr.) [Boquet *et al.*, 1975], common bean (*Phaseolus vulgaris* L.) [Omwega *et al.*, 1990], eggplant (*Solanum melongena* L.) [Fassuliotis and Dukes, 1972; Fassuliotis and Bhatt, 1982], cantaloupe (*Cucumis melo* L.) [Fassuliotis, 1970, 1977a, 1977b] and okra (*Abelmoschus esculentus* (L.) Moench.) [McLeod *et al.*, 1983]. Introduction of root-knot nematode resistance into crop cultivars has only been accomplished till now through conventional breeding procedures [Tanksley *et al.*, 1989; Melchinger, 1990], for instance in tomato [Medina-Filho and Tanksley, 1983], tobacco [Clayton, 1958; Slana *et al.*, 1977; Barker, 1978; Schneider, 1991], wheat [Kaloshian *et al.*, 1989a; 1989b; 1991], common bean [Omwega *et al.*, 1990], soybean [Boquet *et al.*, 1975], and alfalfa [Reynolds, 1955; Stanford *et al.*, 1958; Goplen and Stanford, 1959; Goplen *et al.*, 1959; O'Bannon and Reynolds, 1962; Nigh, 1972; Peaden *et al.*, 1976; Hartman *et al.*, 1979], and in some other crop species [Fassuliotis, 1985; Sasser and Kirby, 1979]. However, classical plant breeding is very time-consuming and not generally applicable, as natural incompatibility barriers may preclude the exploitation of germ-plasm holding inherited resistance against root-knot nematodes. For example, the African horned cucumber, *Cucumis metuliferus* Naud., represents a potential root-knot resistance source for cultivated cucumbers and other

Cucurbitaceae, but interspecific hybridization with *C. melo* failed because of the inviability of the immature hybrid embryo [Fassuliotis, 1977b]. Beside *C. metuliferus*, there is no other, compatible resistance source known.

Recombinant DNA technology offers an alternative possibility for the introduction of hereditary pathogen resistance into susceptible crops [Barton and Brill, 1983; Clark *et al.*, 1991], as it has the potential of circumventing incompatibility barriers. To date, however, this approach is hampered by a general lack of knowledge about the molecular basis of disease resistance. Although its feasibility remains to be proven, 'genetic engineering' using DNA technology seems the most promising approach if there is no compatible, resistant germ-plasm available. Therefore, a study of plant genes and gene products conferring root-knot nematode resistance is desirable.

As yet, the research into pathogen resistance of plants would greatly benefit from model systems. Apart from the possible impact on agriculture, a molecular analysis of root-knot nematode resistance in plants may provide such a profitable model, useful to tackle more complicated resistance mechanisms, thereby contributing to our general understanding of the defence against plant pathogens.

In this introduction, we shall further focus on the resistance against root-knot nematodes which has been found in a wild tomato species, *Lycopersicon peruvianum* (L.) Mill. [Bailey, 1941; Romshe, 1942; Ellis, 1943], as the resistance is genetically simple and well-defined. Furthermore, an acid phosphatase-1 allozyme marker (*Aps-1*^{1}) is known which is tightly linked to the resistance trait, allowing a 'positional cloning' strategy to gain access to the resistance gene (page 25).

Breeding for root-knot nematode resistance in tomato

Bailey [1941] was the first to describe the occurrence of *L. peruvianum* plants 'more tolerant to root knot'. Using embryo culture, Smith [1944] surmounted the inviability of the *L. esculentum* × *L. peruvianum* (P.I. 128657) hybrid embryo, allowing the transfer of the resistance into commercial tomato varieties by repeated backcross breeding. In fact, all currently available nematode-resistant tomato cultivars derive from a single F_1 plant from this original cross [Watts, 1947; Medina-Filho and Tanksley, 1983].

Gilbert and McGuire [1955; 1956] established, that the resistance present in tomato lines derived from *L. peruvianum* P.I. 128657 was determined by a single major, dominant locus, to which they assigned the symbol *Mi*, an acronym taken from the first letters of the root-knot nematode *Meloidogyne incognita*. Although some conflicting data have been reported as to the number of genes involved [Watts, 1947; Frazier and Dennett, 1949; Barham and Sasser, 1956] later reports confirm the monogenic nature of the resistance [Barham and

Winstead, 1957a; 1957b; Winstead and Barham, 1957; Thomas and Smith, 1957], or suggest a block of genes acting as a unit [Harrison, 1960]. The *Mi* locus was mapped on chromosome 6 at position 44 [Gilbert, 1958; see also Koornneef and Zabel, 1990]. *Mi* confers resistance to the prevalent pathotypes of *M. incognita*, *M. arenaria*, *M. javanica* and *M. incognita acrita* [Barham and Winstead, 1957a], but not to *M. hapla* [Hadisoeganda and Sasser, 1982; Ammati *et al.*, 1985; Sidhu and Webster, 1981; Johnson and Fassuliotis, 1984]. Resistance breaks down, however, at temperatures above 28 °C [Dropkin, 1969; Ammati *et al.*, 1986]. Another complication emerged, as carefully performed infectivity tests, using a well-characterized nematode inoculum cultured from a single egg mass, convincingly demonstrated the existence of a minority of *Mi*-compatible populations within species taken as completely *Mi*-incompatible [Triantaphyllou and Sasser, 1960; Netscher, 1970; 1977; Sikora *et al.*, 1973; Viglierchio, 1978; Bost and Triantaphyllou, 1982; Hadisoeganda and Sasser, 1982; Prot, 1984; Ammati *et al.*, 1985; Roberts and Thomason, 1986; 1989; Fargette and Braaksma, 1990; Roberts *et al.*, 1990].

Mi is considered the only locus involved in root-knot nematode resistance of tomato cultivars by some authors [Medina-Filho and Tanksley, 1983]. Genetic data suggest, however, that genes for resistance other than *Mi* are present in at least a few resistant cultivars [Sidhu and Webster, 1975; 1981]. According to these data, the cultivar Small Fry-1, for example, would not possess *Mi*, but a different gene for resistance, $LMiR_2$, that is located on chromosome 6 at a genetic distance of 5.65 map units form the *Mi* locus [Sidhu and Webster, 1975]. Recent molecular analyses [Klein-Lankhorst *et al.*, 1991a; Messeguer *et al.*, 1991a; Ho *et al.*, 1992; Zabel *et al.*, unpublished results; Williamson *et al.*, unpublished results], on the other hand, do not reveal any heterogeneity of the *Mi* chromosomal region among nematode-resistant cultivars. Therefore, it seems unlikely that the genetic constitution of the chromosomal region encompassing *Mi* would differ within the group of resistant tomato cultivars. More likely, the resistant tomato cultivars carry the same gene(s) for resistance at this chromosomal region, the more so, as in all cases the resistance derives from the same *L. peruvianum* P.I. 128657 gene source [Smith, 1944; Medina-Filho and Tanksley, 1983]. Consequently, if genes for resistance other than *Mi* exist within or outside the *Mi* chromosomal region of any tomato cultivar, then both *Mi* and these additional resistance genes should have originated from *L. peruvianum* P.I. 128657. In conclusion, the possibility should be kept in mind, that the root-knot nematode resistance trait designated by the single symbol *Mi*, is in fact governed by (two) tightly linked genes commonly appearing as a single dominant locus.

Resistance to root-knot nematodes apparently different from *Mi* has not only been reported for some commercial tomato cultivars derived from *L. peruvianum* P.I. 128657, but also for other accession numbers within the *L. peruvianum* complex [Ammati *et al.*, 1985; 1986; Roberts *et al.*, 1990]. These accessions carried a type of resistance unlike *Mi*, which was active against *M. hapla* (*L. peruvianum* Acc. No. 270435 and var. *glandulosum* C. H. Mull.

Acc. Nos. 126440 and 126443) or retained its effectiveness at 32 °C (*L. peruvianum* Acc. Nos. 270435, 129152 and LA2157, and var. *glandulosum* Acc. No. 126443). The number of genes involved and their possible allelic or linkage relationship with *Mi* are yet unclear. The introgression of these resistances into adapted tomato cultivars is getting under way [Cap *et al.*, 1991; Scott *et al.*, 1991]. Unfortunately, root-knot nematode populations which can circumvent these new types of resistance have already been found as well [Roberts *et al.*, 1990]. No high resistance level was found in other *Lycopersicon* species [Bailey, 1941; Medina-Filho and Tanksley, 1983; Ammati *et al.*, 1985], although a more recent screening for resistance against *M. incognita* and *M. javanica* [Lobo *et al.*, 1988] indicated a certain level of resistance in some genotypes of *L. peruvianum* and, in addition, in certain genotypes of *L. pennellii*, *L. parviflorum*, *L. chmielewskii*, *L. pimpinellifolium* and *L. hirsutum*.

Mi AS A FAVOURABLE MODEL FOR STUDYING NEMATODE RESISTANCE IN PLANTS

In view of its monogenic, dominant character [Gilbert and McGuire, 1955], the nematode resistance trait conferred by the *Mi* locus presents an attractive model system for studying nematode resistance of plants at the molecular level. Besides, there is a wealth of genetic and biochemical information available about tomato, and elaborate classical and molecular genetic linkage maps [Tanksley and Mutschler, 1990; Koornneef and Zabel, 1990; Klein-Lankhorst *et al.*, 1991a; 1991b] as well as an efficient transformation procedure [Horsch *et al.*, 1985; Fillatti *et al.*, 1987; Koornneef *et al.*, 1987; Hille *et al.*, 1989; Davis *et al.*, 1991]. In addition, a variety of genetic tools have been constructed including morphology marker [Tomato Genetics Stock Center, 1991] and chromosome substitution lines [Rick, 1969; Weide *et al.*, 1993], trisomics [Rick and Barton, 1954; Rick *et al.*, 1964] and pairs of nearly isogenic lines (NILs) that differ only in the *Mi* region on chromosome 6 of tomato [Klein-Lankhorst *et al.*, 1991a]. Recently, other useful genetic tools have become available, as a number of recombinants carrying a crossing-over in the *Mi* chromosomal region were identified [Ho *et al.*, 1992].

Most tomato cultivars holding *Mi* have retained the acid phosphatase-1 allele $Aps\text{-}1^1$, that has been carried along on the introgressed chromosomal segment from the *L. peruvianum* donor genome during the breeding program [Rick and Fobes, 1974; Medina-Filho and Tanksley, 1983]. Linkage between the *Mi* and *Aps-1* loci is so tight that the $Aps\text{-}1^1$ allele has proven to be a convenient and reliable isozyme marker for nematode resistance [Medina-Filho and Tanksley, 1983]. Since no product of the *Mi* gene is known, nor of any other plant disease

resistance gene as a matter of fact, *Mi* is not amenable to isolation through conventional cloning strategies, which are based on gene expression. Instead, since its map position has been established, molecular access to the *Mi* locus might be gained in a so-called 'positional cloning' approach, further specified below, which involves a chromosomal walk between two closely flanking molecular markers. Obviously, the *Aps-1*^{1} gene is a serious candidate as a marker to be isolated for this purpose. Its genetic distance to *Mi* of less than 1 cM [Medina-Filho, 1980] suggests a physical distance in the order of 500 kb, which is within the reach of current long-range cloning and mapping strategies [Burke *et al.*, 1987; Collins, 1988; Poustka and Lehrach, 1988; Rommens *et al.*, 1989]. Moreover, the *Aps-1*^{1} gene itself is accessible to cloning by conventional methods based on the encoded protein product [Paul and Williamson, 1987].

APPROACHES TO THE MOLECULAR CLONING OF THE *Mi* GENE.

Molecular gene cloning strategies are mostly based on knowledge of the (m)RNA or protein product of the gene in question. Techniques using antibodies against the encoded protein [Huynh *et al.*, 1985; Snyder *et al.*, 1987; Sambrook *et al.*, 1989] and oligonucleotides corresponding to nucleotide sequence [Miyada and Wallace, 1987; Sambrook *et al.*, 1989] or amino acid sequence information [Ohtsuka *et al.*, 1985; Lathe, 1985; Wood *et al.*, 1985; Devlin *et al.*, 1988; Sambrook *et al.*, 1989] have now become well-established, making the molecular cloning of a gene encoding a known product to a standard practice for molecular biologists.

On the other hand, when information about the product of a gene is lacking, as is the case for *Mi*, there is a much longer way to go, usually via a nearby flanking marker which has previously been identified by genetic analysis and is by itself amenable to molecular cloning using conventional methods. Subsequently, cloned sequences from the chromosomal region including the gene of interest can be obtained by a chromosomal walk starting with the clone of the closely linked sequence. Since its acid phosphatase-1^{1} protein product (APS-1^{1}) has been identified [Rick and Fobes, 1974] and a protocol for its purification has been described [Paul and Williamson, 1987], the closely linked *Aps-1*^{1} gene meets the requirements to be cloned as a candidate starting point for chromosome walking towards *Mi*. Subsequently, the *Mi* target gene has to be identified among the restricted number of candidate sequences present within the region of the chromosomal walk.

This approach is often referred to as 'reversed genetics' [Orkin, 1986; Goodfellow, 1987; 1989; Rommens *et al.*, 1989], since the nature of the gene product will only be revealed

in the end by inference from its genetic code, rather than the other way round. In a sense, this implies indeed a reversal of the usual order of events in classical or 'forward genetics' [Berg, 1991], namely, the previously known gene product giving entrance to its DNA code. But on the other hand, it is a disputable extension of the original definition of 'reversed genetics' by Charles Weissmann [Berg, 1991], who conceived the term for 'an approach wherein DNA regions are modified at predetermined positions and the effects of these interventions are scored *in vivo* or *in vitro*, in contradiction to classic genetics, where deviant phenotypes are first isolated, and the lesion giving rise to them is identified subsequently' [Müller *et al.*, 1978]. Thus, 'reversed genetics' was originally attributed to the experimental path from a defined deoxyribonucleotide sequence to the corresponding phenotypic characteristic. Therefore, it is inconsequent to use the same term for a gene cloning strategy. 'Positional cloning' [McKusick, 1991a; Camerino and Goodfellow, 1991; Wicking and Williamson, 1991] and 'map-based cloning' [Tanksley *et al.*, 1989] have been proposed as more appropriate alternatives for 'reversed genetics' in this illegitimate sense. In this thesis, 'positional cloning' rather than 'reversed genetics' will be used to designate a gene cloning strategy essentially involving a chromosomal walk starting from a nearby molecular genetic marker.

Below, the different steps of the 'positional cloning' approach will be explained first. Thereafter (page 30), some alternative strategies for product-independent gene cloning will be discussed. Although most of the other methods for product-independent gene cloning have only limited applicability, some were conceivable for the cloning of *Mi*.

'Positional cloning'

Linkage analysis

In general, the gene to be isolated by 'positional cloning' is tracked down to a restricted chromosomal region using segregation studies, cytological analysis of chromosome abnormalities such as deletions, and a diversity of other genetic mapping techniques, including RFLP analysis of animal somatic cell hybrids or plant chromosomal substitution- and nearly isogenic lines (NILs). When the approximate map position of the gene of interest has been determined, flanking markers, preferably on both sides of the target gene, are established, if not yet available. Essentially any tightly linked DNA sequence which is polymorphic and clonable is useful as a marker for the 'positional cloning' approach.

Nowadays, a variety of polymorphic DNA markers are exploited in physical mapping and chromosome walking, including defined gene loci such as allozymic markers, and anonymous DNA markers like RFLPs [Botstein *et al.*, 1980; Wyman and White, 1980; Beckmann and Soller, 1983; Tanksley *et al.*, 1989], VNTRs (Variable Number of Tandem

Repeat) [Nakamura *et al.*, 1987] and SSLPs (Simple Sequence Length Polymorphism) [Tautz and Renz, 1984; Tautz *et al.*, 1986; Tautz, 1989; 1990] also referred to as STRs (Short Tandem Repeat) [Rossiter and Caskey, 1991]. A recently developed method for *in vitro* amplification of specific DNA fragments, the polymerase chain reaction (PCR) [Saiki *et al.*, 1985; 1988; Mullis *et al.*, 1986; Mullis and Faloona, 1987; Ehrlich *et al.*, 1988], promises to be highly useful for developing new types of molecular markers [Evans, 1991; Rose, 1991]. PCR owes its extraordinary potential to the ability to detect different types of DNA polymorphisms, including those giving rise to random amplified polymorphic DNA (RAPD) [Williams *et al.*, 1990; Welsh and McClelland, 1990] and the polymorphisms caused by variation in number of tandemly repeated sequence units [Beckmann and Soller, 1990; D'Ovido *et al.*, 1990; Rossiter and Caskey, 1991]. Therefore, taking full advantage of the PCR could greatly increase the number of reliable and highly polymorphic molecular markers to be used in linkage analysis [Billings *et al.*, 1991; Rossiter and Caskey, 1991].

Most examples of genes approached by a chromosomal walk starting from a nearby marker are found among the genes for human hereditary diseases. The RB locus, determining retinoblastoma, an inheritable tumour of the eye, was found to be tightly linked to an allozymic marker, esterase D [Yunis and Ramsay, 1978; Ward *et al.*, 1984; Sparkes *et al.*, 1983, 1984], which could be cloned using oligonucleotide and antibody probes generated on the basis of the purified esterase D protein [Lee and Lee, 1986; Squire *et al.*, 1986]. Unfortunately, chromosome walking from the esterase D gene towards the RB locus was hampered by the occurrence of highly repetitive sequences between the two loci [Lee *et al.*, 1987a]. Eventually, the RB locus was reached using another tightly linked starting point, the anonymous genomic DNA clone H3-8 [Friend *et al.*, 1986; 1987; Lee *et al.*, 1987a; 1987b; Fung *et al.*, 1987]. Other human genes which were mapped in the vicinity of hereditary disease loci include those encoding the β-subunit of follicle-stimulating hormone (FSHB), erythrocyte catalase (CAT) and a cell surface antigen (MIC1) near the Wilms' tumour locus [Kao *et al.*, 1976; Van Heyningen *et al.*, 1985; Glaser *et al.*, 1986, 1987], the growth hormone GH1 gene near the hyperkalemic periodic paralysis (HYPP) locus [Fontaine *et al.*, 1990] and the creatine kinase gene (CKMM) close to the locus of myotonic dystrophy [Yamaoka *et al.*, 1990].

In addition, tightly linked anonymous DNA markers have been established for all these disease genes. Such markers were also essential in mapping the genes for Duchenne muscular dystrophy [Francke *et al.*, 1985; Monaco *et al.*, 1985; Kunkel *et al.*, 1985, 1986; Van Ommen *et al.*, 1986; Koenig *et al.*, 1987], chronic granulomatous disease [Francke *et al.*, 1985; Baehner *et al.*, 1986; Royer-Pokora *et al.*, 1986], cystic fibrosis (Tsui *et al.*, 1985; White *et al.*, 1985; Wainwright *et al.*, 1985] and many others [Orkin, 1986; McKusick, 1991b; Rossiter and Caskey, 1991; Wicking and Williamson, 1991].

Long-range chromosome walking and restriction mapping

Once DNA markers have been identified and ordered around the target gene, a chromosomal walk can be started from the nearest flanking markers. Usually, the distance in basepairs to be traversed down to the target gene will be quite long, even if the genetic linkage is very tight, that is in the order of only a single map unit. This holds true, in particular, for the genomes of higher eukaryotes like mammals and flowering plants, in which the physical size of the map unit is in the megabase range [Lewin, 1990], because the frequency of crossing-over is relatively low. This implies, that mostly several thousands of kb have to be traversed, which is quite impracticable using the conventional chromosome walking techniques, that allow steps of, on average, only 25 kb [Marx, 1985; Poustka and Lehrach, 1986; Collins, 1988; Sambrook *et al.*, 1989].

Indeed, 'positional cloning' would have been unfeasible without the variant chromosome walking techniques recently developed, permitting 'jumps' over hundreds of kilobases at a time. The essential part of these chromosome 'jumping' or 'hopping' techniques is the circularization of large (> 100 kb) DNA fragments by ligating their ends together and subsequently cloning the joined ends, which are located hundreds of kb apart on the DNA strand along which jumping is performed [Collins and Weissman, 1984; Poustka and Lehrach, 1986; 1988; Poustka *et al.*, 1986; 1987; Smith *et al.*, 1987a; Collins *et al.*, 1987; Collins, 1988]. A complete set of such junction probes is called a jumping library. The use of rare cutters like *Not* I to generate the large DNA fragments [Qiang and Schildkraut, 1984; 1987; O'Connor *et al.*, 1984] permits the jumping library to be applied in conjunction with a complementary linking library, consisting of clones just spanning the ends of contiguous large restriction fragments represented in the jumping library [Smith *et al.*, 1987a; Poustka and Lehrach, 1986; Poustka *et al.*, 1986; Buiting *et al.*, 1988; Pohl *et al.*, 1988; Ito and Sakaki, 1988; Wallace *et al.*, 1989; Brockdorff *et al.*, 1990; Saito *et al.*, 1991]. Alternate screening of the linking and jumping library, respectively using as a probe the jumping or the linking clone identified in the previous step, will result in 'jumping' from a given large restriction fragment to an adjacent one. In this way, a coarse physical map of the passed chromosomal region is drawn and the target gene is approached in long strides rather than by shuffling along overlapping cosmids like in conventional chromosome walking.

The construction of jumping libraries becomes more difficult and expensive with increasing jump size, since an increasing average length of the DNA fragments to be circularized requires a directly proportionally higher input of very high molecular weight DNA, whereas the ligation volume and hence the amount of DNA ligase has to be increased even as the $^3/_2$ power of the average size of the jump [Collins, 1988]. Because of these technical limitations, the average jump size of current jumping libraries is usually in the range of 100 - 200 kb [Collins *et al.*, 1987; Richards *et al.*, 1988], although larger jumps have been reported

[Poustka *et al.*, 1987]. Complementary to chromosome jumping, cloning of large DNA fragments of several hundred kb into yeast artificial chromosomes (YACs) offers a tool for crossing and mapping extensive chromosomal regions [Burke *et al.*, 1987; Ward and Jen, 1990].

The new long-range cloning and mapping strategies are assisted by methods to manipulate high molecular weight DNA molecules without shearing and by recent modifications of conventional agarose gel electrophoresis allowing the separation of very large DNA fragments [Smith and Cantor, 1987; Smith *et al.*, 1988]. These electrophoresis methods, which are capable of separating DNA molecules up to 10 Mb [Anand, 1986; Snell and Wilkins, 1986; Vollrath and Davis, 1987; Smith *et al.*, 1987b; Orbach *et al.*, 1988], have now evolved into a number of variants, such as pulsed field gel electrophoresis (PFGE) [Schwartz *et al.*, 1983; Schwartz and Cantor, 1984], field-inversion gel electrophoresis (FIGE) [Carle *et al.*, 1986; Van Daelen *et al.*, 1989], and contour-clamped homogeneous electric field (CHEF) gel electrophoresis [Chu *et al.*, 1986].

Long-range chromosome walking and mapping techniques have already been successfully applied in the molecular cloning of a number of human hereditary disease genes. Using chromosome jumping, a long-range restriction map was constructed of 280 kb of genomic DNA from the cystic fibrosis (CF) chromosomal region [Collins *et al.*, 1987; Rommens *et al.*, 1989], which subsequently allowed the identification and molecular cloning of the complete CF cDNA sequence [Riordan *et al.*, 1989]. Long-range restriction mapping was found indispensable in producing a physical map of the giant Duchenne muscular dystrophy (DMD) locus which spans at least 2 Mb of DNA [Burmeister and Lehrach, 1986; Van Ommen *et al.*, 1986; Kenwrick *et al.*, 1987; Monaco and Kunkel, 1987; Koenig *et al.*, 1987]. Kenwrick *et al.* (1988) used chromosome jumping to move at least 80 kb from within a large intron sequence in the DMD gene to a region adjacent to an exon of the gene. YAC cloning [Bonetta *et al.*, 1990] and chromosome jumping [Gessler *et al.*, 1990] have assisted in the construction of a physical map of the region on human chromosome 11 spanning the Wilms' tumour locus, which resulted in the identification of transcripts possibly involved in predisposition to, or the development of this tumour [Bonetta *et al.*, 1990; Huang *et al.*, 1990].

These examples demonstrate that it is nowadays practicable to traverse a physical distance in the lower Mb range with these long-range chromosome walking techniques and YAC vectors at hand. This implies that in higher eukaryotes, a linked molecular marker at a genetic distance of 0.5 - 1 cM from the target gene provides a reasonably close starting point for a cloning attempt using the 'positional cloning' approach. In the tomato genome, the distance between *Aps-1*^{1} and *Mi* has been estimated within this range [Medina-Filho, 1980] and therefore, *Aps-1*^{1} is considered suitable to start for *Mi*.

Identification of the target gene

The final step in 'positional cloning' involves the identification of the target gene among the cloned sequences contained within the closest flanking markers. On the basis of the observation that many genes are subjected to evolutionary conservation [Lewin, 1990], a preliminary selection among the candidate sequences could be achieved by screening for cross-hybridization with genomes of other species, of which the more distantly related ones are particularly indicative. Although this is not at all a conclusive test, it may be useful as a first selection [Monaco *et al.*, 1986; Rommens *et al.*, 1989], the more so as it is easily performed by Southern analysis of a 'zoo-blot' in case of an animal gene, or a 'botanic garden-blot' in case of a plant gene. As to a putative *Mi* sequence, conservation will most likely be found in other solanaceous plants. In particular, it should be considered as evidence of the correct sequence, when strong conservation is found in those species, that possess a certain level of resistance to root-knot nematodes, as for example in certain potato cultivars and wild Solanum spp. [Mendoza and Jatala, 1985], and in some tobacco cultivars and wild Nicotiana spp. [Barker and Lucas, 1984].

Unfortunately, it is impossible to give a general and never-failing protocol for further selection. However, the following tests, in one combination or another, are likely to allow the identification of a single candidate gene in the end:

(i) In case of a vertebrate [Bird, 1986; Cedar, 1988] or a higher plant [Antequera and Bird, 1988; Brown, 1989; Messeguer *et al.*, 1991b], a true gene sequence is often preceded at the 5' side by a CpG island. This is a stretch of DNA in which CpG dinucleotides are abundant and non-methylated [Bird, 1986]. As a consequence, the restriction enzyme Hpa II, which cleaves at non-methylated CCGG tetramers, will cut up a CpG island in bits and pieces. Therefore, CpG islands are also referred to as HTF (Hpa II tiny fragment) islands [Bird *et al.*, 1985].

(ii) The selected nucleotide sequence should contain an open reading frame.

(iii) The expression of the candidate gene, as revealed for example by northern blot analysis, should exhibit the expected tissue specificity in accordance with the phenotypic trait controlled by the target gene. In the case of *Mi*, the internal root tissues, especially the vascular and cortical parenchyma and the primary phloem, are the most likely sites of expression.

(iv) A comparison of the versions of the putative target sequence present in genomes carrying different alleles of the target gene should consistently reveal polymorphisms. Accordingly, the candidate *Mi* sequence should be variant or absent in nematode-susceptible tomato cultivars.

(v) Segregation studies should demonstrate absolute co-segregation of the various alleles of the candidate gene with the phenotype they are supposed to confer. Thus, the putative *Mi* sequence should co-segregate with the root-knot nematode resistance trait.

(vi) Complementation of a null mutant following direct or vector-mediated transformation [Rogers and Klee, 1987; Goodfellow *et al.*, 1988; Hille *et al.*, 1989; Hooykaas *et al.*, 1989; Clark *et al.*, 1991] should ultimately allow the identification of a single candidate sequence. To prove its identity as the target gene, the transferred nucleotide sequence should convey the phenotype of the target gene upon expression in the null mutant.

For the cystic fibrosis (CF) gene, such an endeavour has been accomplished. The CF gene was found to encode a protein involved in Cl^- ion secretion and called the cystic fibrosis transmembrane conductance regulator (CFTR) [Riordan *et al.*, 1989; Hyde *et al.*, 1990; Wainwright, 1991]. Transformation of CF airway-cells using a vaccinia virus-CFTR construct resulted in the healing of the defect in the permeability for Cl^- ions in these cells [Rich *et al.*, 1990], as might be expected for an authentic wild type CF cDNA sequence. Similar results were obtained by transformation of pancreatic tumour cells from a CF patient using a recombinant retrovirus carrying a CFTR construct [Drumm *et al.*, 1990]. As efficient transformation procedures have been developed for tomato [Horsch *et al.*, 1985; Fillatti *et al.*, 1987; Koornneef *et al.*, 1987; Hille *et al.*, 1989; Davis *et al.*, 1991], it should accordingly be possible to verify the identity of a putative *Mi* sequence by complementation of a susceptible tomato variety.

Alternative strategies with specific applications

In addition to 'positional cloning', which is, in principle, generally applicable, several other product-independent gene cloning strategies have been developed, most of them serving more specific applications. As some of them represent a conceivable strategy for cloning *Mi* as well, this section will discuss the reasons why these methods were considered less preferable as compared to 'positional cloning'.

Gene tagging

By introducing DNA constructs that will integrate into the genome, it is possible to tag genes with a 'marker sequence' by insertional mutagenesis. The integrating DNA species applied for 'gene tagging' in plants comprise constructs on the basis of the Ti plasmid from the plant-pathogenic bacterium *Agrobacterium tumefaciens* [Koncz *et al.*, 1989; Feldmann *et al.*, 1990], and various transposable elements [Wienand *et al.*, 1982] like the *Ac-Ds* (*Activator-*

Dissociation), *Mu* (*Mutator*) and *Spm/En* (*Suppressor-mutator*, also known as *Enhancer*) elements of maize [Fedoroff, 1989; Döring, 1989] and the *Tam3* element of *Antirrhinum majus* [Sommer *et al.*, 1985; Coen *et al.*, 1989]. Using the cloned insertion mutagen as a probe in Southern blot analysis of a population segregating for the insertion mutation, it is possible to identify a junction fragment showing exclusive co-segregation with the mutant phenotype. Subsequent cloning of this junction fragment would yield cloned sequences from the target gene, which, in turn, should allow the isolation of a clone of the uninterrupted allele.

Examples of genes from higher plants that have been cloned by the 'tagging' approach using transposable elements include the *pallida* anthocyanin biosynthesis locus of *Antirrhinum* [Martin *et al.*, 1985] and a number of maize genes, like the *bronze* anthocyanin biosynthesis loci *bz1* [Fedoroff *et al.*, 1984] and *bz2* [McLaughlin and Walbot, 1987], the chalcone synthetase gene *c2* [Wienand *et al.*, 1986] and the carotenoid biosynthesis locus *y1* [Buckner *et al.*, 1990]. Furthermore, *Arabidopsis thaliana* genes controlling trichome formation [Marks and Feldmann, 1989; Herman and Marks, 1989] and floral development [Yanofsky *et al.*, 1990], have been isolated through tagging with the T-DNA sequence from the *Agrobacterium tumefaciens* Ti plasmid [Velten and Schell, 1985; Feldmann *et al.*, 1990].

Using the transformation procedure available for tomato [Horsch *et al.*, 1985; Fillatti *et al.*, 1987; Koornneef *et al.*, 1987; Hille *et al.*, 1989; Davis *et al.*, 1991], the *Ac* transposable element from maize, has been introduced into tomato and found to be transpositionally active within the tomato genome [Yoder *et al.*, 1988]. Also the introduction of T-DNA sequences into tomato is no problem. Tagging of the *Mi* gene with a transposable element or a T-DNA insertion would therefore be feasible. However, isolation of a T-DNA-tagged *Mi* mutant would require the generation and screening for susceptibility in a root-knot nematode infection assay of hundred thousands of T-DNA-transformed plants. Use of an engineered heterologous transposable element [Haring *et al.*, 1991] might reduce the number of transformants needed to identify an *Mi* insertional mutant, but even then many thousands of plants have probably to be screened. The transposable element has to be delivered in the vicinity of the *Mi* target gene, to begin with, since these elements preferentially transpose to genetically linked sites [Van Schaik and Brink, 1959; Greenblatt, 1968; 1984; Chen *et al.*, 1987]. Transposed *Ac* elements in tobacco, for example, were found inserted mostly within 2 cM from the original integration site [Jones *et al.*, 1990], and similar preference for linked integration was found in tomato [Belzile *et al.*, 1989]. Although convenient RFLP [KLein-Lankhorst *et al.*, 1991a; Messeguer *et al.*, 1991a], RAPD [Klein-Lankhorst *et al.*, 1991b], allozyme (*Aps-1*) and morphological (e.g. *yv*) markers [Rick and Fobes, 1974; Medina-Filho, 1980] are available for rapidly establishing linkage to *Mi*, much effort will still be needed to find a transformant with a copy of the introduced transposon linked to *Mi*. The number of progeny plants of such a transformant to be screened in order to identify a possible *Mi* insertional mutant is expected to be large as well. It is true that this number may be reduced by using a construct containing the transposable

element inserted in an antibiotic resistance gene, allowing at any rate the selection of those progeny plants in which the transposable element has moved from its integration site and therefore is active [Baker *et al.*, 1987; Rommens *et al.*, 1991]. Even then, probably tens of thousands of progeny plants have to be tested for root-knot nematode resistance [Haring *et al.*, 1991].

Indeed, all current examples of successful 'gene tagging' in plants involve genes controlling morphological traits which are readily visible and easily selectable. Even in those cases, phenotypic screening of large numbers of plants required a lot of manpower and extensive greenhouse facilities. As considerably more effort would be needed to perform an assay for root-knot nematode resistance on a large number of plants, cloning of *Mi* by 'gene tagging' seems rather impracticable.

Genomic subtraction

If there is a deletion mutant of the target gene available, then the method of Straus and Ausubel [1990], called 'genomic subtraction', is feasible. This procedure involves the subtraction (removal) of those nucleotide sequences that are common in the wild type and mutant genomes from the total of wild type sequences, thus leaving over the genomic sequences from the wild type that are deleted in the mutant. The authors demonstrate the feasibility of this approach by cloning sequences corresponding to a deletion covering the *lys2* gene of yeast spanning $^1/_{4000}$ th of the genome.

As to *Mi*, this method is currently not practicable due to the absence of a suitable *Mi* deletion mutant. Besides, considerable technical difficulties are to be expected, as the tomato genome ($0.91 - 1.0 \times 10^9$ bp) [Arumuganathan and Earle, 1991] is much more complex than the yeast genome (1.3×10^7 bp) [Lewin, 1990] employed in the model study [Straus and Ausubel, 1990].

Differential cDNA library screening

When pairs of isogenic variants of the organism are available which differ in the expression of a certain hereditary trait, differential screening of a cDNA library [Sambrook *et al.*, 1989] offers an alternative approach to isolate the gene(s) responsible for that trait. cDNA clones representing mRNA sequences that are specifically expressed in association with the relevant phenotype are then selected by duplicate hybridization, using cDNA probes copied from mRNA of each of both variants respectively. However, genotypes which are (nearly) isogenic, except for the target gene, are difficult to construct and therefore, differential screening is mainly applied in those cases where the mRNA populations to be compared are readily

available, as in isolating groups of tissue-specific and conditionally or developmentally regulated genes. As an additional limitation, the method only works for the subset of genes encoding mRNA that is abundantly expressed in one of both variants used for comparison, and present at a substantially lower level or completely absent in the other.

Although nearly isogenic nematode-resistant and susceptible lines of tomato have been described [Klein-Lankhorst *et al.*, 1991a; Messeguer *et al.*, 1991a], differential cDNA library screening for isolating the *Mi* gene is yet not attractive. First, the level of expression of the *Mi* gene is unknown, and besides, the possibility should be considered that the *Mi* gene is not constitutively expressed, but is induced upon infection by root-knot nematodes. This implies, that nematode-challenged tomato plants are to be subjected to the differential screening in order to have the best theoretical chance of success. However, nematode infection will induce expression of a number of pathogenesis-related genes, producing the physiological changes generally observed upon pathogen attack, like, for example, increased phenylalanine ammonia lyase (PAL) activity [Brueske, 1980]. cDNA clones representing these genes will appear positive in addition to an eventual *Mi* clone upon differential library screening. Anyhow, supplementary selection will be needed, as this method will likely identify several other non-isogenic cDNA sequences due to the imperfect quality of the available *Mi/Mi* and *mi/mi* isogenic lines.

Recently, Ho *et al.* [1992] showed the potential of a related approach in identifying sequences associated with the *Mi* region. By screening a pair of nearly isogenic lines with random clones from a cDNA library, a number of *Mi*-linked cDNA clones were identified, one of which (LC379) even happened to map in the *Mi* region of 'Motelle', at present the tomato cultivar carrying the smallest *L. peruvianum* introgressed region.

Evaluation

Whereas the alternative strategies for cloning *Mi* mentioned above suffer from a combination of either the absence of prerequisites, from uncertainties about their efficacy, or from an excessive amount of effort required, there are several arguments in support of the 'positional cloning' approach. These arguments include the availability of a clonable allozyme marker, *Aps-1*^{1}, which is tightly linked to *Mi*, the rapidly increasing experience with long range chromosome walking (page 27), and the feasibility of the positional cloning approach under comparable experimental conditions, as demonstrated by the successful application in the molecular cloning of the complete cystic fibrosis (CF) cDNA and other human hereditary disease gene sequences (page 28).

For these reasons, cloning of *Aps-1*^{1} nucleotide sequences was considered as a constructive step towards the cloning of the *Mi* gene.

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Chapter 2

Purification of tomato acid phosphatase-1[1]

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INTRODUCTION

In tomato (*Lycopersicon esculentum* Mill.) the nematode resistance locus *Mi* is tightly linked to the acid phosphatase-1 locus *Aps-1* [Rick and Fobes, 1974; Medina-Filho, 1980; Medina-Filho and Tanksley, 1983]. Most nematode-resistant cultivars carry the *Aps-1*^{1} allele, encoding an electrophoretically variant allozyme (APS-1^{1}) [Rick and Fobes, 1974; Medina-Filho and Tanksley, 1983]. Since no products of *Mi* expression are known, which could give access to the *Mi* gene at the molecular level, the APS-1^{1} allozyme is of interest as an alternative entrance. Through amino acid sequence information from the encoded protein, the *Aps-1*^{1} gene may be cloned. Given a total map size of 1600 cM for tomato [Ganal *et al.* cited in Messeguer *et al.*, 1991], a haploid genome size of 975 megabase pairs (cv. VFNT cherry [Arumuganathan and Earle, 1991]) and a genetic distance between the *Aps-1* and *Mi* loci of 0.894 cM [Medina-Filho, 1980], an *Aps-1*^{1} clone would provide a marker at an estimated distance of 540 kb from *Mi*, which is sufficiently close to serve as a starting point for long-range chromosome walking techniques [Burke *et al.*, 1987; Collins, 1988]. Besides, an *Aps-1*^{1} clone would provide a molecular probe for convenient nematode resistance genotyping.

As a first step towards cloning *Aps-1*^{1} nucleotide sequences, the encoded APS-1^{1} protein was purified, using the method described by Paul and Williamson [1987] as a guideline.

MATERIALS AND METHODS

Materials

All chemicals were analytical grade. DEAE-Sephacel, Con A-Sepharose, Sephacryl S-200 HR and the Mono Q FPLC-column were purchased from Pharmacia LKB Biotechnology, Uppsala, Sweden; hydroxylapatite was from Bio-Rad, Richmond, California, protein molecular weight standards were from Sigma, St. Louis, Missouri, and DIAFLO YM5 ultrafiltration membranes from Amicon, Lexington, Massachusetts.

Plants and cell cultures

The nematode resistant (*Mi/Mi*, *Aps-1^1/Aps-1^1*) tomato line providing roots for protein extraction were obtained by consecutive selfings of 83M (De Ruiter Seeds, Bleiswijk, The Netherlands), an *L. esculentum* line segregating for *Mi* and *Aps-1* [Klein-Lankhorst *et al.*, 1991]. Three weeks old seedlings were transferred from gravel to Steiner nutrient solution and grown on hydroponics. After 1 month, the roots were harvested and stored frozen at -80 °C.

Cell suspension cultures were derived from L2-14. This is a plant, selected among the progeny that arose from the selfing of L2, a hybrid *L. peruvianum* × *L. esculentum* tomato line, the genome of which is 75% *L. peruvianum* [Thomas and Pratt, 1981]. The cells were cultured at 25 °C in the dark in 1 litre flasks containing 400 ml medium [DuPont *et al.*, 1985] under vigorous shaking. Every 10 - 14 days, one tenth of the suspension was subcultured and the remaining cells were harvested by filtration through a Büchner funnel. Cells were stored frozen at -80 °C. The average yield was 100 - 200 g cells per litre of suspension culture.

Acid phosphatase isozyme analysis

Acid phosphatase (APS) isozyme analysis throughout the protein purification was routinely performed by cellulose acetate membrane (CAM) electrophoresis. Eluate fractions were stamped five times on a cellulose acetate membrane (SM12200, Sartorius, Göttingen, Germany), that had been saturated with the electrophoresis buffer (0.3 M borate buffer pH 8.3) and had been mounted in a Sartophor SM16539 CAM electrophoresis unit (Sartorius, Göttingen, Germany). Electrophoresis was performed for 30 min at 250 V and 4 °C. The membrane was then immediately stained for acid phosphatase activity by soaking overnight at 30 °C under agitation in a staining mixture, freshly prepared by first dissolving 1 mg/ml Fast Black K salt (Serva, Heidelberg, FRG) in 50 mM NaAc pH 5.5, 10 mM $MgCl_2$, and subsequently adding 0.03% (w/v) ß-naphtyl acid phosphate (Sigma, St. Louis, Missouri, USA) immediately before use.

APS isozyme analysis was also performed by APS activity staining of proteins separated by non-denaturing polyacrylamide gel electrophoresis (see below), in particular when levels of APS-1^1 activity below the detection limit of CAM electrophoresis were to be expected.

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed, using a modified Laemmli [1970] discontinuous buffer system. The separating gel (10 × 14.5 × 0.15 cm) contained 12.5% (w/v) acrylamide, 0.1% (w/v)

N,N'-methylenebisacrylamide, 375 mM Tris-HCl pH 8.8 and 0.1% (w/v) SDS. The stacking gel contained 3.9% (w/v) acrylamide, 0.1% (w/v) N,N'-methylenebisacrylamide, 125 mM Tris-HCl pH 6.8 and 0.1% (w/v) SDS. Electrophoresis was performed at a constant voltage of 40 - 175 V in 50 mM Tris, 384 mM glycine, 0.1% (w/v) SDS (pH 8.6).

For non-denaturing PAGE, SDS was omitted from the gel and from the electrophoresis buffer described above. A 10% (w/v) acrylamide / 0.12% (w/v) N,N'-methylenebisacrylamide separating gel and a 3.9% (w/v) acrylamide / 0.1% (w/v) N,N'-methylenebisacrylamide stacking gel were used and electrophoresis was performed at 4 °C for 4 - 6 hours at a constant voltage of 150 V or overnight at 70 V. Subsequently, the gel was equilibrated for 2 × 15 min with staining buffer (50 mM NaAc pH 5.5, 10 mM $MgCl_2$) and stained for APS activity overnight at 30 °C as described above.

Proteins separated by PAGE were silver-stained essentially as described by Morrisey [1981].

Purification of acid phosphatase-1[1]

The purification procedure described below is a modified version of the scheme developed by Paul and Williamson [1987], that is applicable to both roots and suspension cells. The whole procedure was carried out at 0 - 4 °C.

Step 1. Protein extraction

Frozen tomato roots or suspension cells were powdered in liquid nitrogen using a mortar and pestle or a Waring blender. The frozen homogenate was added to extraction buffer (100 mM Tris-HAc pH 8.0, 100 mM KAc, 10% (v/v) glycerol, 2 mM ethylene diamine tetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT), 250 mM Na-ascorbate) at 4 °C, to which 0.5 g Polyclar AT (insoluble polyvinylpyrrolidone) per g fresh weight was added. For roots, 4 ml extraction buffer per g fresh weight was sufficient, but for cells 5 - 6 ml buffer per g fresh weight was needed. After stirring for 1 hour at 4 °C, cell debris and Polyclar AT were removed from the crude extract by centrifugation at 28,000 × g for 30 min. The resulting pellets were re-extracted with 2 ml extraction buffer per g fresh weight using a blender, and clarified by centrifugation as above. The first and second extracts were pooled.

Step 2. $(NH_4)_2$ SO_4 fractionation

A 40 - 75% $(NH_4)_2SO_4$ fraction was prepared from the crude extract by adding solid $(NH_4)_2SO_4$. Precipitation was allowed to proceed for at least 1 hour at 4 °C, under continuous stirring. Precipitates were collected by centrifugation at 28,000 × g for 1 hour and dialysed extensively against TGKEDP buffer (100 mM Tris-HAc pH 8.0, 10% (v/v) glycerol, 100 mM KAc, 2 mM EDTA, 5 mM DTT, 0.1 mM PMSF).

Step 3. DEAE-Sephacel column chromatography

The dialysate obtained in step 2 was loaded on a DEAE-Sephacel column (5.3 cm^2 × 30 cm), pre-equilibrated with TGKEDP. (Protein prepared from 750 g of fresh roots or from 1600 g of fresh suspension cells could be applied at once without overloading the column). After washing the column with at least five column volumes of the same buffer, a linear 100 - 350 mM KAc gradient made up in 1100 ml TGKEDP buffer was applied at a flow rate of 54 ml/hr. Fractions (5 ml) were collected and tested for APS activity using CAM electrophoresis. Those containing the bulk of APS-1[1] activity were pooled.

Step 4. Hydroxylapatite (HAP) column chromatography

The pool of eluate fractions obtained in the previous step was concentrated by ultrafiltration through a DIAFLO YM5 membrane, dialysed against HAP adsorption buffer (10 mM NaP_i pH 7.6, 10% (v/v) glycerol, 5 mM DTT, 0.1 mM PMSF) and loaded on a HAP column (2 cm^2 × 15 cm) equilibrated with HAP adsorption buffer. After washing the column with HAP adsorption buffer, a 500 ml linear 10 - 160 mM NaP_i (pH 7.6) gradient containing 5 mM DTT and 0.1 mM PMSF was applied at a flow rate of 16 ml/hr. Fractions (3 ml) were collected and those containing most of the APS-1[1] activity were pooled.

Step 5. Concanavalin A (Con A)-Sepharose column chromatography

Pooled HAP eluate fractions were concentrated by ultrafiltration through a DIAFLO YM5 membrane and dialysed against Con A adsorption buffer (25 mM Tris-HCl pH 7.4, 50 mM NaCl).

The dialysate was then loaded in portions of 0.5 ml on a freshly prepared Con A-Sepharose column (0.5 cm^2 x 4 cm), pre-equilibrated with Con A adsorption buffer. To allow

optimal adsorption, there was an interval of 15 min between each portion and a pause of 1 hour after the last portion during which column flow was interrupted.

Loosely bound proteins were washed through the column with Con A washing buffer (40 mM Tris-HCl pH 7.4, 200 mM NaCl). A variable proportion (mostly less than 50%) of the APS-1^1 activity eluted during this high salt wash. The tailing part of this broad APS-1^1 peak was collected, as it was sufficiently free from contaminants to be purified completely by the final mono Q FPLC step. After washing with about 10 column volumes of Con A washing buffer, bound APS-1^1 was eluted with Con A elution buffer (40 mM Tris-HCl pH 7.4, 200 mM NaCl, 1% (w/v) α-D-methylglucoside). As soon as the column bed was filled up with elution buffer, flow was interrupted for 1 hour to allow complete desorption [Muller and Carr, 1984] and subsequent elution in a smaller volume. Elution was then continued at a flow rate of 20 ml/hr.

Step 6. Mono Q FPLC

The collected Con A wash fractions and the Con A eluate were pooled, diluted twice with Mono Q adsorption buffer (100 mM Tris-HCl pH 8.0) to adjust the NaCl concentration to 100 mM and subsequently loaded on a pre-equilibrated Mono Q FPLC column. After washing the column with Mono Q adsorption buffer, the adsorbed fraction was eluted with a 55 ml linear, two-stage 0 - 400 mM KAc gradient in 100 mM Tris-HCl pH 8.0. The first section of the gradient from 0 - 100 mM KAc was covered during the first 5 ml. The remaining part of the gradient, from 100 - 400 mM KAc, lasted 50 ml. Fractions (0.75 ml) were collected and the A_{280} of the eluate was continuously monitored using a Pharmacia Monitor UV-M spectrophotometer unit (sensitivity range adjusted to A = 0 - 0.005). Those A_{280} peak fractions containing APS-1^1 activity but no APS-2 activity, as determined by CAM-electrophoresis and non-denaturing PAGE, were pooled. This purified APS-1^1 preparation was concentrated by ultrafiltration and used for amino acid microsequencing as described in chapter 3.

RESULTS

Purification of tomato acid phosphatase-1[1]

The acid phosphatase-1 allozyme (APS-1[1]) encoded by the *Aps-1[1]* allele was purified from both tomato roots and suspension cells as described in detail in Materials and Methods. In using the purification procedure of Paul and Williamson [1987] as a guide-line, no major divergences were met, except for the Con A-Sepharose column chromatography step and the lower yield attained, as specified below. The procedure described here represents a modified and extended version of the Paul and Williamson [1987] protocol and involves the consecutive steps shown in Fig. 1. The chromatographic behaviour of APS-1[1] on the various types of columns used, is summarized in Fig. 1 as well.

tomato roots / suspension cells	competitive component of eluens	applied competitor concentration		
		adsorption	elution	
crude extract			APS-1[1]	APS-2
40 - 75% $(NH_4)_2SO_4$ fractionation				
DEAE - Sephacel	KAc	100 mM	230 mM	215 mM
HAP (hydroxytapatite)	NaP_i	10 mM	50 - 80 mM	35 - 65 mM
Con A - Sepharose (concanavalin A)	α-D-methylglucoside	—	1%	1%
Mono Q (FPLC)	KAc	100 mM[1)]	270 mM	220 mM

1) NaCl

Fig. 1. Flow chart summarizing the purification procedure and chromatographic properties of tomato APS-1[1].

The bulk of contaminating APS isozymes was removed by DEAE-Sephacel column chromatography. APS-1[1] and a portion of the APS-2 activity were adsorbed to the DEAE-Sephacel column at 100 mM KAc, whereas a genetically uncharacterized APS activity passed through almost completely (Fig. 2). By applying a flat KAc gradient (100 - 350 mM), a

considerable portion of the adsorbed APS-2 activity was resolved from the APS-1^1 activity; APS-2 eluted at 215 mM and APS-1^1 at 230 mM (Fig. 2). Further purification was achieved (Fig. 3) by chromatography on hydroxylapatite (HAP). Again, APS-2 eluted at a slightly lower salt concentration (varying between 35 and 65 mM NaP_i) than APS-1^1 (median of peak between 50 and 80 mM NaP_i). The last traces of APS-2 were finally removed by the Mono Q FPLC step. APS-2 eluted at about 220 mM KAc and APS-1^1 at about 270 mM KAc (not shown).

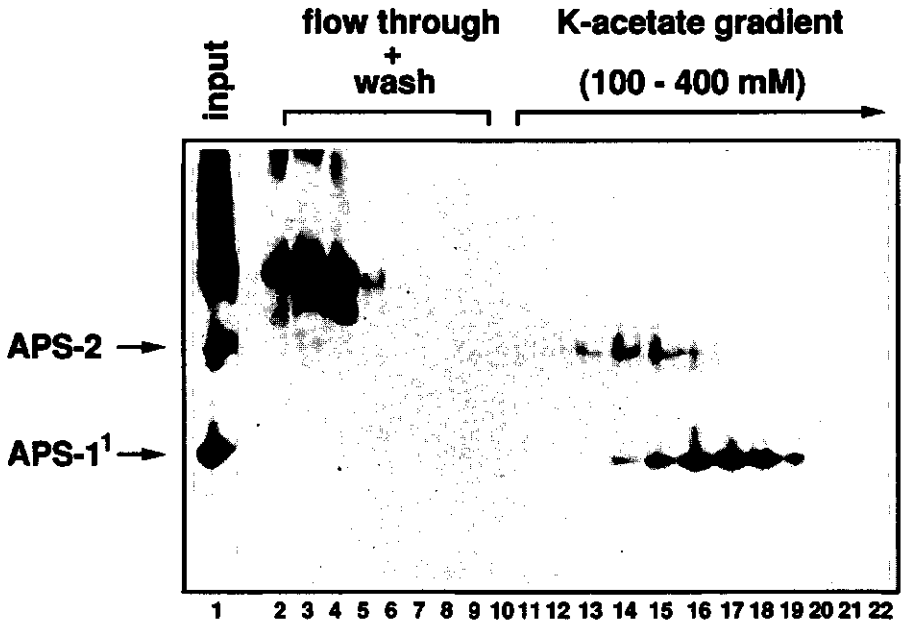

Fig. 2. Separation of tomato APS isozymes by DEAE-Sephacel column chromatography. $(NH_4)_2SO_4$-fractionated proteins from 100 g of tomato (*Aps-1*^{1}/*Aps-1*^{1}) roots were prepared (input) and separated by ion exchange chromatography using a DEAE-Sephacel column (2 $cm^2 \times 23$ cm). Protein extraction, $(NH_4)_2SO_4$-precipitation, loading of the sample and washing of the column were performed according to Materials and Methods. Subsequently, bound proteins were eluted with a 180 ml 100 - 400 mM linear K-acetate gradient in TGKEDP buffer pH 8.0. Column wash and gradient eluate were collected in 3 ml fractions and a 50 µl sample of every third fraction was analysed by non-denaturing PAGE and subsequent APS activity staining, as was 0.5% of the input (lane 1). Only relevant fractions of the flow through and wash (lanes 2 - 9) and of the K-acetate gradient (lanes 10 - 22) are shown, arranged from left to right in consecutive order of elution.

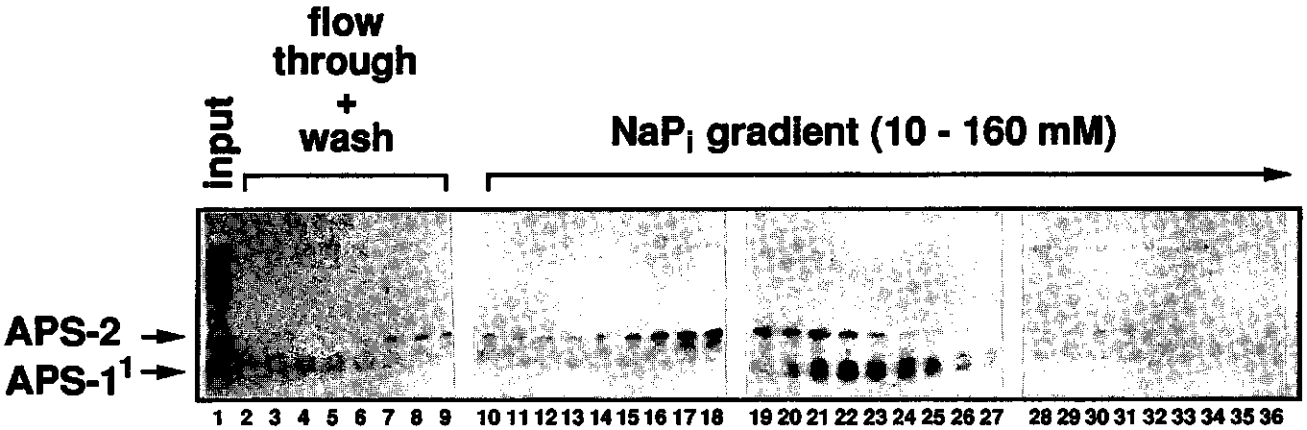

Fig. 3. Separation of tomato APS isozymes by HAP column chromatography. Proteins were extracted from 570 g of L2-14 (*Aps-1¹/Aps-1¹*) suspension cells (step 1) and APS-1¹ was partially purified (steps 2 - 3) according to the purification protocol described in Materials and Methods. The resulting preparation (input) was loaded on a HAP column (2 $cm^2 \times 15$ cm) and unbound proteins were washed through the column with HAP adsorption buffer (containing 10 mM NaP_i pH 7.6). Subsequently, the bound protein fraction was eluted by applying a 300 ml 10 - 160 mM linear NaP_i (pH 7.6) gradient in the same buffer solution. Column wash and gradient eluate were collected in 3 ml fractions and a representative sample of the fractions was analysed by CAM electrophoresis and subsequent APS activity staining, as was the input (lane 1); lanes 2 - 9: consecutive flow through and wash fractions; lanes 10 - 36: consecutive NaP_i gradient fractions. The faint, oval spots between the APS-2 and APS-1¹ bands were stained brownish instead of bluish-purple, and therefore do not represent APS activity. Their appearance during APS activity staining was found to be associated with the presence of reducing compounds (ascorbate and DTT) in the sample subjected to CAM electrophoresis.

The Con A-Sepharose column chromatography step accounted for the largest purification. Upon application of the enzyme preparation at 50 mM NaCl, most of the APS-1^1 activity was bound to the column. In contrast, virtually all protein contaminants appeared in the flow through or were removed in the subsequent washing step using a 200 mM NaCl buffer (Fig. 4). During washing, a proportion (usually less than 50%) of the adsorbed APS-1^1 activity was gradually released from the column in a broad peak, trailing behind the contaminants. The tail of this peak, representing almost pure APS-1^1, was collected for further purification together with the tightly bound fraction, which needed the competition of α-D-methylglucoside to become released (Fig. 4).

In our hands, column 'bleeding' caused contamination of the APS-1^1 preparation with relatively large amounts (up to 10 μg/ml) of Con A (Fig. 4). As a result, SDS-PAGE analysis of the purified APS-1^1 preparation using standard sized (10 cm) 12.5% polyacrylamide gels, was rather deceptive. Due to its similar migration rate in SDS-PAGE, one of the contaminating Con A-associated polypeptides precluded the identification of the apparent APS-1^1 subunit. Only upon using extra long (30 cm) 15% polyacrylamide gels, we were able to visualize the APS-1^1 subunit (Fig. 4, lane 9, 10), which migrated at a slightly higher rate than the Con A contaminant.

In the final Mono Q FPLC purification step, the Con A contaminants were completely removed by the 100 mM KAc wash, yielding a highly purified APS-1^1 enzyme preparation that was directly amenable to amino acid sequence analysis.

As the presence of other APS activities in the partially purified enzyme preparation precluded the APS-1^1 activity to be quantified separately, it was impracticable to evaluate the present purification procedure in terms of increase of specific APS-1^1 activity. Actually, the overall purification factor could only be assessed retrospectively from the yield of purified APS-1^1 protein. From 1.3 kg of roots, about 2 g of total protein was extracted, which yielded approximately 8 μg of purified APS-1^1. Assuming the final APS-1^1 preparation to be virtually pure (Figs. 5, 6), such a yield implies purification of up to 250,000-fold, dependent on and proportional to the actual recovery of APS-1^1 protein. However, only inaccurate estimates of the latter were attainable because of the other APS activities present. Nevertheless, it was apparent from these estimates, that no major loss of the APS-1^1 activity present in the original extract had occurred during any purification step. Since PCR experiments (see chapter 4) confirmed the low abundance of *Aps-1^1* mRNA, the low yield of APS-1^1 protein attained is not likely to be due to inefficient extraction or loss, but rather to the low level of *Aps-1^1* gene expression.

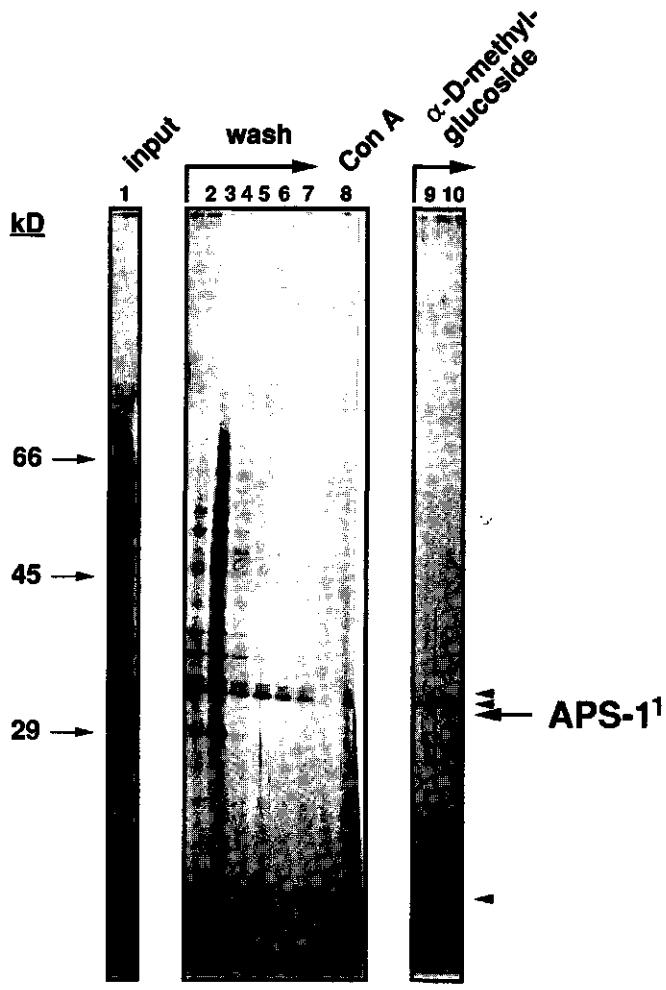

Fig. 4. Purification of tomato APS-1[1] through its affinity for Con A-Sepharose. HAP-purified APS-1[1] (input) was prepared (step 1 - 4) from approximately 250 g of tomato roots and loaded on a Con A-Sepharose column (0.5 cm^2 × 4 cm) as described in Materials and Methods. Proteins with low affinity for Con A were washed through the column using a high salt (200 mM NaCl) washing buffer. Glycoproteins with high affinity were subsequently eluted with high salt buffer containing 1% (w/v) α-D-methylglucoside. Relevant low and high affinity eluate fractions were analysed by SDS-PAGE of 50 µl portions using a 15% (w/v) polyacrylamide / 0.09% (w/v) N,N'-methylenebisacrylamide gel (length 30 cm). The gel was stained for protein with silver. Lane 1: 2% of the input; lanes 2-7: consecutive high salt (200 mM) wash fractions; lane 8: commercial Con A preparation (Pharmacia); lane 9 and 10: two high affinity eluate fractions containing low and high APS-1[1] activity, respectively, as determined by CAM electrophoresis (not shown). Arrow: 31 kD polypeptide, associated with APS-1[1] activity; arrow-heads: Con A-associated bands.

Criteria for purity

Three independent tests were performed to confirm the identity and the homogeneity of the protein that had been purified.

First, gel electrophoresis under non-denaturing conditions showed, that the APS-1^1 preparation consisted of a single homogeneous protein, co-migrating with the APS-1^1 activity (Fig. 5).

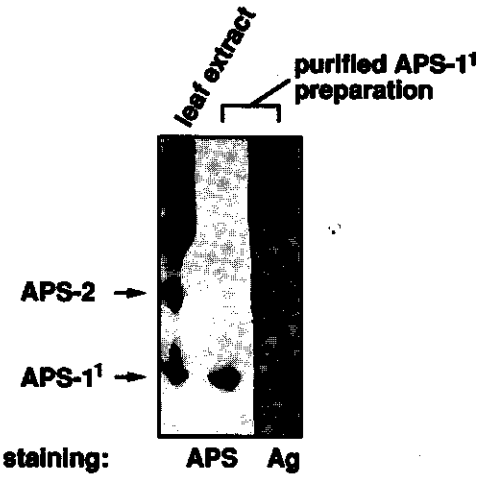

Fig. 5. Co-electrophoresis of APS-1^1 activity with the protein which had been purified. APS-1^1 was purified from tomato roots as described in Materials and Methods, and portions (equivalent to 0.12 and 1.2 g of fresh roots, respectively) of the Mono Q eluate (step 6) were analysed by non-denaturing PAGE, followed by APS activity staining (APS) and silver (Ag) staining (protein), respectively. A crude extract from tomato leaves (*Aps-1^1/Aps-1^1*) was included as an APS isozyme marker.

In a second test, an attempt was made to separate the APS-1^1 activity from the purified protein, using a separation principle that had not been applied in the purification procedure. To this end, the purified APS-1^1 preparation was applied to a Sephacryl S-200 HR gel filtration column and each fraction of the eluate was subjected to both activity and protein staining. As shown in Fig. 6, the elution profile of the APS-1^1 activity completely coincided with the protein profile, confirming the APS-1^1 identity of the purified protein.

The third confirmation was obtained by SDS-PAGE analysis. As shown in Fig. 6, the APS-1^1 preparation consisted of a single 31 kD polypeptide - the apparent APS-1^1 subunit - which, like the native protein, completely co-eluted with the APS-1^1 activity upon gel filtration on the Sephacryl S-200 HR column (Fig. 6).

Sources of APS-1^1 and yields attained

For reasons of economy, no APS-1^1 protein was sacrificed for a standard protein assay. Instead, yields of APS-1^1 were estimated on the basis of the amount of amino acids released

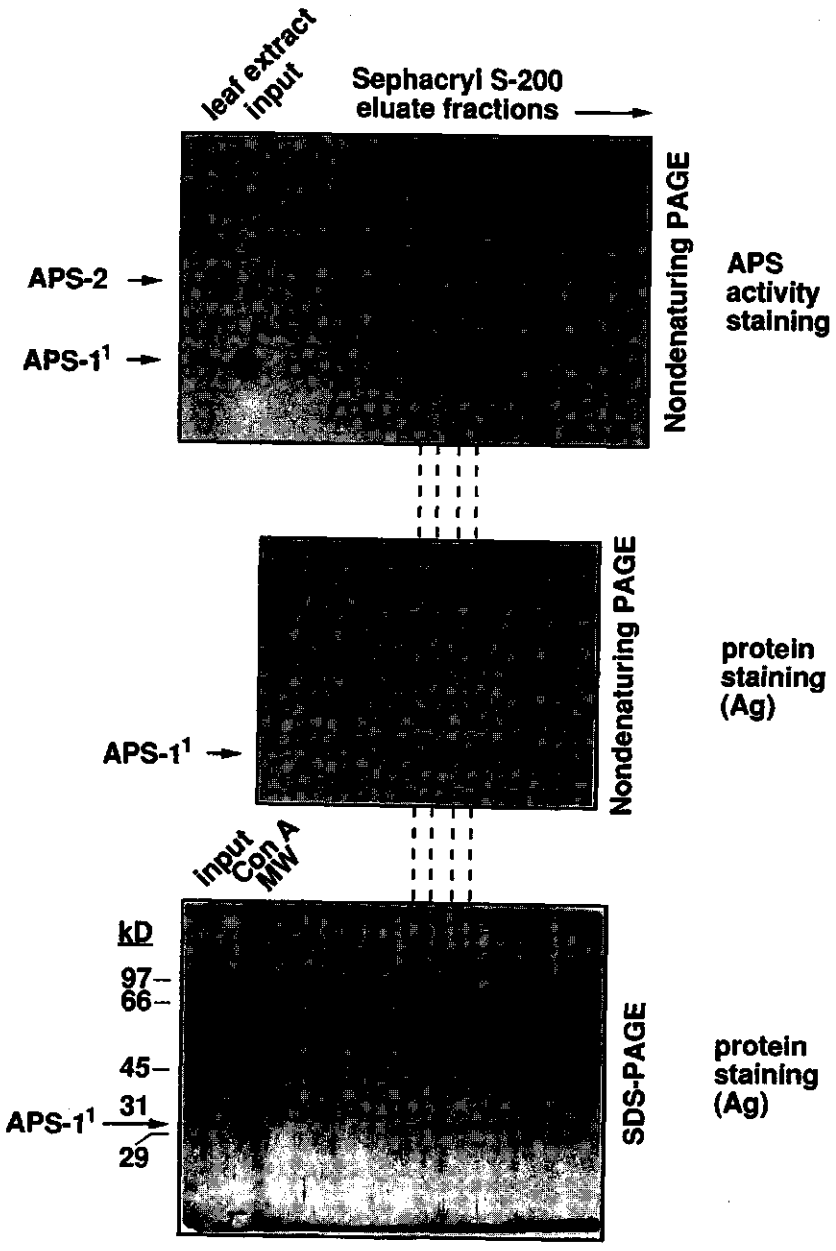

Fig. 6. Co-elution during gel filtration of APS-1[1] activity with the protein which had been purified. Mono Q FPLC-purified APS-1[1] (prepared from 120 g of tomato roots according to step 1 - 6, Materials and Methods) was subjected to gel permeation chromatography using a Sephacryl S-200 HR column (0.79 $cm^2 \times 46$ cm). The column was eluted with 100 mM NH_4HCO_3 pH 8, 5 mM DTT. Portions of consecutive eluate fractions (0.5 ml) were analysed for APS activity (top panel, 1 µl per fraction) and protein composition, using both non-denaturing PAGE (middle panel, 25 µl per fraction) and SDS-PAGE (bottom panel, 25 µl per fraction). Proteins were visualized by silver staining (Ag).

upon complete acidic hydrolysis of a proportion of the APS-1^1 preparations. Per kg of fresh roots, the present purification protocol yielded approximately 6 µg of purified APS-1^1. Suspension cells, on the other hand, yielded only about 2 µg of purified APS-1^1 per kg fresh weight and, hence, were a less favourable source for APS-1^1. However, these data were derived from single determinations, so that their significance remains to be established.

Not only intracellularly, but also in the culture medium, APS-1^1 activity was readily detectable by native PAGE and subsequent APS activity staining, suggesting that the culture medium could serve as a convenient source of APS-1^1 protein.

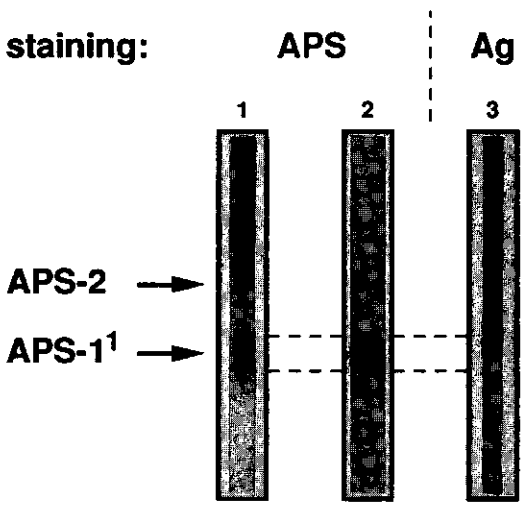

Fig. 7. Yield of extracellular APS-1^1 attained from suspension cell culture medium. One week old L2-14 suspension cell cultures were filtered through a 0.22 µm filter (Millipore) and the cell-free medium (7 litre) was concentrated by ultrafiltration using a DIAFLO YM5 membrane (Amicon). The concentrate was subjected to DEAE-Sephacel (step 3) and Con A-Sepharose (step 5) column chromatography as described in Materials and Methods, and the Con A contamination from the Con A-Sepharose column was removed by a TGKEDP wash, following a second adsorption of the preparation to a DEAE-Sephacel column (0.5 $cm^2 \times 4$ cm). The partially purified extracellular APS-1^1 was then eluted using 400 mM KAc in the same buffer solution. Subsequently, the final preparation was loaded in duplicate (lanes 2 and 3) on a non-denaturing polyacrylamide gel. After electrophoresis, the gel was sliced and lane 2 was stained for APS activity (APS) and lane 3 for protein using silver staining (Ag). Both parts of the gel were equilibrated with distilled water to eliminate the difference in swelling which had occurred during staining. An attempt was made to estimate the amounts of APS-1^1 activity and APS-1^1 protein by reference to the staining intensity of the respective bands. To allow for the detection of minute amounts of APS-1^1 protein by silver staining, a substantial proportion (10%) of the total preparation was loaded in lane 3. However, no clear APS-1^1 protein band was seen, whereas the very intense activity-stained APS-1^1 band in lane 2 was produced by only 4% of the preparation. A crude extract of tomato leaves (*Aps-1^1/Aps-1^1*) was used as an APS isozyme marker (lane 1).

In several attempts, up to 7 litres of culture medium were concentrated by ultrafiltration and subjected to a condensed version of the purification protocol developed for intracellular APS-1^1 as described before. After non-denaturing PAGE of the partially purified extracellular

APS-1[1] preparation and subsequent silver staining, a protein band was visible in the region of the gel where the APS-1[1] activity band was present (Fig. 7). This protein band, however, showed a slightly but significantly lower mobility than the APS-1[1] activity. Hardly any protein was visible exactly at the level in the gel corresponding to the centre of the APS-1[1] activity spot, even when the total APS-1[1] yield from 0.7 litres of medium was loaded into a single slot (Fig. 7, lane 3). Anyhow, the ratio between the intensities of the APS activity staining and the silver staining was much lower as compared to intracellular APS-1[1]. This result was confirmed (not shown) by SDS-PAGE analysis of the extracellular APS-1[1] preparation and subsequent silver staining, as well as by AuroDye (Janssen Life Sciences Products, Beerse, Belgium) staining [Moeremans *et al.*, 1985] of the native proteins following non-denaturing PAGE and subsequent electroblotting onto Immobilon membrane (Millipore, Bedford, Massachusetts). A plausible explanation for the difference between the extracellular and intracellular APS-1[1] preparation as to the ratio of the staining intensities using APS activity staining and silver staining, is, that the the activity of the purified extracellular APS-1[1] was better preserved during purification, resulting in a higher specific activity for the extracellular APS-1[1] preparation as compared to the intracellular preparation.

The use of suspension cell cultures enabled us to study the effect of phosphate starvation, which has been reported to induce excretion of some APS isozymes in tomato plants and suspension cells [Goldstein *et al.*, 1988a; 1988b]. However, preliminary experiments (not shown) indicated, that the concentration of phosphate in the culture medium did not affect the excretion of APS-1[1] activity by L2-14 suspension cells, although, indeed, a certain stimulatory effect of phosphate depletion was found on the activity in the medium of another, anonymous APS isozyme.

These observations led us to the conclusion that culture medium of tomato suspension cells, although of comparatively simple protein composition, does not provide a practicable source of tomato APS-1[1].

DISCUSSION

Acid phosphatases are ubiquitous in living organisms and have been isolated from numerous sources, including various plant species [Felenbok, 1970; Uehara *et al.*, 1974; Chen *et al.*, 1975; Ninomiya *et al.*, 1977; Shinshi and Kato, 1979; Kruzel and Morawiecka, 1982; Ferens and Morawiecka, 1985; Park and Van Etten, 1986; Basboa *et al.*, 1987; Ching *et al.*, 1987; Hefler and Averill, 1987; Paul and Williamson, 1987; Goldstein *et al.*, 1988; Saluja *et al.*,

1989; Kaneko *et al.*, 1990]. They represent a heterogeneous group of proteins differing in molecular mass, subunit structure and carbohydrate content [Fujimoto *et al.*, 1980; Lorenc-Kubis, 1986; Ching *et al.*, 1987; Paul and Williamson, 1987; Saluja *et al.*, 1989]. With respect to the basic features of this class of enzymes, the tomato isozyme APS-1^1 does not differ significantly. Like most acid phosphatases studied to date, the tomato enzyme is a dimeric, glycosylated protein, that displays a 'relaxed' substrate specificity [Paul and Williamson, 1987]. Given the great difficulty of isolating substantial amounts of the APS-1^1 allozyme from tomato and its common features, this enzyme would not have been of particular interest, if the corresponding gene was not closely linked to a disease resistance gene and a conceivable landmark gene in chromosome walking studies.

The present protocol for the purification of APS-1^1 from tomato is a modified version of the procedure developed by Paul and Williamson [1987] , extended with a Mono Q FPLC step. Only minor modifications were made except for the Con A-Sepharose column chromatography step. While these authors reported, that APS-1^1 displayed only a weak affinity for Con A in passing through the Con A-Sepharose column upon initial application, we observed the opposite (Fig. 4). This discrepancy may reflect a great variability in the extent of glycosylation and the sugar composition of the carbohydrate component of APS-1^1, depending on genetic or environmental factors. Another aspect that called for special attention, and has possibly gone unnoticed by Paul and Williamson [1987], was the 'bleeding' of the Con A-Sepharose column, resulting in contamination of the purified APS-1^1 preparation with substantial amounts of Con A (up to 10 µg/ml). As Con A-Sepharose column chromatography constituted the last step in the Paul and Williamson protocol [1987] and, in addition, Con A and APS-1^1 migrated similarly in SDS-PAGE gels, it is questionable, whether the apparent high yield of APS-1^1 (of the order of 1 mg/kg cells) reported by these authors, should not be attributed to a large extent to a contamination with Con A, in particular, as no major loss of APS-1^1 activity was observed throughout the present purification. Anyhow, the Mono Q FPLC step was found imperative to obtain an enzyme preparation, that met the purity criteria required for subsequent amino acid sequencing.

In summary, a purification procedure for tomato APS-1^1 has been developed that provided a highly purified preparation suitable for amino acid microsequencing, as required by the present strategy for cloning *Mi*.

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Chapter 3

Partial amino acid sequencing of tomato acid phosphatase-1[1]

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INTRODUCTION

The acid phosphatase-1[1] (*Aps-1[1]*) gene on chromosome 6 of tomato may provide a nearby starting point for a chromosomal walk towards the agronomically important nematode resistance gene *Mi*, which is estimated to be located at a distance of 540 kb from the *Aps-1[1]* locus [Medina-Filho, 1980; Arumuganathan and Earle, 1991; Ganal *et al.* cited in Messeguer *et al.*, 1991]. Molecular cloning of *Aps-1[1]* is feasible, since the encoded acid phosphatase-1 allozyme (APS-1[1]) has been purified [Paul and Williamson, 1987; Chapter 2] and methods for cloning nucleotide sequences encoding a known polypeptide gene product are currently available. For selection of the cloned sequence are then used specific antibodies [Huynh *et al.*, 1985; Snyder *et al.*, 1987; Sambrook *et al.*, 1989] or oligonucleotide probes, the sequence of which has been derived from the amino acid sequence of the target protein [Ohtsuka *et al.*, 1985; Lathe, 1985; Wood *et al.*, 1985; Devlin *et al.*, 1988; Sambrook *et al.*, 1989].

With a view to cloning the *Aps-1[1]* gene, the purified APS-1[1] preparation described in the previous chapter was used to determine the amino acid sequences of parts of the *Aps-1[1]*-encoded polypeptide chain. For that purpose, protein sequencing has been performed using purified peptides prepared by specific proteolytic cleavage [Aebersold *et al.*, 1987; Prussak *et al.*, 1989]. We did not use protein sequencing starting from the N-terminus, since N-terminal sequencing is often precluded by biosynthetic or artifactual block of the terminal amino group [Hunkapiller *et al.*, 1983a; 1983b; Aebersold *et al.*, 1987]. Given the low amount of purified protein available, no attempt was therefore made to sequence the N-terminus of APS-1[1].

MATERIALS AND METHODS

Concentration of the purified APS-1[1] preparation by ultrafiltration

The Mono Q-FPLC-purified APS-1[1] preparation resulting from step 6 of the purification protocol (chapter 2) was concentrated in portions by repeatedly using a single CENTRICON-10 ultrafiltration device according to the instructions of the manufacturer (AMICON). After each centrifugation run, the sample reservoir, containing the concentrate of the previous ultrafiltration step, was filled up with dilute APS-1[1] preparation and then the concentration step was repeated. This procedure allowed up to 21 ml of APS-1[1] preparation to be concentrated to 40 - 120 µl.

To change the buffer of the final concentrate to 200 mM NH_4HCO_3 (pH 8), four additional cycles of 15-fold dilution and concentration with 200 mM NH_4HCO_3 (passed through a 0.22 µm filter) were performed in the same CENTRICON-10 unit. The concentrated APS-1^1 preparation in approximately 100 µl of 200 mM NH_4HCO_3 was collected by centrifugation of the CENTRICON-10 unit in inverted position for 10 min at 3000 rpm in a bench top centrifuge.

Preparation of APS-1^1 peptides

For preparation of CNBr peptides, 100 pmole of Mono Q-FPLC-purified (step 6) APS-1^1 prepared from roots, was concentrated by lyophilization and redissolved in formic acid. A crystal of CNBr was added and air was removed from the eppendorf reaction tube by flushing with nitrogen gas. The tube was capped air-tight and incubated overnight at 4 °C.

To prepare tryptic peptides, 100 pmole (~3 µg) of APS-1^1, purified from suspension cells (chapter 2), was concentrated into 100 µl 200 mM NH_4HCO_3 by ultrafiltration and 0.01% (w/v) SDS was added. Subsequently, the protein was digested with 1 µg of HPLC-purified trypsin (Boehringer, Mannheim) at 37 °C for 16 hours.

Peptides were separated by reversed-phase HPLC (Applied Biosystems, model 130A), using an AquaporeRP-300 column (2.1 × 30 mm) and a linear gradient of 0 - 70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA) to elute the peptides. The effluent was monitored at 220 nm.

Peptide sequencing

HPLC-purified peptides which showed a symmetrical elution peak were lyophilized, redissolved in 30 µl formic acid and bound to polybrene-pretreated, TFA-activated glass fibre filters. For sequencing of the peptides by automated Edman degradation, a pulsed-liquid phase sequencer was used (Applied Biosystems, model 477A). Sequencing was performed as described [Beyreuther *et al.*, 1983].

RESULTS

Partial amino acid sequencing of tomato APS-1[1]

Because of the low amount of APS-1[1] protein available (chapter 2) and the risk of dealing with a protein that is blocked at its N-terminus, no attempt was made to determine the N-terminal amino acid sequence. Instead, amino acid sequencing of APS-1[1] was performed on peptides, generated by proteolytic cleavage and purified by reversed-phase HPLC. The first batch of APS-1[1], obtained from roots, was cleaved with CNBr and a second batch, purified from suspension cells, was cleaved by tryptic digestion.

Two CNBr and seven tryptic cleavage products were recovered sufficiently pure and in adequate amounts to allow determination of their respective (partial) amino acid sequences (Table 1). Together, these peptides revealed 61 residues of amino acid sequence information from the APS-1[1] polypeptide chain. Assuming about 280 amino acid residues to be contained within a 31 kD polypeptide, this corresponds to approximately 22% of the APS-1[1] amino acid sequence.

Table 1. Partial amino acid sequence of tomato APS-1[1]. Deduced Met residues, preceding CNBr peptides I and II, are shown in parentheses. The CNBr peptides are boxed within the tryptic peptides where they are part of (VII and IX, respectively).

I : (Met)-Tyr - Tyr - Ile - Leu

II : (Met)-Val - Gly - Pro - Gly - Tyr - Lys

III : Phe - Val - Pro - Glu - Thr - Asn - Leu - ? - Asn - Arg

IV : Ile - Val - Gly - Asn - Ser - Gly - Asp - Gln - Trp - Ser - Asp - Leu - Leu - Gly - (Arg)?

V : Leu - Ile - Leu - Arg

VI : Val - Phe - Leu - Leu - Thr - Gly - Arg

VII : ? - Phe - Lys - Leu - Pro - Asn - Pro - Met - [Tyr - Tyr - Ile - Leu] - (Arg)?

CNBr peptide I

VIII : Leu - Tyr - Gln - Glu - Val - Leu - Lys - Phe - (Lys / Arg)

IX : Ala - Met - [Val - Gly - Pro - Gly - Tyr - Lys]

CNBr peptide II

Among the seven tryptic peptides sequenced, two (VII and IX) were found to overlap (Table 1) the amino acid sequence established for the respective CNBr peptides (I and II). Since the CNBr and the tryptic peptides were derived from independent APS-1[1] preparations, isolated from two different sources, this observation lends support to the reliability of the established amino acid sequence information.

DISCUSSION

As a consequence of the low level of expression of the APS-1[1] protein, the purification protocol presented in chapter 2 resulted in a very dilute APS-1[1] preparation, which needed to be concentrated for microsequencing purposes into a volume applicable to a glass fibre filter. In fact, up to 21 ml had to be concentrated into about 100 µl. At first, for the batch of APS-1[1] protein isolated from hydroponic roots, we had chosen lyophilization to reduce the volume, as this method principally allows the sample to be redissolved in whatever suitable volume. After lyophilization however, the dehydrated APS-1[1] had become insoluble in trypsin digestion buffer. Therefore, the lyophilized APS-1[1] was only amenable to CNBr cleavage, as this reaction may be performed in formic acid, in which lyophilized APS-1[1] was readily soluble.

Both CNBr peptides we found, were very short (4 and 6 amino acid residues, respectively) and together accounted for only a small fraction of the APS-1[1] polypeptide chain (a total of ±280 amino acid residues). As CNBr breaks peptide bonds on the carboxyl side of methionine [Gross, 1967] and this amino acid occurs relatively rarely in natural polypeptide chains [Nikodem and Fresco, 1979; Scott *et al.*, 1988], large peptides are usually produced [Gross, 1967; Nikodem and Fresco, 1979; Scott *et al.*, 1988; Plaxton and Moorhead, 1989]. Thus, the missing of the larger cleavage products would easily be accounted for, if APS-1[1] would contain only few methionine residues and the large cleavage products are not recovered, because the large peptides will resemble the complete denatured protein in being highly insoluble at moderate pH conditions.

Fortunately, ultrafiltration proved a successful by-pass of the low solubility of lyophilized APS-1[1], making tryptic digestion feasible. Refilling the sample reservoir of the ultrafiltration unit with the dilute APS-1[1] preparation after each concentration step and repeating the concentration procedure allowed a reduction in volume from 21 ml to ±100 µl. During this concentration procedure, the introduction of dust into the essentially dust-free Mono Q-FPLC-purified APS-1[1] preparation was avoided by refilling the ultrafiltration unit in a sterile hood and using membrane-filtered ammonium bicarbonate exchange buffer. These precautions were

taken, because preliminary experiments had indicated, that the ultrafiltration membrane may become impermeable upon repeated use of the unit, probably by clogging of minute particles on the membrane surface.

As a result, the purity level of the APS-1[1] preparation allowed up to 14 consecutive amino acid residues (peptide IV) to be determined with great certainty. The largest peptides, IV and VII, both contain a stretch of three amino acid residues (Asp - Gln - Trp and Met - Tyr - Tyr, respectively) with only fourfold codon degeneracy. From such sequences oligonucleotides with comparatively small degeneracy can be derived.

After the completion of this work Tanaka *et al.* [1990] confirmed that tomato APS-1[1] is N-terminally blocked in showing that treatment with pyroglutamylpeptidase renders tomato APS-1[1] amenable to Edman degradation, allowing the 14 N-terminal amino acid residues to be sequenced. The sequence determined by these authors did not overlap, however, with any of the APS-1[1] amino acid sequences described in this chapter.

The presence of Lys residues in some of our tryptic APS-1[1] peptides (VII and VIII) showed that only incomplete digestion had been attained in spite of the rigorous digestion procedure applied. Recently, Erion *et al.* [1991] published similar experiments using high levels of trypsin and endoprotease Lys-C. They still found incomplete digestion of their APS-1[1] preparation, even when they added a ratio of more than 1:10 protease to APS-1[1] (w/w), which lends support to our observation that tomato APS-1[1] shows a certain level of protease resistance.

In summary, we conclude that APS-1[1] amino acid sequence information has been obtained, allowing the design of oligonucleotides useful in cloning the *Aps-1[1]* gene.

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Chapter 4

Specificity of degenerate PCR primer pools corresponding to APS-1[1] amino acid sequence information

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For the purpose of cloning *Aps-1*[1] nucleotide sequences, degenerate oligonucleotide pools, synthesized on the basis of the known APS-1[1] amino acid sequences (chapter 3), can be applied either as a probe to screen a cDNA library, or as primers to direct a polymerase chain reaction (PCR). The latter approach would appear more discriminatory, because amplification by PCR intrinsically involves co-operative annealing of two different oligonucleotide primers and thus combines the selectivity of both, whereas selection of positive clones from a cDNA library is based on the annealing of only one single oligonucleotide probe at a time. Moreover, cDNA library screening using degenerate pools of oligonucleotide probes under low-stringency hybridization conditions usually results in numerous false positives. Moreover, taking into account that the APS-1[1] protein is low abundant (chapter 2), identification of an *Aps-1*[1] cDNA clone would likely demand the screening of a very large number of clones. On the basis of these considerations, PCR-mediated amplification of *Aps-1*[1] nucleotide sequences was chosen.

Oligonucleotide pools encompassing all possible nucleotide sequences encoding the parental amino acid sequence [Girgis *et al.*, 1988; Mack and Sninsky, 1988; Hahn *et al.*, 1989; Horikoshi *et al.*, 1989; Larrick *et al.*, 1989a; 1989b; Nunberg *et al.*, 1989; Wilks *et al.*, 1989] as well as pools with restricted complexity [Knoth *et al.*, 1988; Lee *et al.*, 1988; Wilks *et al.*, 1989; Patil and Dekker, 1990] have been applied as PCR primers. The number of different oligonucleotide sequences needed to obtain an effective primer pool can be restricted by incorporating the essentially unselective nucleotide deoxyinosine monophosphate (dIMP) at ambiguous positions [Knoth *et al.*, 1988; Ehlen *et al.*, 1989; Patil and Dekker, 1990] and besides, by choosing among the common deoxynucleotides on the basis of an 'educated guess' [Orlandi *et al.*, 1989] as will be pointed out below. The latter approach, however, is carrying the risk that the target sequence will not be primed, in particular when applied to the 3' end of the primers [Sommer and Tautz, 1989].

In view of the experience that degenerate oligonucleotide pools generally show low specificity if used as a probe in library screening [Wood *et al.*, 1985; Devlin *et al.*, 1988], degenerate PCR primer pools may likewise direct the amplification of non-target fragments. This problem may be overcome by using shorter primers, which comprise only part of the parental APS-1[1] peptide. Such a primer design would allow the identification of the target fragment among any co-amplified sequences on the basis of the nucleotide sequences flanking the primers. In the correct amplification product, these flanking nucleotide sequences are expected to encode the additional amino acids known from the amino acid sequence analysis. On the other hand, the presence of target-related sequences within the template DNA may interfere with this strategy and hamper the final identification of the target fragment.

In this chapter, the successful application of degenerate PCR primer pools of restricted complexity level for the amplification of an apparent *Aps-1*^{1} genomic sequence is described. The necessity to have other tests available complementary to the nucleotide and amino acid sequence match, is illustrated with the performance of *Aps-1*^{1} primer pools that were made shorter than the parental APS-1^{1} peptide.

MATERIALS AND METHODS

Plants and cell cultures

Seeds of commercial tomato cultivars and breeding lines containing different *Aps-1* alleles were kindly provided by several Dutch seed production companies. *L. esculentum* lines 83M71392 and 83M71398 form a pair of nearly isogenic lines (NILs), differing only in the chromosome 6 region containing the *Aps-1* and *Mi* loci [Klein-Lankhorst *et al.*, 1991]. The nematode-sensitive line 83M71392 carries the *Aps-1*^{3} and *mi* alleles. The nematode-resistant line 83M71398 carries the *Aps-1*^{1} and *Mi* alleles introgressed from *L. peruvianum*.

LA 1641 is a *L. esculentum* chromosome substitution line in which the *L. esculentum* chromosome 6 has been replaced by chromosome 6 from *L. pennellii* LA 716, except for the very distal end of the long arm encompassing RFLP marker TG193. This line has been developed from a *L. esculentum* × *L. pennellii* cross by Rick [1969]. A detailed molecular and genetic characterization of this substitution line has been accomplished (Weide *et al.*, 1993). Both *L. pennellii* LA 716, and *L. esculentum* LA 1641 were obtained from Dr. C.M. Rick, University of California, Davis, California.

Plants were grown in a greenhouse at temperatures between 20 ° and 35 °C. For DNA extraction young, still expanding leaves were harvested, frozen in liquid nitrogen and stored at -80 °C.

L2-14 cell suspension cultures were derived from a plant, selected among the progeny that arose from the selfing of L2, which is a hybrid *L. peruvianum* × *L. esculentum* line containing 75% *L. peruvianum* [Thomas and Pratt, 1981]. The cells were cultured at 25 °C in the dark in 1 l flasks containing 400 ml medium [DuPont *et al.*, 1985] under vigorous shaking. Every 10 - 14 days one tenth of the suspension was subcultured; the remaining cells were then harvested by filtration using a Büchner funnel, and were stored frozen at -80 °C. The average cell yield was 100 - 200 g/l.

Synthesis of oligodeoxyribonucleotides

Pools of oligodeoxyribonucleotides were synthesized on a Biosearch Cyclone model no. 8400 DNA synthesizer using β-cyanoethyl phosphoramidite chemistry.

Construction of a cDNA library

A cDNA library of root poly(A)+RNA was constructed essentially as described by Huynh *et al.* [1985]. If not specified, materials were purchased from Boehringer or GIBCO BRL.

RNA was isolated from hydroponic roots of the nematode-resistant line 83M71398 (*Aps-1*^{1}*/Aps-1*^{1}, *Mi/Mi*) by extraction with phenol and precipitation from 2 M LiCl [De Vries *et al.*, 1982]. The poly(A)-containing RNA fraction was purified by a single adsorption to oligo(dT)-cellulose (Type 2, Collaborative Research) and reverse transcribed using an oligo(dT)$_{12\text{-}18}$ primer (Pharmacia) and avian myeloblastoma virus (AMV) reverse transcriptase (Life Science). Primers for second strand synthesis were generated by RNAase H treatment of the mRNA:cDNA hybrid and the second cDNA strand was synthesized through replacement of the RNA strand using DNA polymerase I [Okayama and Berg, 1982; Gubler and Hoffman, 1983]. The double-stranded cDNA was treated with T4 DNA polymerase and *Eco*RI linkers were ligated to the blunt ends using T4 DNA ligase (50 U/µl, Boehringer). The linkers were cut with *Eco*RI and the released linker fragments removed by Bio-Gel A-50 (Bio-Rad) column chromatography. Phage λgt11 DNA was digested with *Eco*RI and treated with alkaline phosphatase from calf intestine (CIP) to prevent self-ligation. Then *Eco*RI-linked cDNA and *Eco*RI-digested vector DNA were ligated and the ligation products packaged into phage particles using a commercial packaging mix (Promega). From a titration on the *Escherichia coli* Y1090 host strain in the presence of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), it was estimated that the unamplified library contained 1% of wild-type and 9×10^5 pfu of recombinant phages. Before screening, the library was amplified once by plating on *E. coli* Y1090.

Library screening using degenerate oligonucleotide pools

The amplified cDNA library was plated on NZCYM-agar (10 g/l NZ-amine, 5 g/l NaCl, 5 g/l yeast extract, 1 g/l casamino acids, 2 g/l $MgSO_4 \cdot 7\ H_2O$, pH 7.5) at a density of 33,500 pfu per petri dish (diameter 17 cm) using *E. coli* Y1090 as a host. Plaques were grown for 6-8 hours at 37 °C, and replicas were made in triplicate on HATF filter membranes (Millipore).

Phage DNA was released and bound to the membranes using standard procedures [Sambrook *et al.*, 1989].

Filters were prehybridized in 1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate for 2 hours at 65 °C. Subsequently, 10-20 ng/ml oligonucleotide probe was added, that had been labeled to a specific activity of 0.2-1.7 × 10^8 dpm/µg using bacteriophage T4 polynucleotide kinase and γ-^{32}P-dATP [Sambrook *et al.*, 1989]. Hybridization was performed overnight at a temperature 15-40 °C below the T_m as calculated according to Lathe [1985]. The filters were washed in 6 × SSC (0.9 M NaCl, 0.09 M sodium citrate pH 7.0) at the same temperature during 15 min.

Autoradiography was performed for 2-8 days at -80 °C using Kodak Xomat AR-5 film and an intensifying screen.

Polymerase chain reaction (PCR)

The reaction mixture for the amplification of tomato genomic DNA sequences contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % (w/v) gelatin, 0.2 mM of each dNTP, 2.5 U AmpliTaq (Perkin Elmer Cetus), 1 µg of genomic DNA and 4 µg of each of two appropriate primer mixtures in a 100 µl reaction volume. The reaction mixture was overlaid with 100 µl paraffin. In general, amplification was performed for 30 cycles through a regime of 1 min template denaturation at 92 °C, followed by a 1 min primer annealing at 55 °C and a 3 min primer extension at 72 °C, using an automatic thermal cycler (PREM III, Lep Scientific). As a positive control, we used a pair of perfectly matching [Chase and Williams, 1986] *Adh-2* primers (5'-GTCGACTACTGTAGGCCAA-3' and 5'-ATCCAACATGAACCACG-3'), which had been found to direct the amplification of a 0.7 kb and a 476 bp tomato *Adh-2* sequence, respectively with genomic and cDNA as a template.

Amplification of *Aps-1*^{1} mRNA sequences was performed as follows: After heating for 3 min at 85 °C, L2-14 RNA (1 µg) and 4 µg antisense *Aps-1*^{1} primer pool IV.23a or VII.23a (Table 1) were allowed to anneal at 37 °C for 15 min in a volume of 10 µl containing 10 mM Tris-HCl pH 8.3, 250 mM KCl, 1 mM EDTA. Then, 15 µl of a mixture was added containing 25 mM Tris-HCl pH 8.3, 16.7 mM $MgCl_2$, 8.3 mM DTT, 0.42 mM of each dNTP, 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) and 20 U of RNasin, and cDNA was synthesized at 42 °C for 15-30 min. For subsequent amplification of *Aps-1*^{1} cDNA sequences by PCR, the reaction mixture was adjusted to 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01 % (w/v) gelatine and each dNTP was replenished with 1 µl of a 10 mM stock, resulting in a final reaction volume of 100 µl. Reaction conditions were similar as described for the amplification of genomic *Aps-1*^{1} sequences, except

for a slower increase of temperature during primer extension, resulting from an additional 10 sec of heating at 65 °C prior to the 3 min 72 °C step.

PCR products were analysed by agarose gel electrophoresis, using 1.5% or 2.5% (w/v) agarose gels, and visualized by ethidium bromide (EtBr) staining.

Southern blot analysis

Genomic DNA from various tissues was isolated according to Murray and Thompson [1980] with the omission of the final CsCl - EtBr centrifugation step. Restriction enzyme-digested DNA (3 µg) was loaded on a 1% (w/v) agarose gel. After electrophoresis, restriction fragments were nicked by a 5 min treatment with short wave UV irradiation and blotted onto Gene Screen Plus membrane, as recommended by the manufacturer (DuPont). DNA was then cross-linked to the membrane by UV irradiation for 1 min, followed by baking at 80 °C for 1 hour.

Hybridization was performed overnight in 50 mM Tris-HCl pH 7.5, 1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate, 0.1 mg/ml salmon sperm DNA at 65 °C using 25 ng of the 2,4 kb PCR product, labeled (10^8-10^{10} dpm/µg) by random priming [Feinberg and Vogelstein, 1984], as a probe. Blots were washed at a final stringency of 0.5 x SSC (75 mM NaCl, 7.5 mM sodium citrate pH 7.0) at 65 °C. Autoradiography was performed at -80 °C for 1-3 days using Kodak XAR film and an intensifying screen.

Nucleotide sequence analysis

DNA fragments to be sequenced were subcloned into pTZ18R or pTZ19R. Sequence analysis was performed according to the dideoxynucleotide chain-termination method [Sanger *et al.*, 1977], either using double-stranded template or single-stranded template DNA as described in the text. Single-stranded template DNA was prepared using M13KO7 helper bacteriophages and an *E. coli* DH5α F' host strain as described by Vieira and Messing [1987].

RESULTS

Screening of a cDNA library using degenerate oligonucleotide pools

The partial amino acid sequence of APS-1^1 (chapter 3) enabled us to develop oligonucleotide probes for the *Aps-1^1* gene. Initially, degenerate oligonucleotide pools corresponding to APS-1^1 peptides II, III, IV and VII (chapter 3, Table 1) were synthesized which essentially contained all possible nucleotide sequences encoding the parental peptide. In order to limit the number of different oligonucleotide species per pool to at most 512, deoxyinosine (dIMP) was used at certain ambiguous positions within oligonucleotides corresponding to long amino acid sequences. Subsequently, the oligonucleotide pools were utilized as probes to screen a library of about 200,000 cDNA clones representing mRNA sequences from nematode resistant (*Aps-1^1/Aps-1^1*) tomato roots. A couple of initially positive clones were found, none of which, however, remained positive upon plaque purification. On the other hand, five positive *Adh-2* clones were readily identified upon screening 33,500 plaques, using a perfectly matching 17-mer oligonucleotide probe corresponding to position 462 - 478 within the 5' part of the *Adh-2* coding region (1140 bp). This indicates that the negative result with the *Aps-1^1* oligonucleotides was not likely due to the mere absence of 5' mRNA sequences from the cDNA library, but rather reflected the extreme rareness of the *Aps-1^1* mRNA and the technical difficulties inherent to library screening with complex oligonucleotide mixtures.

The negative results obtained thus far using the degenerate *Aps-1^1* oligonucleotide probes and the theoretical superiority of the PCR as compared to library screening prompted us to pursue the PCR approach for isolating *Aps-1^1* nucleotide sequences.

PCR using highly complex primer pools.

In a first attempt to amplify *Aps-1^1* nucleotide sequences by PCR, oligonucleotide primers were synthesized corresponding to (a part of) APS-1^1 peptides III, IV, VII and IX, in both orientations, using the same strategy to deal with codon degeneracy as applied in oligonucleotide probe design. Generally, this resulted in highly complex mixtures, containing up to 512 different primer species; some representative examples are included in Table 1 and 2. The amplification products synthesized under the direction of these complex primer pools were analysed by agarose gel electrophoresis and visualized by ethidium bromide (EtBr) staining.

With most combinations of primer pools, a variety of amplification products was found using both genomic (some representative examples are shown in Fig. 1) and cDNA templates (not shown), especially with pools of the shorter primers, as for example the IIs + IIIa primer

combination in Fig. 1. Most of these products were unlikely to represent the target fragment, since they were also present in the minus template control or in the unpaired primer reactions, that contained only one of both primer pools needed to produce the target fragment. The specificity attained with the complex primer pools was low, even though reaction conditions were used, 2.5 mM Mg^{2+} and 2.5 U/100µl Taq polymerase, demonstrated to be optimal in preliminary PCR experiments testing the influence of Mg^{2+} (Fig. 2) and Taq polymerase concentration (data not shown) and involving a pair of perfectly matching *Adh-2* primers described in Materials and Methods.

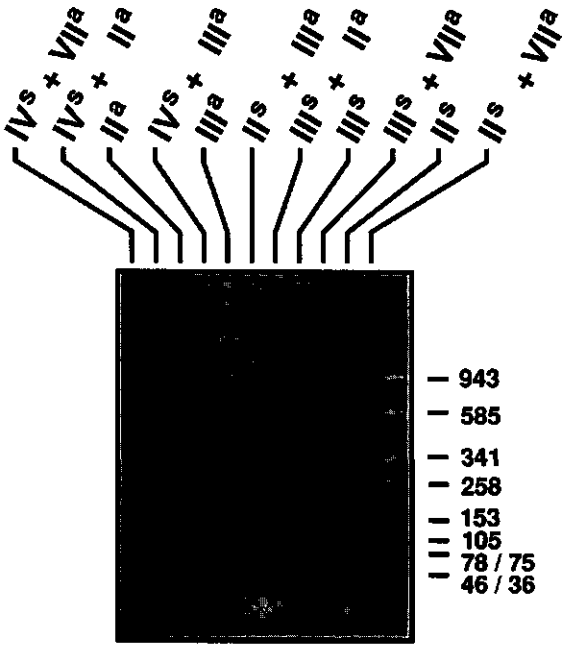


Fig. 1. PCR products amplified under the direction of highly complex *Aps-1*^{1} primer pools using tomato genomic DNA (*Aps-1*^{1}/*Aps-1*^{1}) as a template. The combination of *Aps-1*^{1} primer pools used in the amplification reaction is referred to with the Roman numerals of the parental APS-1^1 peptides. The orientation of the primers is shown in superscript (s = sense; a = antisense). The marker lane shows the size in base pairs of *Sau*3AI restriction fragments of pUC18 DNA.

As subtractive comparisons of the paired primer reactions with the corresponding unpaired primer and minus-template controls did not indicate a single putative *Aps-1*^{1} fragment, we have subjected the possible target fragments to RFLP analysis, using a pair of NILs holding

different *Aps-1* alleles, to determine whether the product originated from the *Aps-1*^{1}*/Mi* chromosomal region. As far as tested, this additional selection did neither provide a clue to the presence of an *Aps-1*^{1} target fragment among the pool of amplified products. Apparently, none of these primer pools, in any combination, was of practical use for the amplification of *Aps-1*^{1} nucleotide sequences.

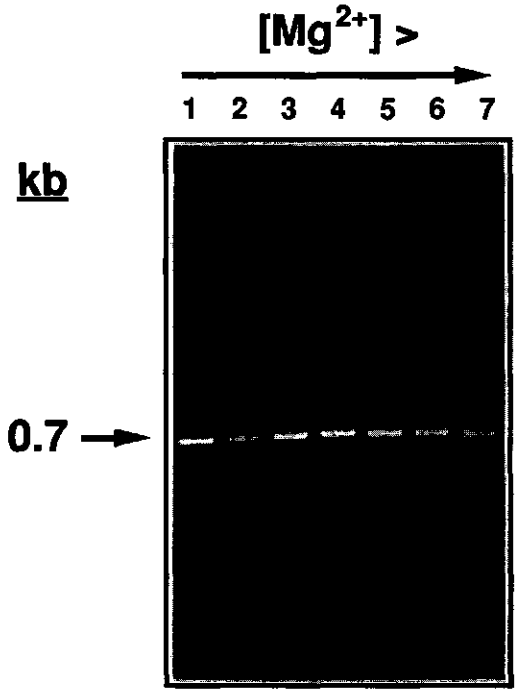


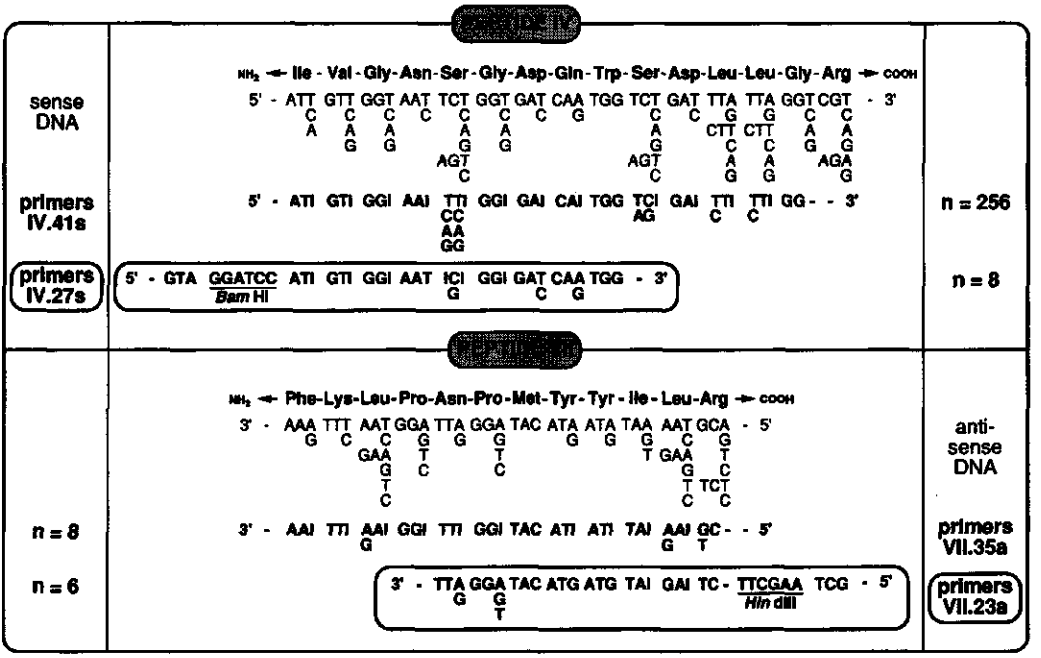
Fig. 2. Effect of the Mg^{2+} concentration on the specificity of the amplification reaction directed by a pair of *Adh-2* primers using tomato genomic DNA as a template. Twenty-five cycles of PCR were performed in the presence of 3.4 U of Taq polymerase (Amersham) and increasing amounts of Mg^{2+} ion: 1.5 mM (lane 1), 2.0 mM (lane 2), 2.5 mM (lane 3), 3.0 mM (lane 4), 3.5 mM (lane 5), 4.0 mM (lane 6) and 5.0 mM (lane 7). Other reaction conditions were as described in Materials and Methods. The 0.7 kb amplification product is the target fragment expected for *Adh-2*. Note the appearance of larger and smaller aspecific products at high Mg^{2+} concentrations.

PCR using primer pools with reduced complexity

In a second attempt to amplify *Aps-1*^{1} nucleotide sequences, we redesigned the 5' region of the primers in order to obtain less complex primer mixtures. We continued to incorporate all possible nucleotide sequences encoding the three amino acids which determined the 3' terminus of the primers in order to ensure a perfect match at the 3' end, which is a prerequisite for efficient elongation [Sommer and Tautz, 1989]. In the 5' part of the primers, dIMP was used at ambiguous positions whereas, at certain twofold ambiguities, the most stabilizing common nucleotide was chosen (T at T/C ambiguities and G at G/A ambiguities, considering that a G-T mismatch is more stable than a G-I base pair [Jaye *et al.*, 1983; Martin *et al.*, 1985]). In case of amino acids for which codon usage in dicots [Murray *et al.*, 1989] indicated possible strong

codon preference, we chose the most probable nucleotide(s), in particular within the 5' proximal region. Whenever possible, the primers were made shorter than the parental APS-1[1] peptide to permit the identification of the target fragment among possible, co-amplified sequences on the basis of the colinear remainder of the amino acid sequence information. To facilitate cloning of the resulting PCR products, restriction sites were added to the 5' ends of the primers (Tables 1 and 2).

Table 1. Degenerate PCR primer pools of high and reduced complexity level corresponding to the amino acid sequence of APS-1[1] peptides IV and VII.



The code of the primers is composed of a Roman numeral indicating the APS-1[1] peptide from which it was derived, followed by the number of nucleotides corresponding to APS-1[1] amino acid sequence information and the orientation of the primer (*s = sense; a = antisense*); *n indicates the number of different nucleotide sequences* contained within the primer pool. The combination of primer pools which successfully directed the amplification of the *Aps-1*[1] target fragment is boxed.

The adjustments thus made to the primer pools appeared to produce a decisive increase of the specificity of the PCR. When this alternative primer design was applied to the longest APS-1[1] peptides, peptides IV and VII, the combination of primers IV.27s and VII.23a (Table 1) was found to direct the amplification of a single major ±105 bp product with both a

genomic (Fig. 3) and a cDNA template (Fig. 4). Unlike the products amplified under the direction of the first generation of complex primer pools (Fig.1), the amplification of the ±105 bp product was reproducible and exhibited the correct amplification characteristics. No product at all was detectable in the unpaired primer and minus-template controls (Figs. 3 and 4).

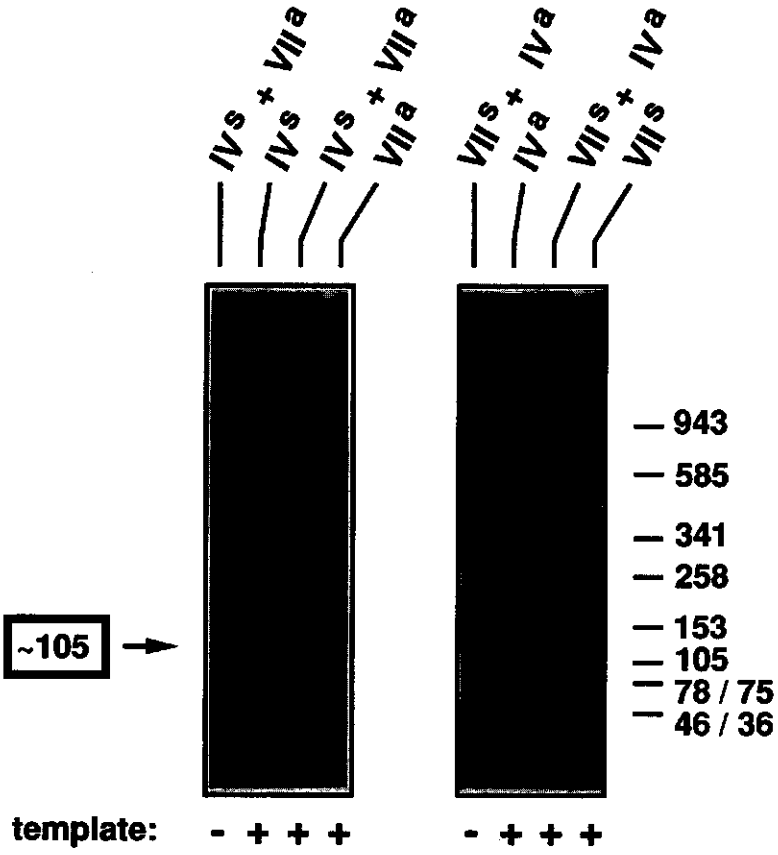


Fig. 3. PCR products from a tomato (*Aps-1*^{1}/*Aps-1*^{1}) genomic template amplified under the direction of degenerate primer pools derived from APS-1^1 peptides IV and VII and designed with reduced complexity. The *Aps-1*^{1} primer pools used in the amplification reaction are indicated with the Roman numerals of the parental APS-1^1 peptides. The orientation of the primers is shown in superscript (s = sense; a = antisense). Fragment sizes are shown in base pairs.

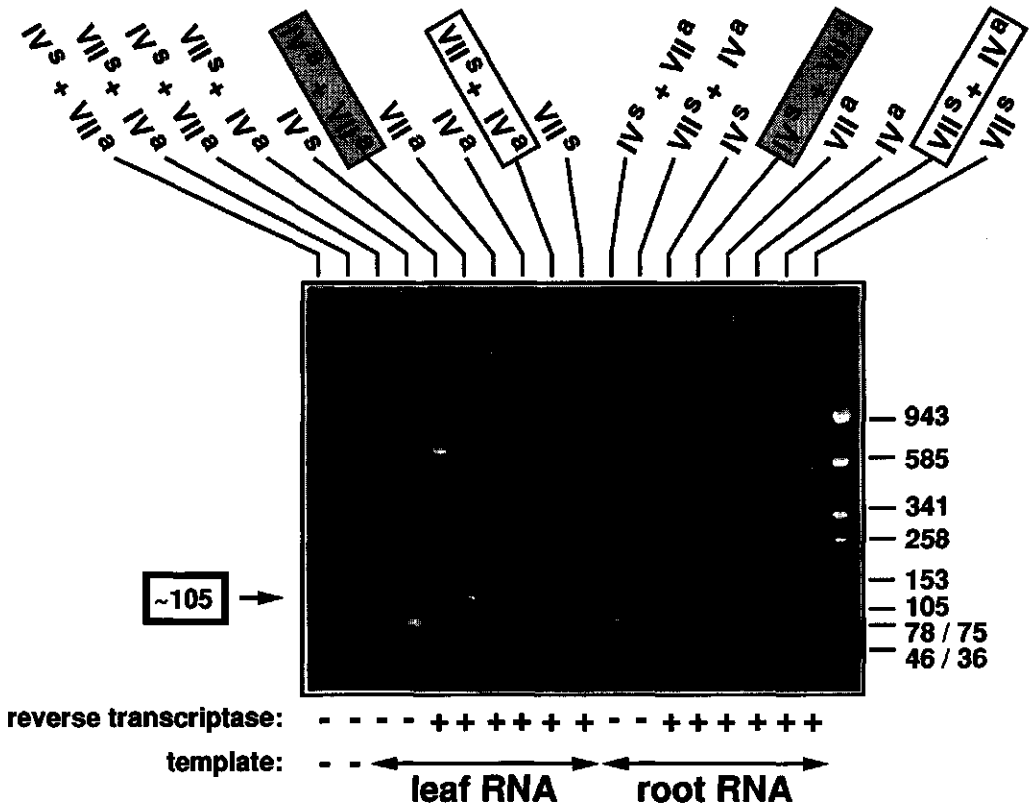


Fig. 4. PCR products from a tomato (*Aps-1*^{1}/*Aps-1*^{1}) cDNA template amplified under the direction of degenerate primer pools derived from APS-1^1 peptides IV and VII and designed with reduced complexity. The *Aps-1*^{1} primer pools used in the amplification reaction are indicated with the Roman numerals of the parental APS-1^1 peptides. The orientation of the primers is shown in superscript (s = sense; a = antisense). The primer combination which produced the *Aps-1*^{1} target fragment is placed in a shaded box. The target fragment is absent when the opposite primer orientation is used (blank box) and in the unpaired primer controls. Fragment sizes are shown in base pairs.

Sequence heterogeneity of the IV.27s/VII.23a-directed ±105 bp PCR product

The origin of the IV.27s/VII.23a-directed amplification product was investigated by direct nucleotide sequencing. To this end, the gel-purified PCR product was cloned into pTZ18R, using the restriction endonuclease cleavage sites added to the 5' ends of the primers (Table 1). Sequence analysis of four clones demonstrated, that the product was heterogeneous (Fig. 5), although all clones sequenced definitely represented a two-primer product of 115 or 116 bp

length. Therefore, this diversity is not likely to represent an irrelevant PCR artefact, but rather a family of related tomato nucleotide sequences.

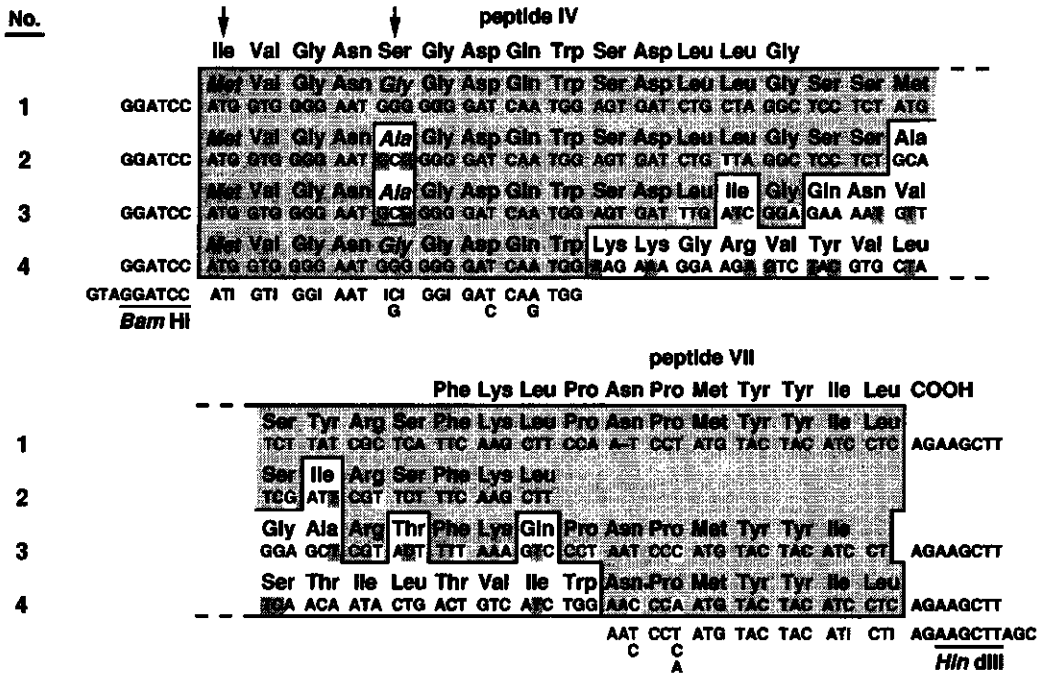


Fig. 5. Nucleotide sequence heterogeneity of the IV.27s/VII.23a directed PCR product. The gel-purified amplification product was cloned into pTZ18R and found to contain nucleotide sequences 1 - 4 upon sequence analysis. The sequences were aligned to sequence 1, and to the PCR primers used and their parental APS-1[1] peptides. Amino acid residues or nucleotides that each sequence had in common with sequence 1 were shaded. Arrows mark positions where the deoxyinosine in the PCR primer had caused another amino acid to be encoded by the cloned amplified sequence than the one present in the corresponding APS-1[1] peptide. Sequence 3 was found truncated at an internal cleavage site for the *Hin*dIII restriction enzyme used in cloning.

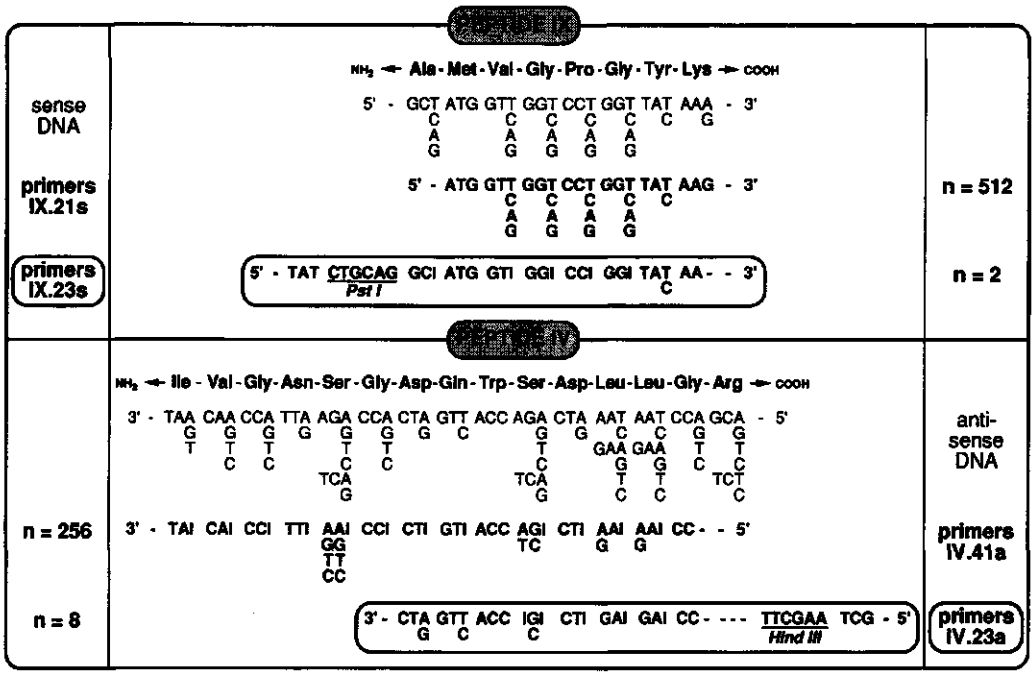
Although the sequences 3 and 4 (Fig. 5) could be sorted out on the basis of the mismatches with the expected APS-1[1] amino acid sequences, two nucleotide sequences, labeled 1 and 2 in Fig. 5, remained obvious *Aps-1[1]* candidates in showing complete colinearity with both known APS-1[1] amino acid sequences flanking the primer regions.

As the candidate sequences 1 and 2 showed a poor performance as a probe in Southern blot hybridization, it was not possible to establish which of the two candidates represented the *Aps-1[1]* target sequence by RFLP analysis of a pair of NILs holding different *Aps-1* alleles .

Amplification of a 2.4 kb genomic *Aps-1*^{1} nucleotide sequence

Another set of PCR primers tested was derived from *Aps-1*^{1} peptides IV and IX (Table 2). In using as a template genomic DNA from the suspension cell line L2-14, carrying the *Aps-1*^{1} allele, the combination of primer pools IX.23s and IV.23a directed the synthesis of a major 2.4 kb amplification product (Fig. 6A, lane 3). This product was found neither in the single primer controls (Fig. 6A, lane 1 and 2) nor in the minus template control (not shown). Moreover, the 2.4 kb product was not formed when mixtures of primers in the opposite, apparently incorrect orientation were combined (Fig. 6A, lane 7), as might be expected for a correct amplification product.

Table 2. Degenerate PCR primer pools of high and reduced complexity level corresponding to the amino acid sequence of APS-1^{1} peptides IV and IX.



The code of the primers is composed of a Roman numeral indicating the APS-1^{1} peptide from which it was derived, followed by the number of nucleotides corresponding to APS-1^{1} amino acid sequence information and the orientation of the primer (s = sense; a = antisense); n indicates the number of different nucleotide sequences contained within the primer pool. The combination of primer pools which successfully directed the amplification of the *Aps-1*^{1} target fragment is boxed.

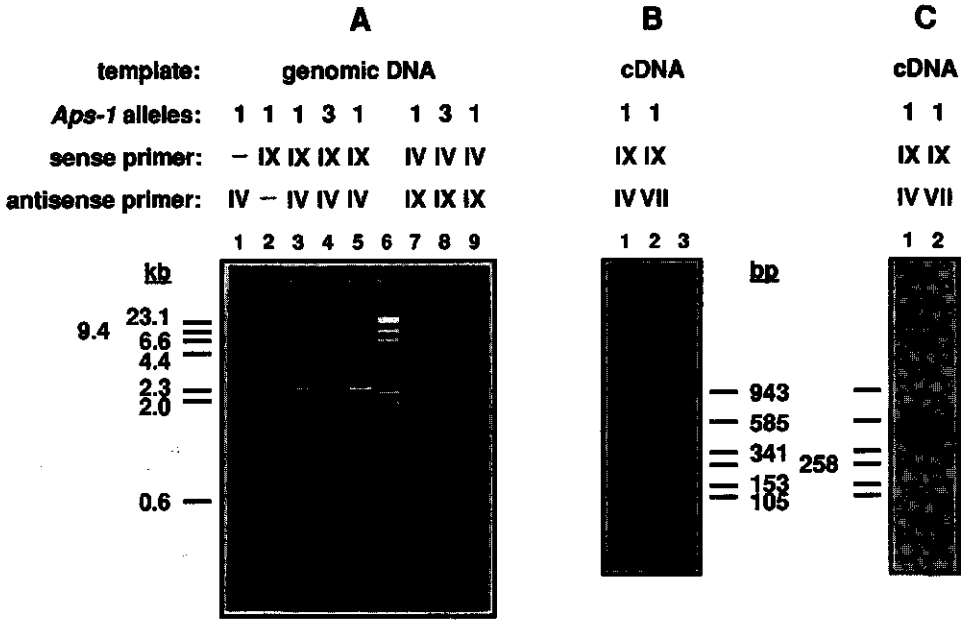


Fig. 6. Amplification products of a PCR directed by oligonucleotide primers corresponding to tomato APS-1^1 peptides IV and IX. PCR reactions were primed with either the IV.27s/IX.24a or the reverse IX.23s/IV.23a primer combination, using as a template either genomic DNA (A), or cDNA reverse transcribed from L2-14 (*Aps-1*^{1}) mRNA (B and C); IV and IX represent the primer pools in the sense orientation (IV.27s, IX.23s) or antisense orientation (IV.23a, IX.24a) as indicated above each lane. Two genomic templates (panel A) carrying the *L. peruvianum Aps-1*^{1} allele (L2-14, lanes 3, 7 and 83M71398, lanes 5, 9), and one carrying the *L. esculentum Aps-1*^{3} allele (83M71392, lanes 4, 8), were tested as indicated. Appropriate single primer (A, lanes 1, 2) and minus template controls were included, some of which are shown. None of the controls yielded any detectable amplification products. Panels B and C also illustrate (lanes 2) the amplification of a cDNA sequence related to the 2.4 kb genomic amplification product, using primers IX.23s in combination with a pool of primers (VII.23a, Table 1) corresponding to APS-1^1 peptide VII. cDNA-directed PCR products, which were undetectable by ethidium bromide staining following electrophoresis through a 2.5% gel (B), were detected by Southern blot analysis (C) using the 2.4 kb genomic PCR product (A) as a probe. Hind III-digested λ DNA (A, lane 6) and *Sau*3AI-digested pUC18 DNA (B, lane 3) were included as molecular size markers.

To our surprise, synthesis of the 2.4 kb PCR product was allele-specific and dependent on the presence of the *Aps-1*^{1} allele at the target locus in the template DNA. This was evident from experiments, using as a template genomic DNA from a pair of NILs, that only differed in the *Aps-1* alleles (*Aps-1*^{1}/*Aps-1*^{1} *vs. Aps-1*^{3}/*Aps-1*^{3}) and a small region of flanking DNA,

containing the *Mi* locus (Fig. 6A, lanes 5 and 9 *vs.* 4 and 8). Later experiments demonstrated, that also the *Aps-1*$^+$ allele did not direct the synthesis of the 2.4 kb genomic PCR product nor of any other product, like the *Aps-1*^{3} allele (data not shown). Apparently, on a heterologous *Aps-1*^{3} or *Aps-1*$^+$ template the annealing of one or both primers is impaired due to sequence divergency from the homologous *Aps-1*^{1} template. The allele specificity of the IX.23s/IV.23a primer combination provides another piece of evidence indicating that the low specificity of the IV.27s/VII.23a primer pair is not the rule with carefully designed, degenerate PCR primer pools, but is rather a template-dependent phenomenon, determined by the presence of a family of target-related sequences within the template DNA, as will be argued in chapter 5.

Unlike genomic DNA, cDNA reverse transcribed from L2-14 (*Aps-1*^{1}) mRNA was a poor template in the PCR primed by IX.23s and IV.23a. Whereas the 2.4 kb genomic target fragment was easily detectable by ethidium bromide staining following 30 cycles of PCR (Fig. 6A), the cDNA-directed amplification product could only be observed by Southern blot analysis (Fig. 6C, lane 1). Using the 2.4 kb genomic DNA-directed PCR product as a probe, a 490 bp sequence was detected. Priming of cDNA with oligonucleotide pool IX.23s in combination with primers corresponding to APS-1^1 peptide VII, resulted in the synthesis of a 550 bp PCR product, that again was only visible following Southern blot analysis (Fig. 6C, lane 2). The poor amplification of the *Aps-1*^{1} mRNA sequences was not due to the quality of the cDNA preparation, but rather reflected the low abundance of the messenger RNA, as appeared from control experiments with oligonucleotide primers designed to allow the specific amplification of the alcohol dehydrogenase(*Adh*)-2 mRNA. In that case, the cDNA preparation directed the synthesis of an *Adh-2* mRNA-specific PCR product of the proper size, that was readily stainable with EtBr following 30 cycles (data not shown). Taking into account the very low yield of APS-1^1 protein, we conclude, that the *Aps-1*^{1} gene in tomato is expressed at a very low level.

Genetic map position of the amplified 2.4 kb genomic sequence

To further verify the *Aps-1*^{1} origin of the 2.4 kb PCR product, its chromosomal location was determined using restriction fragment length polymorphism (RFLP) analysis. To this end, a Southern blot of the pair of NILs (described above), and an *L. esculentum* chromosome substitution line (LA 1641) carrying chromosome 6 from *L. pennellii* was hybridized with the 2.4 kb PCR product. If the 2.4 kb product represents an authentic *Aps-1*^{1} sequence, then it should map on chromosome 6 and exhibit an *L. pennellii*-specific hybridization pattern in the *L. esculentum* chromosome 6 substitution line. Similarly, the 2.4 kb PCR product is expected to reveal RFLPs between the pair of NILs, that only differ in the *Aps-1/Mi* region on

chromosome 6. These genetic predictions about the PCR product were met as shown in Fig. 7. The chromosomal region encompassing the amplified sequence, appeared to be highly polymorphic. Of 8 restriction enzymes tested, 6 produced an RFLP between the NILs and between *L. esculentum* and *L. pennellii*. Furthermore, by using the 2.4 kb genomic PCR product as a probe in library screening, a cDNA clone was isolated, which will be demonstrated to present an *Aps-1*^{1} cDNA clone in chapter 5. Using the cDNA clone thus isolated as a probe

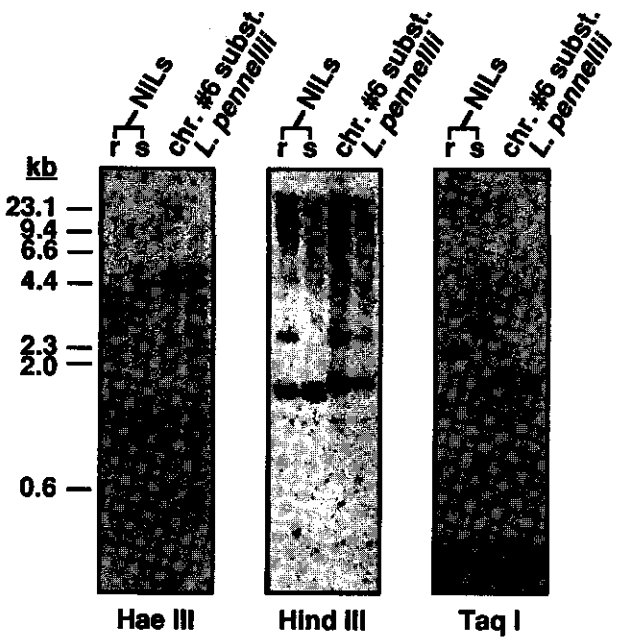


Fig. 7. Mapping of the 2.4 kb PCR product in the *Aps-1*^{1}*/Mi* region on chromosome 6 by RFLP analysis. *Hae*III-, *Hin*dIII- and *Taq*I-digested DNA from a pair of nearly isogenic, nematode resistant (r) (83M71398, *Aps-1*^{1}*/Aps-1*^{1}) and susceptible (s) (83M71392, *Aps-1*^{3}*/Aps-1*^{3}) tomato lines (NILs), the *L. esculentum* chromosome 6 substitution line (LA 1641) and its *L. pennellii* (LA 716) progenitor were subjected to Southern blot analysis using the 2.4 kb PCR product as a probe.

in Southern blot analysis of a panel of commercial tomato cultivars carrying different *Aps-1* alleles, RFLPs between the *Aps-1*^{1}, *Aps-1*^{3} and *Aps-1*$^{+}$ alleles were revealed, allowing these particular *Aps-1* alleles to be recognized (Fig. 8) and suggesting that the 2.4 kb genomic PCR product is at least related to *Aps-1*^{1}.

Altogether, these RFLP mapping data provide genetic evidence indicating, that the 2.4 kb PCR product probably represents a portion of the *Aps-1*^{1} gene located on chromosome 6.

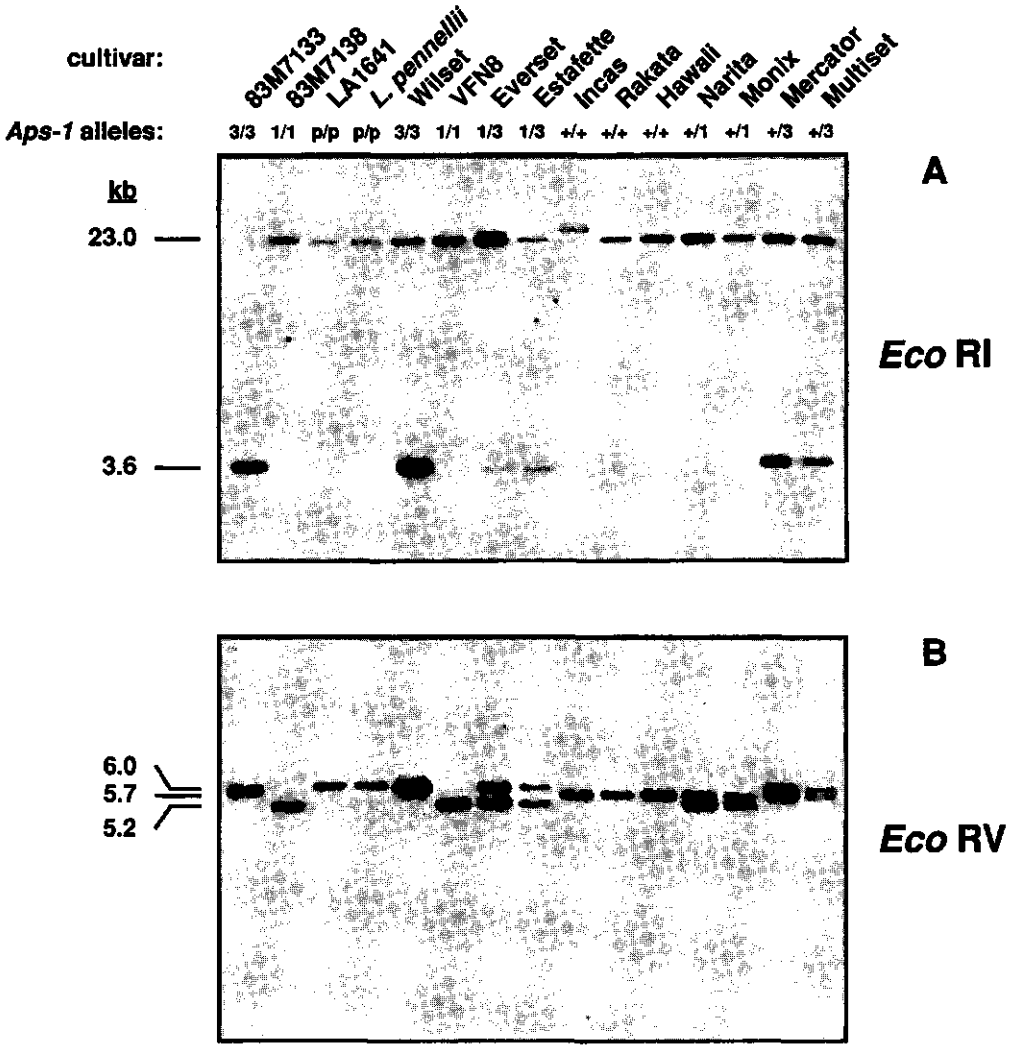


Fig. 8. RFLP analysis of a panel of tomato cultivars and lines carrying different *Aps-1* alleles. Genomic *Eco*RI (panel A) and *Eco*RV (panel B) restriction fragments of the indicated tomato genotypes were Southern blotted and hybridized with a cDNA clone (Chapter 5) corresponding to the 2.4 kb genomic *Aps-1*^{1} sequence amplified under the direction of primers IX.23s/IV.23a (Fig. 6). The *Aps-1* genotype is shown above each lane, + = *Aps-1*$^{+}$, 1 = *Aps-1*^{1}, 3 = *Aps-1*^{3} and p = *L. pennellii Aps-1* allele. The *Aps-1*^{3} allele produces a specific *Eco*RI fragment of about 3.6 kb, which is absent from the *Aps-1*$^{+}$ and *Aps-1*^{1} and *L. pennellii Aps-1*^{p} alleles. The *Aps-1*^{3} and *L. pennellii Aps-1*^{p} alleles give rise to the upper, the *Aps-1*$^{+}$ allele produces the intermediate, and the *Aps-1*^{1} allele the lower band of a triplet of *Eco*RV bands visible around 5.7 kb.

DISCUSSION

To identify *Aps-1*^{1} nucleotide sequences by using oligonucleotides synthesized on the basis of amino acid sequence information from the APS-1^1 protein, there are two obvious approaches, namely cDNA library screening and PCR. Theoretical considerations indicate that PCR is expected to be the more discriminatory alternative, as this method exploits the combined selectivity of two different oligonucleotides. In particular this will matter, if the involved gene is expressed at low level. The results obtained with both approaches confirmed our expectations about the superiority of the PCR.

Exhaustive screening of about 200,000 cDNA clones with various oligonucleotides corresponding to APS-1^1 amino acid sequence information resulted in a lot of false positives, but no consistently positive clone was found.

PCR, on the other hand, readily amplified a 2.4 kb fragment from the *Aps-1*^{1} gene. The amount of product after only 30 reaction cycles was sufficient to allow easy visualization of the target fragment by ethidium bromide staining, using only 10% of the reaction mixture. Moreover, the amplification of the *Aps-1*^{1} genomic fragment was specific, as no additional amplification products were visible.

Crucial to the success of the PCR was the use of degenerate primer mixtures containing deoxyinosine incorporated at ambiguous codon positions. Deoxyinosine-containing oligonucleotides have been successfully applied in screening cDNA and genomic libraries [Ohtsuka *et al.*, 1985; Takahashi *et al.*, 1985; Sambrook *et al.*, 1989], but as yet only scarcely in priming PCR on cDNA [Knoth *et al.*, 1988] or genomic DNA [Ehlen and Dubeau, 1989; Patil and Dekker, 1990]. The advantage of the use of deoxyinosine is probably that the sequence complexity of the degenerate primer pools is kept at a low level, so that the proportion of primers which is effective in priming the target sequence is increased. Incorporation at ambiguous positions of the nucleotide that is most probable on the basis of codon usage frequencies in dicotyledonous plants [Murray *et al.*, 1989], and choice of the most stabilizing nucleotide at some twofold ambiguities further contributed to restricting the complexity of the *Aps-1*^{1} primer pools. Choosing a particular nucleotide among the ones which are possible, carries the risk, that the priming of the target sequence might be adversely affected or even totally abolished, especially when applied to the 3'-terminal region of the primers [Sommer and Tautz, 1989]. On the other hand, it may suppress aspecific priming, as the chances of an occasional incomplete match between the primers and non-target template sequences are diminished. As a consequence, less competition for polymerase molecules between aspecific amplification products and the target fragment will occur. In comparison with degenerate primer pools containing all possible nucleotide sequences encoding the parental amino acid sequence, these restrictive adjustments have the additional advantage of allowing a relatively long stretch

of amino acid sequence to be represented in the primers. This is considered beneficial, since the specificity of a primer generally increases with its length. Wilks *et al.* [1989] designed PCR primer mixtures on the basis of amino acid sequence motifs conserved within the protein tyrosine kinase family, and compared highly complex pools of relatively long primers with moderately complex mixtures of shorter primers. Their results as well as ours indicate, that the degenerate primer pools of moderate complexity and short length are the most effective ones.

A perfect match of the three consecutive 3'-terminal nucleotides of a PCR primer was found to be prerequisite for efficient priming [Sommer and Tautz, 1989]. To make quite sure we therefore took care, that all possibilities for the three 3'-terminal codons were incorporated in our PCR primer pools. However, we have not checked whether such stringent precautions are necessary. This implies that, in general, the performance of the primer pools might still be improved by minimizing the number of positions at the 3'-end of the primers for which all possible nucleotides are incorporated into the pool, resulting in a further reduction of the complexity of the pool.

The described adjustments to the degenerate *Aps-1*^{1} primer pools produced a dramatic increase of their specificity. Amplification was not only found to be confined to the target locus, but the combination of primers IX.23s and IV.23a even showed specificity for amplifying nucleotide sequences of the particular allele (*Aps-1*^{1}) to which these primers had been directed. The heterologous *Aps-1*$^+$ and *Aps-1*^{3} alleles were not primed by one or both of these primer pools, as shown by the absence of any detectable PCR product. Degenerate oligonucleotide probes corresponding to amino acid sequence information from the protein product of the target gene are well-known for the high level of aspecific hybridization in library screening. Our results challenge this reputation in showing, that careful design of degenerate oligonucleotide pools can produce highly specific PCR primers, which might even possess allele specificity.

The extent of the divergence between the *Aps-1* alleles is unknown, but the allele specificity of the IX.23s/IV.23a primer combination and the high level of restriction fragment length polymorphism found in Southern blot analysis of the *Aps-1*^{1}, *Aps-1*$^+$ and *Aps-1*^{3} alleles indicate considerable differences between these alleles. Probably the differences are not confined to a single point mutation, although such a minor difference would yet be sufficient to explain the variation in electrophoretic mobility of the protein products of these alleles.

Our results illustrate, that target-related sequences within the template may give rise to by-products in a PCR directed by degenerate oligonucleotide primer pools, a complication which may necessitate several independent tests to achieve unambiguous identification of the target fragment. In case of the IV.23s/VII.23a-directed PCR, amplification of several related nucleotide sequences occurred, two of which met, and thereby disqualified our first identification criterion, the colinearity with the determined APS-1^1 amino acid sequence of the nucleotide stretch following the primer regions of the PCR product. A second criterion, RFLP analysis of a pair of NILs holding different *Aps-1* alleles, was made useless by the low quality

of cloned IV.23s/VII.23a-directed amplification products as a probe, which is possibly due to their small length and to the presence of mismatched G/C base pairs, incorporated in exchange for I/C pairs in the primer regions during molecular cloning. Finally, isolation of an *Aps-1*^{1} cDNA clone with the help of the 2.4 kb *Aps-1*^{1}-specific PCR product allowed the discrimination of the *Aps-1*^{1} target fragment through nucleotide sequencing of the region of the *Aps-1*^{1} gene which had served as the template for the amplification of the IV.23s/VII.23a-directed *Aps-1*^{1} target fragment (Chapter 5).

Williamson and Colwell [1991] recently reported the isolation of an *Aps-1*^{1} cDNA clone from a cDNA library. Among the approximately 350,000 plaques screened, a single *Aps-1*^{1} cDNA clone was identified. Taken together, these data provide strong indications for the low expression level of the *Aps-1*^{1} gene, which, in turn, could explain the low yield of purified APS-1^1 protein attained (chapter 2) . The results of the PCR using cDNA as a template were consistent with the results obtained in cDNA library screening. The poor amplification of *Aps-1*^{1} cDNA observed with primer IX.23s in combination with primers IV.23a and VII.23a in spite of the notorious amplifying power of the PCR presents a striking illustration of the extremely low concentration of the *Aps-1*^{1} mRNA in tomato suspension cells.

It is tempting to conclude from the comparison made in this chapter, that PCR is preferable whenever a gene is to be cloned on the basis of amino acid sequence information from the encoded protein, although we are aware of the limited value of an isolated example. Even when the isolation of a complete cDNA clone is the final objective, it may pay to prepare a PCR product first, as it allows the use of stringent hybridization conditions in subsequent library screening. When the available amino acid sequence information is sufficient for only a single primer, the PCR approach could still be pursued by using the 'anchored' variant [Frohman *et al.*, 1988; Loh *et al.*, 1989], in which a vector sequence or another 'anchor' sequence ligated to the template serves as the second primer.

Until now, the isolation of nucleotide sequences corresponding to amino acid sequence information by means of the PCR amplification technique has rather been the exception. Model studies showing the feasibility of the approach have been reported [Knoth *et al.*, 1988; Girgis *et al.*, 1988] and, in addition, a few examples of its successful application. Lee *et al.* [1988] were among the first to employ degenerate PCR primer pools corresponding to amino acid sequence information. Both primer pools they used were derived from the N-terminal amino acid sequence of porcine liver urate oxidase and consisted of 32 different oligonucleotide sequences only containing the four common bases. The target fragment was easily identified by Southern blot analysis of the reaction mixture using an internal oligonucleotide probe which incorporated the most frequently used codons. Degenerate primer pools on the basis of N-terminal amino acid sequence information have also been employed to produce by PCR a perfectly matching probe for the gene encoding the yeast transcription factor TFIID, allowing the subsequent cloning of the gene [Hahn *et al.*, 1989; Horikoshi *et al.*, 1989]. Furthermore, degenerate PCR

primer pools designed on the basis of common amino acid sequence motifs in the respective protein families have been applied in isolating nucleotide sequences from the feline herpesvirus thymidine kinase gene [Nunberg *et al.*, 1989], from hepadna virus reverse transcriptase genes [Mack and Sninsky, 1988], immunoglobulin variable region genes [Larrick *et al.*, 1989a; 1989b; Orlandi *et al.*, 1989] and protein tyrosine kinase genes [Wilks *et al.*, 1989].

The results presented in this chapter add to these literature reports in showing, that the route from protein to gene via PCR, although time-consuming and indirect, is one of great security, in particular when the gene involved, encodes a low abundant mRNA. The more so, as careful translation of amino acid sequence information into oligonucleotide pools can produce highly specific PCR primers, as illustrated by the success of the simplifying adjustments made to our complex *Aps-1*[1] primer pools.

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Chapter 5

Isolation of an *Aps-1*^{1} cDNA clone using a highly selective probe generated by PCR

Jac M.M.J.G. Aarts, Jan G.J.Hontelez, Henk J. van de Kamer, Inca C. Kusters,

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INTRODUCTION

In the previous chapter it was described how amino acid sequence information from the APS-1^1 protein was used to design PCR primers which specifically primed tomato DNA carrying the *Aps-1*^{1} allele and directed the amplification of a 2.4 kb genomic nucleotide sequence. This amplification product probably represented an *Aps-1*^{1} sequence as judged from its genetic map position. In this chapter, the nucleotide sequence of parts of the 2.4 kb fragment will be presented, which provides conclusive evidence that the 2.4 kb fragment originates from the *Aps-1*^{1} allele.

The 2.4 kb fragment was found to possess the expected selectivity as a probe in the screening of a cDNA library, allowing the isolation of an *Aps-1*^{1} cDNA clone and the subsequent determination of a partial *Aps-1*^{1} cDNA sequence. The amino acid sequence deduced from the nucleotide sequence of this *Aps-1*^{1} cDNA clone revealed a striking homology with a vegetative storage protein from soybean (*Glycine max*) [Mason *et al.*, 1988; Staswick, 1988; 1989a]. The co-amplification of target-related nucleotide sequences under the direction of the degenerate *Aps-1*^{1} primer pools IV.27s and VII.23a described in the previous chapter is re-examined in the light of the protein homology found.

MATERIALS AND METHODS

cDNA library screening

An amplified cDNA library in phage λgt11 from tomato roots of the nematode-resistant nearly isogenic line (*Aps-1*^{1}/*Aps-1*^{1}, *Mi/Mi*) was constructed as described in chapter 4. The library was plated on NZCYM-agar (10 g/l NZ-amine, 5 g/l NaCl, 5 g/l yeast extract, 1 g/l casamino acids, 2 g/l $MgSO_4 \cdot 7\ H_2O$, pH 7.5) at a density of 33,500 pfu per petri dish (diameter 17 cm) using *E. coli* Y1090 as a host. Plaques were grown for 6-8 hours at 37 °C, and replicas were made in triplicate on HATF filter membranes (Millipore). Phage DNA was released and bound to the membranes using standard procedures [Sambrook *et al.*, 1989].

The library was screened using as a probe the 2.4 kb PCR product that was amplified under the direction of degenerate *Aps-1*^{1} primer pools IX.23s and IV.23a. The probe DNA was labeled to a specific activity of 3×10^8 dpm/µg by random primer labeling [Feinberg and Vogelstein, 1984] using α-^{32}P-dATP.

Filters were prehybridized in 1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate for 2 hours at 65 °C, before 10-50 ng/ml of the ^{32}P-labeled probe DNA was added.

Hybridization was allowed to proceed overnight at 65 °C. Subsequently, the filters were once washed in 6 × SSC (0.9 M NaCl, 0.09 M sodium citrate pH 7.0) at the same temperature during 15 min. Autoradiography was performed for 2-8 days at -80 °C using Kodak Xomat AR-5 film and an intensifying screen.

Restriction mapping

Genomic DNA was isolated from leaves essentially according to Murray and Thompson [1980], but with omission of the final CsCl - EtBr centrifugation step. Restriction enzyme-digested DNA was prepared and analysed by agarose gel electrophoresis following standard methods as described in Sambrook *et al.* [1989].

Nucleotide sequence analysis

Nucleotide sequence analysis was performed as described in Chapter 4.

Database screening

The amino acid sequences encoded by the nucleotide sequences stored in the EMBL and GenBank nucleic acid databases were screened for homology with APS-1[1] amino acid sequences using the TFASTA program, which performs a search according to Pearson and Lipman [1988]. The program was run on a VAX computer using the VMS operating system.

RESULTS

Nucleotide sequence data showing the *Aps-1[1]* origin of the 2.4 kb amplification product

RFLP analysis had shown that the 2.4 kb amplification product, synthesized under the direction of primer pools IX.23s and IV.23a, met the genetic mapping requirements of an *Aps-1[1]* sequence (Chapter 4). Here, this indirect, genetic evidence is supplemented by an analysis of its nucleotide sequence.

First, the 2.4 kb PCR product (Chapter 4) was subcloned in pTZ18R. Seven subclones were partially sequenced (Fig. 1A), yielding the nucleotide sequence of the termini and of four internal regions of the 2.4 kb PCR product (Fig. 1B), together comprising about 61% of its

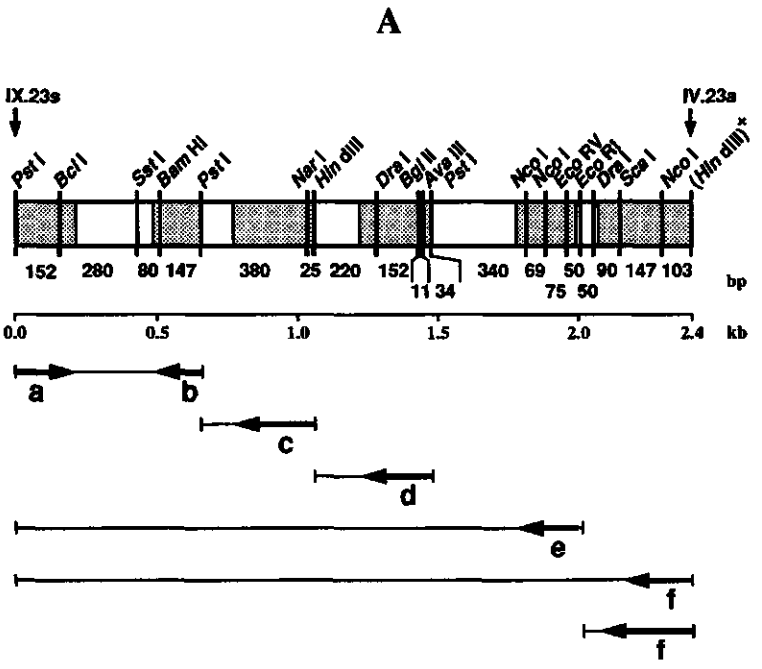
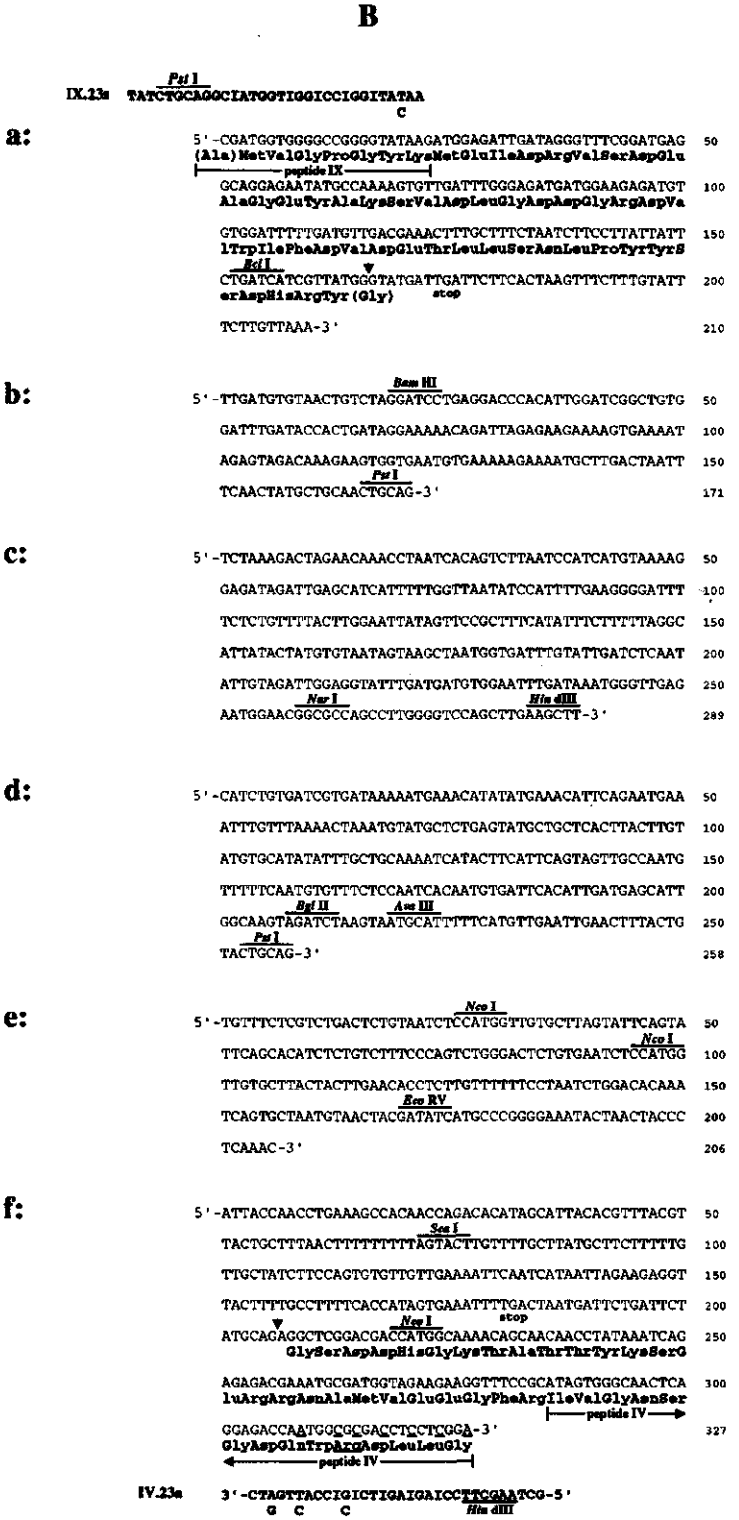


Fig. 1. Partial nucleotide sequence of the 2.4 kb genomic PCR product synthesized under the direction of degenerate *Aps-1*[1] primer pools IX.23s and IV.23a.

A) The physical map determined by restriction analysis and nucleotide sequencing of the PCR product. Subclones of the indicated restriction fragments were partially sequenced according to the arrows, yielding the nucleotide sequence of the shaded regions. + = *Hin*dIII site supposed to be contributed by primers IV.23a, but found to be absent. When originally present in the incorporated primer, then this site was apparently lost in the cloning procedure, as vector sequences followed after the 3'-terminal A residue of region f.

B) (opposite page) Nucleotide sequences determined for the regions corresponding to the letter codes. The deduced amino acid sequence in the exon regions at the termini is shown up to the putative exon/intron junctions (arrow heads) as judged from the currently operational consensus sequence [Lewin, 1990]. The primers used in the amplification reaction were aligned with their annealing site to show the positions corresponding to deoxyinosine in the primers, where a cytidyl residue was incorporated irrespective of the nucleotide present in the original *Aps-1*[1] template sequence. As a consequence, an Arg residue (underlined) was encoded in the region corresponding to primers IV.23a instead of the Ser residue known to be present in APS-1[1] peptide IV from the amino acid sequence data (Chapter 3). The underlined nucleotides are aberrant from the partial *Aps-1*[1] cDNA sequence represented by clone A in Fig. 2, and were apparently determined by the primer incorporated into the particular PCR product cloned and sequenced here.

B


IX.23a
Pst I
TATCTGCAGGCIATGGTIGGICCIGGITATAA
C
a:
5'-CGATGGTGGGGCCGGGGTATAAGATGGAGATTGATAGGGTTTCGGATGAG 50
(Ala)MetValGlyProGlyTyrLysMetGluIleAspArgValSerAspGlu
peptide IX
GCAGGAGAATATGCCAAAAGTGTTGATTTGGGAGATGATGGAAGAGATGT 100
AlaGlyGluTyrAlaLysSerValAspLeuGlyAspAspGlyArgAspVa
GTGGATTTTTGATGTTGACGAAACTTTGCTTTCTAATCTTCCTTATTATT 150
lTrpIlePheAspValAspGluThrLeuLeuSerAsnLeuProTyrTyrS
EcI I
CTGATCATCGTTATGGGTATGATTGATTCTTCACTAAGTTTCTTTGTATT 200
erAspHisArgTyr(Gly) stop
TCTTGTTAAA-3' 210
b:
Bam HI
5'-TTGATGTGTAACTGTCTAGGATCCTGAGGACCCACATTGGATCGGCTGTG 50
GATTTGATACCACTGATAGGAAAAACAGATTAGAGAAGAAAAGTGAAAAT 100
AGAGTAGACAAAGAAGTGGTGAATGTGAAAAAGAAAATGCTTGACTAATT 150
Pst I
TCAACTATGCTGCAACTGCAG-3' 171
c:
5'-TCTAAAGACTAGAACAAACCTAATCACAGTCTTAATCCATCATGTAAAAG 50
GAGATAGATTGAGCATCATTTTTGGTTAATATCCATTTTGAAGGGGATTT 100
TCTCTGTTTTACTTGGAATTATAGTTCCGCTTTCATATTTCTTTTTAGGC 150
ATTATACTATGTGTAATAGTAAGCTAATGGTGATTTGTATTGATCTCAAT 200
ATTGTAGATTGGAGGTATTTGATGATGTGGAATTTGATAAATGGGTTGAG 250
Nar I
Hin dIII
AATGGAACGGCGCCAGCCTTGGGGTCCAGCTTGAAGCTT-3' 289
d:
5'-CATCTGTGATCGTGATAAAAATGAAACATATATGAAACATTCAGAATGAA 50
ATTTGTTTAAAACTAAATGTATGCTCTGAGTATGCTGCTCACTTACTTGT 100
ATGTGCATATATTTGCTGCAAAATCATACTTCATTCAGTAGTTGCCAATG 150
TTTTTCAATGTGTTTCTCCAATCACAATGTGATTCACATTGATGAGCATT 200
Bgl II
Ase III
GGCAAGTAGATCTAAGTAATGCATTTTTCATGTTGAATTGAACTTTACTG 250
Pst I
TACTGCAG-3' 258
e:
Nco I
5'-TGTTTCTCGTCTGACTCTGTAATCTCCATGGTIGTGCTTAGTATTCAGTA 50
Nco I
TTCAGCACATCTCTGTCTTTCCCAGTCTGGGACTCTGTGAATCTCCATGG 100
TTGTGCTTACTACTTGAACACCTCTTGTTTTTTCCTAATCTGGACACAAA 150
Eco RV
TCAGTGCTAATGTAACTACGATATCATGCCCGGGGAAATACTAACTACCC 200
TCAAAC-3' 206
f:
5'-ATTACCAACCTGAAAGCCACAACCAGACACATAGCATTACACGTTTACGT 50
Sca I
TACTGCTTTAACTTTTTTTTTAGTACTTGTTTTGCTTATGCTTCTTTTTG 100
TTGCTATCTTCCAGTGTGTTGTTGAAAATTCAATCATAATTAGAAGAGGT 150
TACTTTTGCCTTTTCACCATAGTGAAATTTTGACTAATGATTCTGATTCT 200
stop
Nco I
ATGCAGAGGCTCGGACGACCATGGCAAAACAGCAACAACCTATAAATCAG 250
GlySerAspAspHisGlyLysThrAlaThrThrTyrLysSerG
AGAGACGAAATGCGATGGTAGAAGAAGGTTTCCGCATAGTGGGCAACTCA 300
luArgArgAsnAlaMetValGluGluGlyPheArgIleValGlyAsnSer
peptide IV
GGAGACCAATGGCGCGACCTCCTCGGA-3' 327
GlyAspGlnTrpArgAspLeuLeuGly
peptide IV
IV.23a
3'-CTAGTTACCIGICTIGAIGAICCTTCGGAATCG-5'
G C C
Hin dIII

total length. The data did not indicate any nucleotide sequence heterogeneity among the 2.4 kb amplified material, and confirmed in this respect the conclusion drawn from restriction analysis (not shown). The nucleotide sequence of the 2.4 kb fragment linked up with the primer IV.23a was found to encode (Fig. 1B) the successive amino acids of peptide IV not represented in the primer (COOH <-- Gly-Ser-Asn-Gly-Val-Ile --> NH_2). Similarly, the expected G was found (Fig. 1B) next to primer IX.23s, completing the codon for the C-terminal Lys residue of peptide IX. Taken together, these nucleotide sequence data are in accordance with the *Aps-1*^{1} origin of the 2.4 kb PCR product.

Integration of the nucleotide sequence and restriction analysis data from the amplified 2.4 kb *Aps-1*^{1} fragment produced the partial restriction map of the *Aps-1*^{1} gene shown in Fig. 1.

Isolation of an *Aps-1*^{1} cDNA clone

Subsequently, we used the 2.4 kb PCR product as a probe to screen a cDNA library. Among 315,000 clones tested, two positives were found. Nucleotide sequence analysis demonstrated that one of them, clone A in Fig.2, perfectly matched with the probe except for the IV.23a primer region. This clone apparently contained the C-terminal part of the *Aps-1*^{1} gene, as

Fig. 2 (opposite page). Nucleotide sequence of two cDNA clones related to the 2.4 kb PCR product amplified under the direction of degenerate *Aps-1*^{1} PCR primer pools IX.23s and IV.23a.

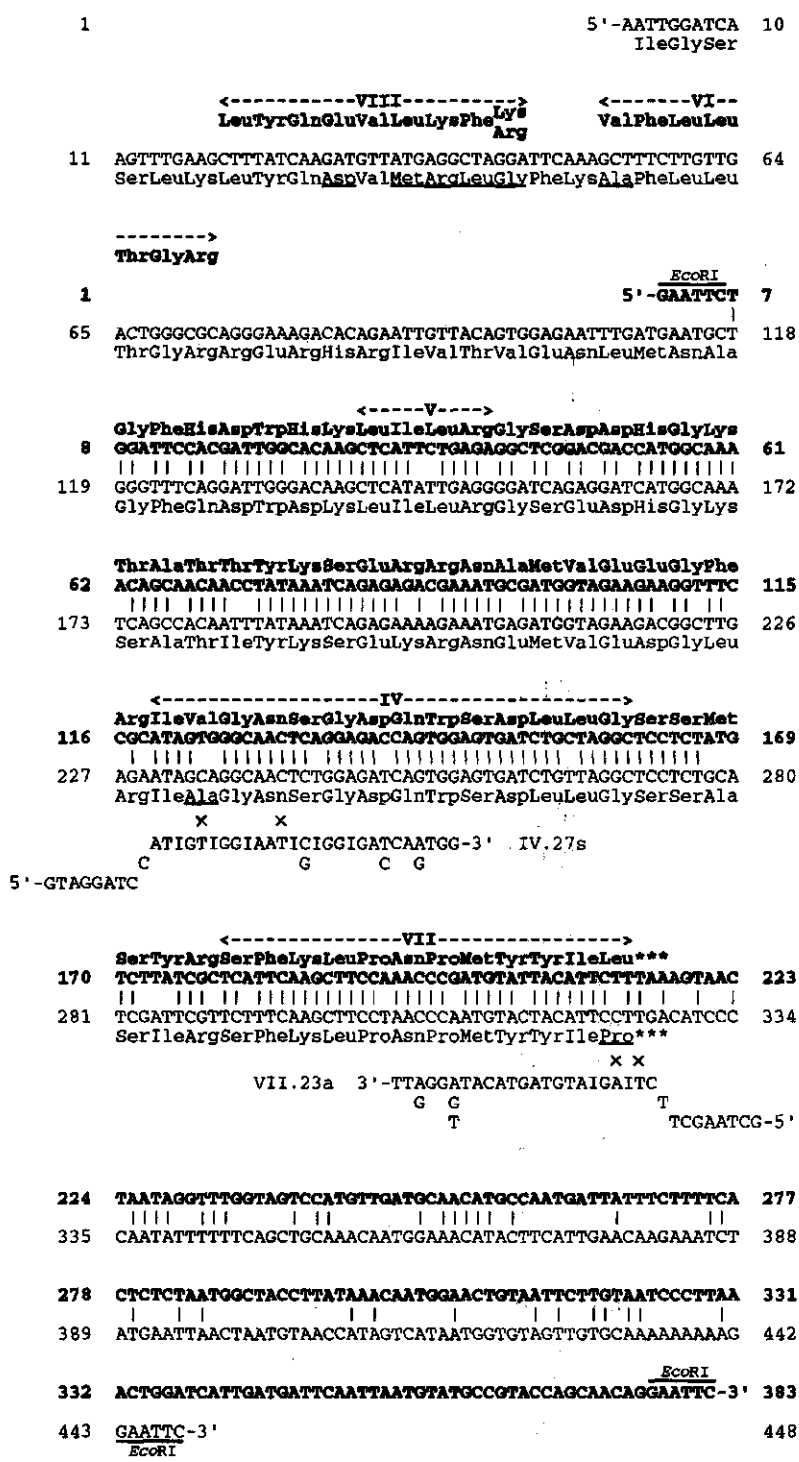
Clone A corresponds to the downstream exon region of the 2.4 kb PCR product and encodes the complete amino acid sequence of APS-1^1 peptides IV, V and VII, as previously determined (Chapter 3). This clone was therefore considered to represent an *Aps-1*^{1} cDNA clone.

Clone B apparently encodes a protein with extensive homology to the putative product of clone A. However, clone B is unlikely to represent an *Aps-1*^{1} cDNA clone, as the supposed protein product contains two amino acid residues (underlined) aberrant from the previously determined APS-1^1 amino acid sequences (Chapter 3), in the regions corresponding to either APS-1^1 peptide IV and VII.

The clones were aligned according to the position of the stop codon (***) in the reading frame. Identical nucleotides are connected by vertical lines to show the homology at the nucleic acid level (63.3% identical nucleotides within total overlap; 80.6% identity within the protein coding region). The homology between clone A and B apparently extended beyond the 5' end of clone A, as the additional nucleotide sequence present in clone B was found to encode amino acid sequences showing homology with APS-1^1 peptides VI and VIII at the indicated positions (aberrant amino acid residues encoded by clone B are underlined).

Primers IV.27s and VII.23a were aligned with their presumed annealing sites in clone B to show that they could readily prime the corresponding nucleotide sequences within tomato cDNA and genomic DNA (see text).

cDNA clone :


B : 1 5'-AATTGGATCA 10
IleGlySer
<----------VIII----------> <--------VI--
LeuTyrGlnGluValLeuLysPheLys Arg ValPheLeuLeu
B : 11 AGTTTGAAGCTTTATCAAGATGTTATGAGGCTAGGATTCAAAGCTTTCTTGTTG 64
SerLeuLysLeuTyrGlnAsnValMetArgLeuGlyPheLysAlaPheLeuLeu
--------> ThrGlyArg
EcoRI
A : 1 5'-GAATTCT 7
B : 65 ACTGGGCGCAGGGAAAGACACAGAATTGTTACAGTGGAGAATTTGATGAATGCT 118
ThrGlyArgArgGluArgHisArgIleValThrValGluAsnLeuMetAsnAla
<-----V----> GlyPheHisAspTrpHisLysLeuIleLeuArgGlySerAspAspHisGlyLys
8 GGATTCCACGATTGGCACAAGCTCATTCTGAGAGGCTCGGACGACCATGGCAAA 61
119 GGGTTTCAGGATTGGGACAAGCTCATATTGAGGGGATCAGAGGATCATGGCAAA 172
GlyPheGlnAspTrpAspLysLeuIleLeuArgGlySerGluAspHisGlyLys
ThrAlaThrThrTyrLysSerGluArgArgAsnAlaMetValGluGluGlyPhe
62 ACAGCAACAACCTATAAATCAGAGAGACGAAATGCGATGGTAGAAGAAGGTTTC 115
173 TCAGCCACAATTTATAAATCAGAGAAAAGAAATGAGATGGTAGAAGACGGCTTG 226
SerAlaThrIleTyrLysSerGluLysArgAsnGluMetValGluAspGlyLeu
<----------IV----------> ArgIleValGlyAsnSerGlyAspGlnTrpSerAspLeuLeuGlySerSerMet
116 CGCATAGTGGGCAACTCAGGAGACCAGTGGAGTGATCTGCTAGGCTCCTCTATG 169
227 AGAATAGCAGGCAACTCTGGAGATCAGTGGAGTGATCTGTTAGGCTCCTCTGCA 280
ArgIleAlaGlyAsnSerGlyAspGlnTrpSerAspLeuLeuGlySerSerAla
ATIGTIGGIAATICIGGIGATCAATGG-3' IV.27s
5'-GTAGGATC
<----------VII----------> SerTyrArgSerPheLysLeuProAsnProMetTyrTyrIleLeu***
170 TCTTATCGCTCATTCAAGCTTCCAAACCCGATGTATTACATTCTTTAAAGTAAC 223
281 TCGATTCGTTCTTTCAAGCTTCCTAACCCAATGTACTACATTCCTTGACATCCC 334
SerIleArgSerPheLysLeuProAsnProMetTyrTyrIlePro***
VII.23a 3'-TTAGGATACATGATGTAIGAITC
TCGAATCG-5'
224 TAATAGGTTTGGTAGTCCATGTTGATGCAACATGCCAATGATTATTTCTTTTCA 277
335 CAATATTTTTTCAGCTGCAAACAATGGAAACATACTTCATTGAACAAGAAATCT 388
278 CTCTCTAATGGCTACCTTATAAACAATGGAACTGTAATTCTTGTAATCCCTTAA 331
389 ATGAATTAACTAATGTAACCATAGTCATAATGGTGTAGTTGTGCAAAAAAAAAG 442
EcoRI
A : 332 ACTGGATCATTGATGATTCAATTAATGTATGCCGTACCAGCAACAGGAATTC-3' 383
B : 443 GAATTC-3' 448
EcoRI

APS-1^1 peptides IV (14 amino acids), V (4 amino acids) and VII (12 amino acids) (see Chapter 3) were found to be encoded within the reading frame shown in Fig. 2. Moreover, peptide VII, which turned out to represent the C-terminus of APS-1^1, and peptide IV were spaced within clone A in accordance with the difference in electrophoretic mobility between the corresponding cDNA-directed PCR products, indicating about 60 bp difference in length (see Chapter 4, Fig. 7C, lanes 1 and 2). Together, we consider these DNA sequencing results conclusive evidence as to the *Aps-1*^{1} origin of the 2.4 kb PCR product and the corresponding cDNA clone A.

Clone B, on the other hand, although obviously *Aps-1*^{1}-related (Fig. 2), is unlikely to represent an *Aps-1*^{1} nucleotide sequence, as it encoded 2 amino acid residues aberrant from the known amino acid sequences of APS-1^1 peptides IV and VII, whereas clone A revealed a perfect match. In addition, clone B encoded amino acid sequences homologous, but not identical to APS-1^1 peptides VI and VIII in the region extending beyond the overlap with clone A, which also argued against clone B representing *Aps-1*^{1}.

Amino acid sequence homology

Decoding of the nucleotide sequence of the isolated *Aps-1*^{1} cDNA clone revealed a total of 69 amino acid residues of the C-terminal part of the APS-1^1 polypeptide chain (Fig. 2). In the 2.4 kb amplified genomic *Aps-1*^{1} fragment, a stretch of 154 nucleotides following primer IX.23s has been determined ending with a stop codon, which indicated that an exon/intron junction had been passed, presumably at the position indicated by an arrowhead in Fig. 1. Decoding of this supposed *Aps-1*^{1} exon sequence yielded 48 residues of putative APS-1^1 amino acid sequence (Fig. 1) beside the 8 known residues of peptide IX. Three additional stretches of in total 24 residues were already known from direct amino acid sequence analysis of APS-1^1 peptides III, VI and VIII (Chapter 3).

Upon screening nucleic acid data bases (EMBL, GenBank) with these partial APS-1^1 amino acid sequences, some interesting homologies were found. For example, the peptide element Gly-Pro-Gly-Tyr-(Lys), which is part of the (overlapping) APS-1^1 peptide II and IX, occurs in alkaline phosphatases of rat [Misumi *et al.*, 1988; Thiede *et al.*, 1988], mouse [Terao and Mintz, 1987], bovine [Garattini *et al.*, 1987] and human [Henthorn *et al.*, 1986; Weiss *et al.*, 1986; Berger *et al.*, 1987; Kishi *et al.*, 1989; Watanabe *et al.*, 1989] origin and in the repressible acid phosphatase genes *PHO3* and *PHO5* from baker's yeast [Bajwa *et al.*, 1984]. Except for this conserved peptide element, no other significant homologies with (acid) phosphatases were found.

Surprisingly, however, a striking homology exists between tomato APS-1^1 and a putative vegetative storage protein (VSP) from soybean (*Glycine max*) [Mason *et al.*, 1988;

Staswick, 1988; 1989a], in particular within the regions comprising peptides IV and VII. Over the 69 C-terminal amino acid residues readable from the cDNA clone so far, the highest level of homology (54% identity) was found with the β-subunit of VSP (Fig. 3B). The tentative APS-1^1 amino acid sequence flanking peptide IX and deduced from the nucleotide sequence of the 2.4 kb PCR product was also aligned with VSP-β (Fig. 3A). By introduction of a gap of two residues in the VSP-β chain, a decreased but still significant level of homology (34% identity) was revealed in another region of the APS-1^1 amino acid chain, located more towards the N-terminus, suggesting that a considerable homology might be sustained throughout the APS-1^1 polypeptide chain.

Co-amplification of *Aps-1*^{1}-related nucleotide sequences under the direction of degenerate *Aps-1*^{1} oligonucleotide primer pools

As described in chapter 4, the *Aps-1*^{1} primer pair IV.27s/VII.23a directed the amplification of a set of variant, but related ±115 bp sequences from both genomic DNA and cDNA of tomato (Fig. 6 in Chapter 4). A comparison with the obtained partial *Aps-1*^{1} cDNA sequence finally allowed the *Aps-1*^{1} target sequence to be identified within the amplified 115 bp sequence family. Sequence 1 (Fig. 6 of Chapter 4) was found to exhibit complete accordance with the *Aps-1*^{1} cDNA clone (A in Fig. 2), whereas sequence 2 (the other remaining *Aps-1*^{1} candidate on the basis of colinearity with APS-1^1 amino acid sequence information) diverged from the *Aps-1*^{1} cDNA clone at the nucleotide and amino acid sequence level. Therefore, sequence 1 apparently represents the *Aps-1*^{1} target sequence.

The sequence heterogeneity of the 115 bp PCR product suggested the presence within the tomato genome of a diverse family of sequences related to *Aps-1*^{1}, which are all primed by the IV.27s/VII.23a oligonucleotides. Accordingly, the 2.4 kb *Aps-1*^{1} sequence recognized, in addition to *Aps-1*^{1} clone A, a different cDNA clone (B in Fig. 2) which appeared to correspond with anonymous sequence 2 of the 115 bp family (Fig. 6, Chapter 4). This suggests, that clone B is representative of the template which had actually directed the amplification of sequence 2. Accordingly, the nucleotide sequences found within clone B at the potential annealing sites for *Aps-1*^{1} primers IV.27s and VII.23a showed in fact, that no more than two mismatches (indicated by × in Fig. 2) in the 5' part of each primer had to be tolerated in the amplification of sequence 2, mismatches which are known [Sommer and Tautz, 1989] to be less interfering with the amplification reaction than those in the 3' part. The sequence data from clone B support the theory of a high level of homology among the *Aps-1*^{1}-related sequence family at the primer annealing sites explaining the co-amplification of non-target sequences under the direction of the IV.27s/VII.23a *Aps-1*^{1} primers.

Furthermore, the high level of homology between the C-terminal part of tomato APS-1^1 and soybean VSP, which is believed to play a role as a vegetative storage protein for temporary nitrogen storage [Mason *et al.*, 1988; Staswick, 1989b; 1989c; Mason and Mullet, 1990], suggested that one of the *Aps-1^1*-related PCR products might encode a tomato homologue of this soybean protein. However, alignment of the amino acid sequences possibly encoded by the co-amplified tomato sequences with the amino acid sequences of the VSP subunits, did not reveal any striking homologies in the relevant part between the primer regions. Moreover, the partial amino acid sequence (in the reading frame producing maximal homology) encoded by *Aps-1^1*-related cDNA clone B was found even slightly less homologous (51%) to VSP-β than the corresponding APS-1^1 amino acid sequence (54%).

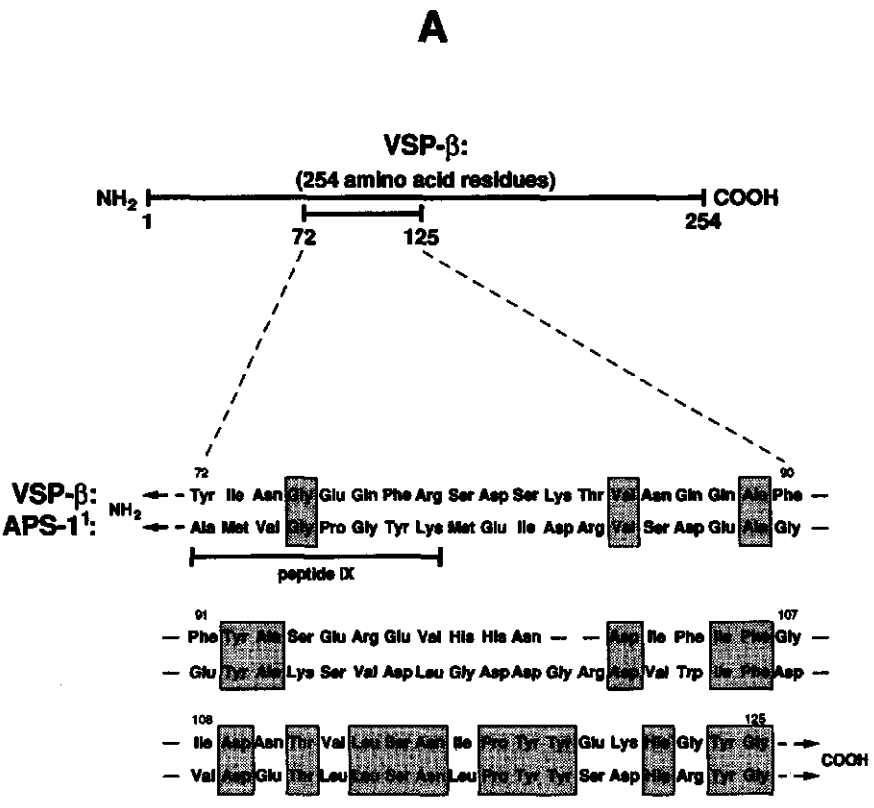
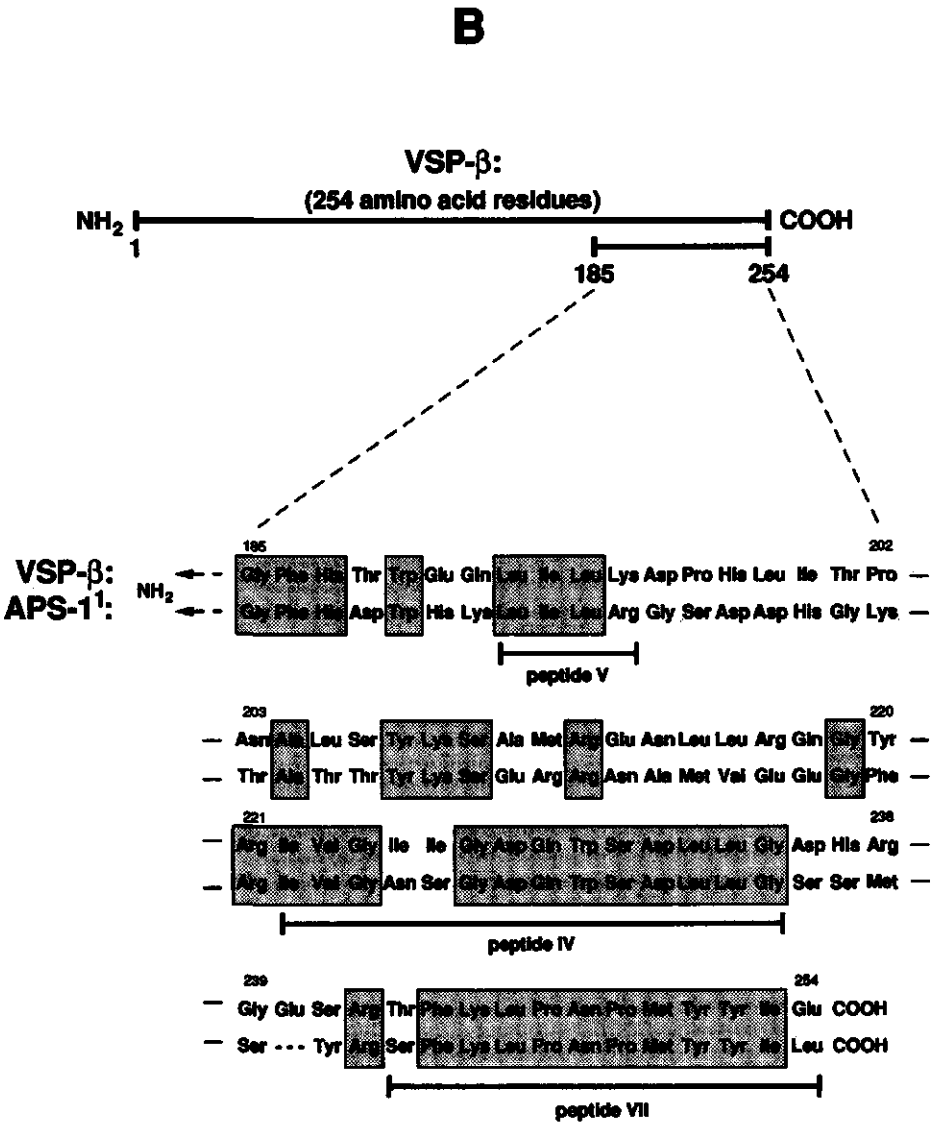


Fig. 3. Amino acid sequence homology between tomato APS-1^1 and soybean vegetative storage protein VSP-β. APS-1^1 and VSP-β amino acid sequences [Mason *et al.*, 1988; Staswick, 1988; 1989a] were aligned as accomplished by the TFASTA database search computer program [Pearson and Lipman, 1988]. The 56 residues of APS-1^1 amino acid sequence encoded by the upstream exon of the 2.4 kb genomic PCR product revealed 34% identity with amino acids 72-125 of the VSP-β polypeptide chain (panel A). The 69 C-terminal amino acids of APS-1^1 deduced from *Aps-1^1* cDNA (clone A, Fig. 2) showed an even greater homology with the C-terminus of VSP-β, comprising 54% identical residues between amino acids 185-254 of VSP-β (panel B, opposite page).

DISCUSSION

PCR under the direction of degenerate *Aps-1*[1]-targeted primer pools was found to amplify the synthesis of a 2.4 kb genomic DNA sequence (Chapter 4). Unlike the degenerate oligonucleotide pools corresponding to APS-1[1] amino acid sequence information, which performed very poorly in library screening (see Chapter 4), this amplification product served as a highly specific probe in the identifying an *Aps-1*[1] cDNA clone.



We believe that the sequence of the isolated cDNA clone (A in Fig. 2), encoding 29 amino acid residues colinear with the known APS-1^1 amino acid sequences, together with the circumstantial evidence given in the previous chapter fully confirm its identity as an *Aps-1^1* cDNA clone. Therefore, we conclude that our indirect approach, using PCR to go from protein to gene, was completely justified.

Tomato APS-1^1 exhibits a surprising amino acid sequence homology with soybean vegetative storage protein VSP [Mason *et al.*, 1988; Staswick, 1988; 1989a]. Whereas soybean VSP expression is regulated by many different factors [Staswick, 1990; Mason and Mullet, 1990], such as developmental stage, sink removal (depodding), wounding, nitrogen nutrition, water deficit and jasmonic acid, no regulatory factors of tomato APS-1^1 expression are known so far. Tomato APS-1^1 is a low abundant protein and the expression of APS-1^1 in tomato suspension cells was found to be independent from the concentration of inorganic phosphate and on that ground, one might argue that APS-1^1 is not obviously involved in the phosphate metabolism of tomato. Thus, tomato APS-1^1 may serve other functions, and therefore, a regulated increase of APS-1^1 expression for the purpose of nitrogen storage under as yet unknown conditions cannot be totally excluded. The more so, as the example of patatin, a vegetative storage protein from potato exhibiting lipid acyl hydrolase activity [Andrews *et al.*, 1988], indicates that vegetative storage proteins can possess enzymatic activity. Similarly, a much higher acid phosphatase activity has recently been found in extracts of soybean leaves after induction of VSP expression by depodding (Staswick, personal communication). On the surface, such analogies may suggest functional similarity of tomato APS-1^1 with soybean VSP, but, on the other hand, they may just as well represent only the relicts of the evolution from a common ancestor, which has resulted in quite different biological functions.

The co-amplification of *Aps-1^1*-related sequences which occurred during PCR under the direction of degenerate primer pools IV.23s and VII.23a indicated the existence of an *Aps-1^1*-related sequence family in the tomato genome. Accordingly, an *Aps-1^1*-related cDNA clone (B in Fig. 2) was found, which hybridized with the 2.4 kb genomic *Aps-1^1* amplification product and corresponded to one of the co-amplified *Aps-1^1* related sequences (number 2 in Fig. 6 of Chapter 4). However, neither the DNA fragments co-amplified in the IV.23s/VII.23a-directed PCR, nor the *Aps-1^1*-related cDNA clone B encoded an amino acid sequence that resembled the VSP amino acid sequence substantially more than APS-1^1. Therefore, none of these *Aps-1^1*-related sequences seems an obvious candidate to represent the homologous tomato (vegetative storage) protein.

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Chapter 6

General discussion

THE PURIFICATION OF TOMATO APS-1[1] IN RETROSPECT

With the final goal of isolating the root-knot nematode resistance gene *Mi* of tomato through a map-based cloning approach, nucleotide sequences from the tightly linked *Aps-1[1]* gene were cloned [this thesis; see also Aarts *et al.*, 1991]. As acid phosphatase proteins have been purified from a variety of organisms including many plant species [Felenbok, 1970; Uehara *et al.*, 1974a; Chen *et al.*, 1975; Ninomiya *et al.*, 1977; Shinshi and Kato, 1979; Fujimoto *et al.*, 1980; Kruzel and Morawiecka, 1982; Ferens and Morawiecka, 1985; Lorenc-Kubis, 1986; Park and Van Etten, 1986; Basboa *et al.*, 1987; Ching *et al.*, 1987; Hefler and Averill, 1987; Paul and Williamson, 1987; Goldstein *et al.*, 1988; Saluja *et al.*, 1989; Kaneko *et al.*, 1990], it was anticipated that the purification of the enzyme encoded by the *Aps-1[1]* allele from tomato would be rather trivial. However, the extreme low abundance of the APS-1[1] protein turned out to pose a serious problem. As a result, much more effort than expected was needed to obtain a sufficient amount of protein for microsequencing (Chapter 3) of the APS-1[1] amino acid chain.

Unlike lyophilisation, concentration of the purified APS-1[1] protein by ultrafiltration kept the protein soluble at moderate pH. Trypsin was found preferable to CNBr for generating APS-1[1] peptides (Chapter 3), as most of the CNBr cleavage products were lost during the subsequent HPLC purification step. Presumably, the majority of the cleavage products had become insoluble in the HPLC loading buffer as a result of irreversible denaturation during lyophilisation and exposure to formic acid.

Recently, several reports appeared which describe the purification of the APS-1[1] protein of tomato [Tanaka *et al.*, 1990; Aarts *et al.*, 1991; Erion *et al.*, 1991; Williamson and Colwell, 1991] and the subsequent cloning of *Aps-1[1]* nucleotide sequences [Williamson and Colwell, 1991; Erion *et al.*, 1992; Tanaka *et al.*, 1992]. These reports confirm our observation that APS-1[1] is a low abundant protein (0.005 to 0.01% of the total soluble protein in cv. VFNT suspension cells according to Erion *et al.* [1991]), although either of the new purification protocols described results in a substantially higher yield of APS-1[1] protein (up to 50 µg/l VFNT suspension cells [Williamson and Colwell, 1991]) than ours (about 0.3 µg/l L2-14 suspension cells). These authors have used extraction buffers both with [Williamson and Colwell, 1991] and without [Erion *et al.*, 1991; Tanaka *et al.*, 1990] added detergent, as well as widely varying chromatographic techniques, suggesting that there is no technical reason for the observed discrepancy in yield. On the other hand, all of these purification protocols take the cv. VFNT, either suspension cells [Williamson and Colwell, 1991; Erion *et al.*, 1991] or leaves [Tanaka *et al.*, 1990], as the source of APS-1[1] protein, whereas we have used L2-14 suspension cells and roots from the nematode resistant nearly isogenic line 83M, respectively. Since the only consistent divergence between our and their purification protocols is in the *L. esculentum* cultivar used, we believe that a genetically determined difference in the expression

level of APS-1^1 between the respective tomato cultivars is the most likely explanation for the higher yield attained with the VFNT cultivar.

The analysis of a complete *Aps-1^1* cDNA [Erion *et al.*, 1991; Williamson and Colwell, 1991] and genomic clone [Erion *et al.*, 1992] showed, that tomato APS-1^1 has 43% overall amino acid sequence homology with soybean VSP-β [Mason *et al.*, 1988; Staswick, 1988, 1989], that the *Aps-1^1* gene is comprised of three exons, like the soybean VSP gene, and that the intron/exon junctions occur at identical positions [Rapp *et al.*, 1990]. In contrast, the homology with other (acid) phosphatases is only minimal [Chapter 5; Williamson and Colwell, 1991]. Therefore, tomato APS-1^1 should be considered as a representative of a distinct class of plant acid phosphatases, in which soybean VSP would probably have to be included as well, since purified VSP from depodded soybean plants was recently found to exhibit acid phosphatase activity [Erion *et al.*, 1991]. Other remarkable differences with some plant phosphatases is the extremely low pH optimum of tomato APS-1^1 (pH 3.5 - 4.0 according to Paul and Williamson, [1987]), and the lack of inhibition by phosphate ions [Tanaka *et al.*, 1991] [compare Uehara *et al.*, 1974b; Shinshi and Kato, 1979; Mizuta and Suda, 1980; Ferens and Morawiecka, 1985; Giordani *et al.*, 1986]. In some other respects, however, tomato APS-1^1 [Chapter 2; Paul and Williamson, 1987] resembles various acid phosphatases from plant origin, such as in glycosylation [Shinshi and Kato, 1979; Fujimoto *et al.*, 1980; Kruzel and Morawiecka, 1982; Park and Van Etten, 1986; Ching *et al.*, 1987], (homo)dimer subunit structure [Uehara *et al.*, 1974a; Shinshi and Kato, 1979; Park and Van Etten, 1986; Basboa *et al.*, 1987; Lynn and Clevette-Radford, 1987] and relaxed substrate specificity [Uehara *et al.*, 1974b; Kruzel and Morawiecka, 1982; Ferens and Morawiecka, 1985; Park and Van Etten, 1986].

FROM PROTEIN TO GENE VIA THE POLYMERASE CHAIN REACTION. AN EVALUATION WITH REGARD TO TOMATO *Aps-1^1*.

The amino acid sequence information obtained from the APS-1^1 peptides was translated into degenerate oligonucleotide pools, which were applied as a probe in screening a cDNA library and, alternatively, as primers in the polymerase chain reaction (Chapter 4). After the design of the primer pools had been optimized, PCR readily allowed the amplification of a 2.4 kb genomic *Aps-1^1* fragment, whereas a serious attempt to isolate a cDNA clone from a library failed. In comparing the two approaches, we feel, that the route via PCR is preferable, since it allows subsequent library screening using the amplified target sequence as a highly selective

probe. As expected, our 2.4 kb genomic amplification product readily identified an *Aps-1*^{1} cDNA clone encoding the C-terminal 69 amino acid residues of APS-1^1 (Chapter 5).

Co-amplification of *Aps-1*^{1}-related nucleotide sequences under the direction of degenerate primer pools (IV.27s and VII.23a), indicated the presence within the tomato genome of a family of *Aps-1*^{1}-related nucleotide sequences, some of which were found to be transcribed (Chapter 5). Co-amplification of target-related nucleotide sequences might represent a problem that will often arise when using degenerate PCR primer pools. Quite stringent selection criteria may be required to enable the identification of the target fragment among the related by-products, as is illustrated by our preconceived selection criteria. These turned out insufficiently stringent, although they included, among others, the requirement of considerable nucleotide sequence colinearity with known APS-1^1 amino acid sequences. Even selection for the presence of 5 and 4 colinear codons adjacent to the respective PCR primers appeared to be insufficient to identify the *Aps-1*^{1} target fragment.

Maybe it is not merely a coincidence that the co-amplification of target-related sequences occurred with primers derived from the C-terminal region of APS-1^1, where the sequence homology with soybean VSP is particularly high, and did not occur in the amplification of the 2.4 kb fragment, involving a primer (IX.23s) corresponding to an APS-1^1 region of lower homology level [Williamson and Colwell, 1991]. Instead, this coincidence might indicate, that a tomato protein with extensive homology to soybean VSP is encoded by one or more of the co-amplified *Aps-1*^{1}-related nucleotide sequences, though possibly at a lower level as compared to the homology between APS-1^1 and VSP (Chapter 5).

Aps-1^1 AS THE GATEWAY TO *Mi*

Now that the tightly linked *Aps-1*^{1} allozyme marker has been turned into a starting point for chromosome walking towards the agronomically important root-knot nematode resistance gene *Mi*, it has become clear, which price in time and effort had to be paid for the molecular cloning of *Aps-1*^{1}. Therefore, an evaluation of the benefits of *Aps-1*^{1} as an entrance to *Mi* , in the light of the input needed, is possible now and seems appropriate, the more so as considerable progress has recently been made in mapping RFLP [Klein-Lankhorst *et al.*, 1991a; Messeguer *et al.*, 1991; Ho *et al.*, 1992] and RAPD [Klein-Lankhorst *et al.*, 1991b; Dr. V. M. Williamson, personal communication] markers in the *Mi* chromosomal region. In addition, the physical distance between *Aps-1*^{1} and *Mi* has to be reassessed in view of the suppression of

meiotic recombination apparently occurring in this chromosomal region [Loh *et al.*, 1987; Messeguer *et al.*, 1991; Ho *et al.*, 1992].

Medina-Filho [1980] reported the absence of any recombinant between *Aps-1* and *Mi* in a population of 513 plants segregating for the *Mi* and *Aps-1* loci. From these segregation data, the genetic distance between the *Aps-1* and *Mi* loci was calculated to be less than 0.894 cM. Given a C-value of 975 Mb for tomato [Arumuganathan and Earle, 1991] and a genetic map comprising 1,600 cM [Ganal *et al.* cited in Messeguer *et al.*, 1991], 1 cM corresponds, on an average, to approximately 600 kb in tomato. On the basis of this mean value, a physical distance between the *Aps-1* and *Mi* loci of less than 540 kb is estimated from their genetic distance. On the other hand, in studying mitotic recombination within the *Mi* chromosomal region in tissue culture, Loh *et al.* [1987] found as much as 15 recombinants (29%) between the *Aps-1* and *yv* loci among a population of 51 plants regenerated from tissue culture, suggesting that the low rate of recombination between *Aps-1*^{1} and *Mi* observed during meiosis is not in proportion to the physical distance between these loci. As a consequence, the physical distance between *Aps-1*^{1} and *Mi* would have been severely underestimated, the more so, since crossing-over occurs much less frequent during mitosis than during meiosis. Recently, Messeguer *et al.* [1991] compared the frequencies of recombination between a number of molecular and classical genetic markers around the *Mi* gene in a cross of a nematode-susceptible *L. esculentum* genotype with *L. pennellii*, and in a second cross with the nematode-resistant VFNT Cherry tomato cultivar, containing *L. peruvianum* DNA in the *Mi* chromosomal region. Recombination between *L. esculentum* DNA and *L. peruvianum* DNA in the *Mi* region was found to occur at least five times less than between *L. esculentum* and *L. pennellii* DNA of that region.

These results indicate that the physical distance between the *Aps-1* and *Mi* loci may turn out to be much larger than the several hundreds of kilobases expected, and if so, *Aps-1*^{1} might be too far away from *Mi* to serve as a practicable starting point for a chromosomal walk. Moreover, an RFLP marker, *LC379* [Ho *et al.*, 1992], and a RAPD marker, *Rex1* [Dr. V.M. Williamson, personal communication], have been mapped more close to *Mi* than *Aps-1*^{1} and would therefore offer starting points that seem preferable to *Aps-1*^{1}.

On the other hand, the exact position of *LC379* and *Rex1*, whether proximal or distal to *Mi*, is still unknown. This implies that *Aps-1*^{1} might still be important to determine the direction of a chromosomal walk, in particular, when both new markers map at the same side of *Mi*. In respect of the distance to *Mi*, *LC379* and *Rex1* may seem more suitable indeed as a starting point than *Aps-1*^{1}, yet not as such, since both of them represent a polymorphism which was found by Southern blot analysis to be part of a repetitive sequence [Ho *et al.*, 1992; Dr. V.M. Williamson, personal communication]. Therefore, single copy nucleotide sequences flanking these markers have to be isolated first, whereas *Aps-1*^{1}, as a single copy gene, is directly

applicable for starting a chromosomal walk. Anyhow, *Aps-1*^{1} will serve, at least, as an additional useful landmark in the *Mi* chromosomal region.

Taking a retrospective view, one could argue that the amount of effort needed to obtain amino acid sequence information from the APS-1^1 protein and to clone the *Aps-1*^{1} gene has been outweighed by the relative ease by which tightly linked RAPD and RFLP markers could be established in the *Mi* region [Klein-Lankhorst *et al.*, 1991a; 1991b; Messeguer *et al.*, 1991; Ho *et al.*, 1992]. At the onset of this research however, RFLP and PCR technology was just emerging, rendering the molecular cloning of the *Aps-1*^{1} gene more appropriate.

It is hard to predict on the basis of our present knowledge about the organization of the *Mi* chromosmal region, whether the distance from the more closely linked markers *LC379* or *Rex1* to *Mi* will be traversable by current long-range chromosome walking techniques, the more so as these markers and *Mi* might still be far more apart than the 540 kb originally estimated for the distance from *Aps-1*^{1} to *Mi* on the basis of the segregation data of Medina-Filho [1980]. In theory, these techniques can cover very large distances, although this may require a great amount of effort. They even have the potential to traverse the 2500 kb or more, which is the estimated distance between *Aps-1*^{1} and *Mi* according to the genetic data of Messeguer *et al.* [1991], and probably a more realistic approximation than that of Medina-Filho [1980]. But in practice, much will depend on the organization of repetitive nucleotide sequences in the *Mi* region. The availability of YAC clones comprising several hundred kilobases of tomato DNA will hopefully allow to cross chromosomal regions refractory to chromosome jumping. Recently, important progress was made towards a complete library of mainly large YAC clones, as Van Daelen *et al.* [personal communication] accomplished the construction of a partial tomato genomic library of YAC clones containing up to 700 kb of inserted DNA and 150 kb on average.

In summary, if these considerations imply, that *Aps-1*^{1} does not represent any more the main gateway to *Mi*, it still offers a useful landmark along the route to the *Mi* gene. Hence, the cloning of *Aps-1*^{1} nucleotide sequences described in this thesis might, eventually, contribute to the unraveling at the molecular level of a pathogen resistance response in the plant kingdom, a matter which is of great concern to agriculture, but so far only poorly understood.

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Samenvatting

SAMENVATTING

Het wortelstelsel van tomateplanten (*Lycopersicon esculentum* Mill.) kan worden aangetast door wortelknobbelaaltjes (*Meloidogyne* spp.). Dit zijn kleine wormvormige organismen (0,4 - 1,4 mm lang) welke in de bodem voorkomen en als parasieten leven op de wortels van vatbare planten. Infectie met wortelknobbelaaltjes gaat gepaard met de vorming van karakteristieke knobbeltjes op het worteloppervlak en belemmert het functioneren van het wortelstelsel. De opname van water en mineralen wordt verstoord, waardoor de planten kleiner blijven, sneller verwelken, vatbaarder worden voor infectie met andere pathogenen en minder opbrengst geven. Resistentie tegen wortelknobbelaaltjes is aanwezig in de wilde tomatesoort *Lycopersicon peruvianum* (L.) Mill. en werd in de cultuurtomaat ingekruist.

De resistentie tegen wortelknobbelaaltjes in wilde en cultuurtomaten berust op de aanwezigheid van een enkel dominant gen, gelegen op chromosoom 6 en aangeduid met het symbool *Mi*. Het *Mi* gen blijkt genetisch nauw gekoppeld te zijn (0.89 cM) aan het *Aps-1*^{1} allel van het zure fosfatase-1 gen. De schattingen van het aantal bp (baseparen, de "bouwstenen" van het DNA) gelegen tussen *Aps-1*^{1} en *Mi* lopen uiteen van minder dan $5{,}5 \times 10^{5}$ tot $2{,}5 \times 10^{6}$ bp (Dit is minder dan 0,26% van de totale hoeveelheid DNA per tomategenoom, die $9{,}75 \times 10^{8}$ bp bedraagt). Een dergelijke afstand ligt binnen het bereik van recentelijk ontwikkelde technieken voor het isoleren van genen door middel van lange-afstands "chromosome walking". Hierbij worden vanuit een nauw gekoppeld, goed gedefinieerd startpunt (in dit geval het *Aps-1*^{1} gen) opeenvolgende stukken DNA gekloneerd, totdat, zo "lopend" langs het chromosomale DNA, ook DNA-klonen met daarop het *Mi* gen zijn verkregen. Wanneer het *Mi* gen eenmaal is gekloneerd, kunnen op gemakkelijke wijze grote aantallen kopieën van het DNA van dit gen worden verkregen en kan voldoende *Mi* DNA worden geproduceerd om de werking van het gen te kunnen bestuderen.

In het kader van het isoleren van *Mi*-DNA via "chromosome walking" werd een *Aps-1*^{1} kloon geïsoleerd via het *Aps-1*^{1} genprodukt (het APS-1^{1} eiwit). Het *Aps-1*^{1} genprodukt werd gezuiverd uit de wortels van op watercultuur gekweekte tomateplanten en ook uit *in vitro* als suspensie gekweekte cellen van tomaat. Hierbij werd gebruik gemaakt van conventionele lage-druk kolomchromatografische technieken. De vierde zuiveringsstap, concanavaline A (Con A)-Sepharose-kolomchromatografie, bleek bijzonder effectief en resulteerde in een vrijwel zuiver APS-1^{1} preparaat. Tijdens deze stap raakte het APS-1^{1} preparaat weliswaar verontreinigd met Con A, dat in kleine hoeveelheden losliet van de kolom, maar in de daaropvolgende en laatste Mono Q-FPLC stap werd de verontreiniging met Con A weer verwijderd. De beschreven zuiveringsprocedure resulteerde in een APS-1^{1} preparaat dat zich bij polyacrylamidegel-electroforese (PAGE) homogeen gedroeg, zowel onder denaturerende als niet-denaturerende condities. Bovendien bleek het gezuiverde eiwit co-elutie en co-electroforese te vertonen met de

APS-1^1 enzymactiviteit, wanneer het werd onderworpen aan respectievelijk gelfiltratie en polyacrylamidegel-electroforese onder niet-denaturerende omstandigheden. De opbrengst van de zuiveringsprocedure bedroeg slechts enkele microgrammen APS-1^1 per kg wortels of suspensiecellen. De oorzaak van deze lage opbrengst moet worden gezocht in het lage expressieniveau van het *Aps-1*^{1} gen, aangezien bij geen van de zuiveringsstappen aanzienlijke verliezen aan APS-1^1 activiteit werden waargenomen.

Het gezuiverde APS-1^1 eiwitpreparaat werd gesplitst in peptiden door behandeling met cyanogeenbromide (CNBr) of met trypsine. Na zuivering van de APS-1^1 peptiden met behulp van "high performance liquid chromatography" (HPLC) werd van twee CNBr peptiden en van zeven tryptische peptiden de aminozuurvolgorde geanalyseerd, waardoor in totaal 61 aminozuren van de APS-1^1 polypeptideketen konden worden bepaald.

Twee APS-1^1 peptiden van respectievelijk 8 en 14 aminozuren bevatten de meest geëigende aminozuursequentie-informatie voor de synthese van corresponderende oligonucleotidenmengsels voor toepassing als "primer" in de "polymerase chain reaction" (PCR). De PCR is een techniek waarmee een segment ("target fragment") van een bepaald stuk DNA enkele miljoenen malen gekopieerd ("geamplificeerd") kan worden met behulp van twee korte enkelstrengs DNA-fragmentjes (de zogeheten "primers") welke overeenkomen met sequenties aan de respectievelijke uiteinden van het te vermeerderen DNA-segment.

Wanneer PCR-primers worden gebaseerd op de aminozuurvolgorde gecodeerd door het te amplificeren DNA-segment, dan moet bij het ontwerpen van de primers rekening worden gehouden met de degeneratie van de genetische code. In het algemeen zal om die reden met mengsels van PCR-primers worden gewerkt. Het bleek echter van doorslaggevend belang voor het effectief laten verlopen van de PCR reactie om het aantal verschillende nucleotidensequenties in een primer-mengsel beperkt te houden. Dit werd bereikt door op gedegenereerde posities in het 5'-gedeelte van de primers niet alle theoretisch mogelijke nucleotiden in te bouwen, maar of alleen het meest waarschijnlijke nucleotide, of desoxyinosinemonofosfaat (dIMP). Tevens werd een efficiënte verlenging gewaarborgd door elke mogelijke combinatie voor de drie 3'-terminale codons in het mengsel van primers op te nemen.

Met behulp van aldus ontworpen primer-mengsels, gebaseerd op de aminozuurvolgorde van bovengenoemde APS-1^1 peptiden, kon in een PCR-reactie met genomisch DNA als matrijs een DNA-fragment met een lengte van 2.4 kilobasenparen (kb) worden geamplificeerd. Dit 2.4 kb PCR-produkt werd alleen geproduceerd, indien DNA waarop het *Aps-1*^{1} allel voorkomt, als matrijs werd gebruikt. Als de matrijs het *Aps-1*^{3} of *Aps-1*$^+$ allel bevatte werd geen enkel produkt gevormd. Bovendien detecteerde het 2.4 kb PCR-produkt restrictiefragment-lengtepolymorfismen (RFLPs) tussen twee "nearly isogenic lines" (NILs) van tomaat die alleen verschillen in het *Aps-1*^{1}*/Mi* gebied van chromosoom 6. Het gegeven dat het 2.4 kb produkt alleen werd gevormd indien een matrijs werd gebruikt die het *Aps-1* allel bevatte waarvan de

primers waren afgeleid, alsmede de genetische positie op chromosoom 6, geven aan, dat het bij dit produkt hoogstwaarschijnlijk gaat om een *Aps-1*[1] nucleotidensequentie. Wanneer dezelfde primer-mengsels werden gebruikt in een PCR reactie met cDNA als matrijs werd een 490 bp *Aps-1*[1] fragment geamplificeerd. Er werd een overlappende cDNA sequentie van 550 bp gevonden, wanneer in combinatie met dezelfde "upstream primers" "downstream primers" werden gebruikt die correspondeerden met het C-terminale tryptische peptide van het APS-1[1] eiwit. De hoeveelheid die in 30 ronden PCR van het 490 bp en het 550 bp cDNA fragment werd geproduceerd was zo gering, dat deze alleen kon worden gedetecteerd door middel van Southern blot hybridisatie met het 2.4 kb genomische PCR-produkt als "probe". Dit geeft aan dat er slechts een geringe hoeveelheid mRNA voor APS-1[1] aanwezig was en vormt opnieuw een aanwijzing voor het lage niveau waarop het *Aps-1*[1] gen tot expressie komt.

Naast de 2.4 kb *Aps-1*[1] sequentie werd met behulp van twee andere primer-mengsels, zowel met genomisch DNA als met cDNA als matrijs, ook een ±115 bp produkt verkregen. Dit tweede PCR-produkt bleek meerdere verwante nucleotidensequenties van nagenoeg gelijke lengte te bevatten. De genetische positie van deze nucleotidensequenties kon niet worden vastgesteld, doordat de klonen van deze sequenties slecht bleken te functioneren als "probe".

Uit een cDNA-bank van tomaat, welke klonen van de eiwit coderende nucleotidensequenties bevat, werden aan het 2.4 kb PCR produkt homologe cDNA-klonen geïsoleerd. De geamplificeerde 2.4 kb *Aps-1*[1] sequentie werd daartoe radio-actief gemerkt en vervolgens konden via moleculaire hybridisatie met deze "probe" de *Aps-1*[1], en aan *Aps-1*[1] verwante cDNA-klonen in de bank worden geïdentificeerd.

Er werden twee cDNA-klonen met verwante nucleotidensequenties geïsoleerd. Een van beide was kennelijk een *Aps-1* cDNA-kloon, aangezien in de nucleotidenvolgorde van deze kloon sequenties coderend voor drie APS-1[1] peptiden (peptide IV, VI en VII, te zamen 30 aminozuren) werden aangetroffen. Deze *Aps-1*[1] cDNA-kloon codeerde voor 69 aminozuren van de APS-1[1] polypeptideketen inclusief de C-terminus, welke overeen bleek te komen met tryptisch peptide VII.

Een ander deel van de APS-1[1] aminozuursequentie werd afgeleid uit de nucleotidensequentie volgend op de "upstream primer" in het 2.4 kb genomische *Aps-1*[1] amplificatieprodukt. In dit gedeelte van de APS-1[1] polypeptideketen werden in totaal 56 aminozuren opgehelderd inclusief peptide IX.

De APS-1[1] aminozuurvolgorde voor zover die door ons werd bepaald, vertoonde geen significante homologie met andere (zure) fosfatases in de GenBank en EMBL databanken. Wel werd een opvallende aminozuursequentiehomologie gevonden met een vegetatief opslageiwit ("vegetative storage protein"), VSP-β, van sojaboon (*Glycine max* [L.] Merr.), dat zich ophoopt in de bladeren na het verwijderen van de peulen.

De detectie van een aan *Aps-1*[1] verwante cDNA-kloon door de 2.4 kb genomische *Aps-1*[1] probe alsmede de heterogeniteit in nucleotidensequentie binnen het 115 bp PCR product

vormen aanwijzingen voor het bestaan van een familie van aan *Aps-1*^{1} verwante nucleotidensequenties binnen het tomategenoom. Een van de vier gevonden nucleotidensequenties die deel uitmaakte van het 115 bp PCR produkt, bleek identiek te zijn aan 115 bp van de *Aps-1*^{1} sequentie. Geen van de overige drie sequenties, noch de aan *Aps-1*^{1} verwante cDNA-kloon, vertoonde op aminozuurniveau meer homologie met het VSP-β van sojaboon dan met APS-1^{1} van tomaat. Daarom is het onwaarschijnlijk, dat een van deze sequenties het homoloog van VSP-β in tomaat representeert.

De beschreven procedure voor het isoleren van een *Aps-1*^{1} cDNA-kloon illustreert, dat het mogelijk is om gedegenereerde PCR-primer-mengsels te ontwerpen met een hoge graad van specificiteit. Wanneer een gen wordt gekloneerd via het gecodeerde eiwit lijkt het derhalve raadzaam om eerst te proberen of via PCR een kwalitatief hoogwaardige "probe" kan worden gegenereerd, alvorens men zijn toevlucht neemt tot het directe gebruik van deze minder selectieve oligonucleotidenmengsels als "probe".

De *Aps-1*^{1} cDNA-kloon, beschreven in dit proefschrift, vormt een belangrijk oriëntatiepunt bij "chromosome walking" naar het *Mi* locus van tomaat en kan daarmee bijdragen aan het kloneren van een agronomisch belangrijk nematodenresistentiegen.

Nawoord

Promotie-onderzoek doen is zo nu en dan wel een eenzame, maar zeker geen eenmans-aangelegenheid, ook al vermeldt de titelpagina van een proefschrift gewoonlijk slechts één auteur. Velen hebben mij bij het tot stand brengen van dit proefschrift ondersteund. Allereerst wil ik mijn ouders noemen, die mij hebben grootgebracht en hun belangstelling voor biologie en natuurwetenschappen overtuigend op mij wisten over te dragen. Hen wil ik op deze plaats, evenals mijn zus, hartelijk bedanken voor hun steun in velerlei opzicht en voor hun interesse in hetgeen ik allemaal aan het doen was.

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Curriculum vitae

Jacobus Martinus Maria Joseph Gertrudis Aarts werd geboren te Horst op 11 november 1958. In 1977 behaalde hij het diploma gymnasium-β aan het St.-Thomascollege te Venlo. Aansluitend studeerde hij planteziektenkunde (N-14) aan de toenmalige Landbouwhogeschool te Wageningen. De propaedeuse werd behaald in 1978 en het kandidaatsexamen in 1982. De ingenieursstudie omvatte een verzwaard hoofdvak virologie (prof. dr. ir. J.P.H. van der Want), een hoofdvak moleculaire biologie (prof. dr. A. van Kammen), en een bijvak fytopathologie (prof. dr. ir. J. Dekker). De praktijktijd buiten de Landbouwhogeschool werd doorgebracht bij het Nederlands Kanker Instituut (Antonie van Leeuwenhoekhuis) te Amsterdam (prof. dr. P. Borst). In 1986 behaalde hij het ingenieursdiploma en werd hij als promotie-assistent aangesteld bij de vakgroep Moleculaire Biologie van de Landbouwhogeschool. De resultaten van dit promotie-onderzoek zijn beschreven in dit proefschrift. Sinds januari 1992 is hij als post-doctoraal medewerker verbonden aan de vakgroep Toxicologie van de Landbouwuniversiteit.