Positional Cloning of a Gene for Nematode Resistance in Sugar Beet

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The *Hs1*$^{pro-1}$ locus confers resistance to the beet cyst nematode (*Heterodera schachtii* Schmidt), a major pest in the cultivation of sugar beet (*Beta vulgaris* L.). The *Hs1*$^{pro-1}$ gene was cloned with the use of genome-specific satellite markers and chromosomal breakpoint analysis. Expression of the corresponding complementary DNA in a susceptible sugar beet conferred resistance to infection with the beet cyst nematode. The native *Hs1*$^{pro-1}$ gene, expressed in roots, encodes a 282–amino acid protein with imperfect leucine-rich repeats and a putative membrane-spanning segment, features similar to those of disease resistance genes previously cloned from higher plants.

Crops are attacked by viruses, bacteria, fungi, and nematodes as pathogens. Genes involved in the resistance to viruses, bacteria, and fungi have been identified initiating the elucidation of the respective resistance pathways (*1*). However, the mechanisms conferring resistance to parasitic nematodes have remained obscure. Nematodes cause an estimated loss of ~$100 billion per year in agriculture. Economically most important are sedentary nematodes of the genera *Meloidogyne*, *Heterodera*, and *Globodera*, which become permanently fixed in the host roots after inducing specific feeding structures. The beet cyst nematode (BCN) *Heterodera schachtii* Schmidt has a broad host range that includes many species from different plant families such as *Chenopodiaceae* and *Brassicaceae*. It is a severe pest in sugar beet (*Beta vulgaris* L.) cultivation. Typical symptoms are wilted leaves and a whiskered appearance of the roots. The nematode's life cycle is divided into four juvenile stages (J1 through J4). After death of the females, cysts are formed in which the infective J2 juveniles, retained within the eggshells, can survive in the soil for up to 10 years. Because the application of nematicides is restricted on account of environmental concerns, the current measures to control BCN and other sedentary nematodes include crop rotations or growing catch crops (such as nematode-resistant *Raphanus sativus* var. *oleiformis*). However, the most favored strategy is the breeding of resistant cultivars.

Genes that confer resistance to nematodes are lacking in cultivated *Beta* species. The only sources of resistance are the wild species *B. procumbens* and related species *B. webbiana* and *B. patellaris* (*2*). Plants carrying the nematode resistance gene *Hs1*$^{pro-1}$ on chromosome 1 of *B. procumbens* display an incompatible reaction between host and parasite (*2*). The roots of plants carrying this gene are invaded by J2 juveniles, but most of the nematodes die in the late J2 stage because of degradation of the initiated feeding structure (syncytium). In rare cases, females develop, albeit abnormally, displaying a transparent appearance because of the absence of eggs. Thus, the nematodes cannot complete their life cycle.

The *Hs1*$^{pro-1}$ resistance gene has been transferred to sugar beet by species hybridization and backcrossing (*3*). The beets exhibiting complete resistance could be classified as follows: (i) monosomic addition lines ($2n = 19$) carrying the entire wild beet chromosome 1, (ii) fragment addition lines ($2n = 19$) with a fragmented wild beet chromosome, and (iii) a euploid ($2n = 18$) line carrying a translocation from the wild beet *B. procumbens* encompassing the *Hs1*$^{pro-1}$ locus at the end of linkage group IV (*4*). All these lines exhibit resistance to infection, although they suffer from reduced resistance transmission rates and poor agronomical performance, making molecular cloning of the *Hs1*$^{pro-1}$ gene an important target. Because of the absence of chromosome pairing between sugar beet and wild beet chromosomes, the translocations are inherited as a whole. No recombination between sugar beet chromosomes and the wild beet translocations has been observed in meiotic studies or through molecular marker analysis (*5*). Therefore, our strategy for cloning *Hs1*$^{pro-1}$ differed from commonly used map-based cloning approaches. We relied on mapping chromosomal breakage points rather than on recombination mapping.

For cloning the *Hs1*$^{pro-1}$ gene, closely linked marker loci have been identified (*5*). The *B. procumbens*–specific satellite pRK643 sequence proved to be helpful in identifying the translocation line with the smallest wild beet segment (number A906001) among a panel of chromosomal mutants (Fig. 1A) (*5*). This line was chosen for the positional cloning of the gene. The marker pRK643 cosegregated perfectly with resistance in a segregating F_2 population of 241 individuals. Using this satellite marker as a probe, we extracted three clones, span-

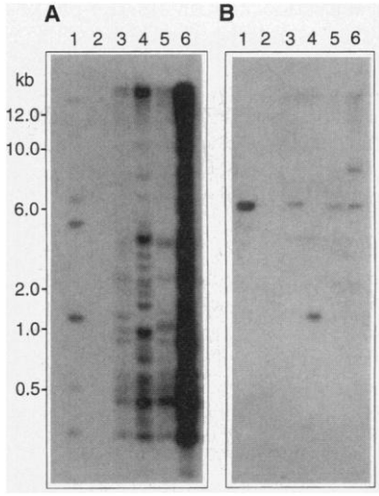

Fig. 1. DNA blot analysis of susceptible and resistant beets probed with the wild beet–specific satellite marker pRK643 (**A**) and the cDNA clone 1832 (**B**). For (A) and (B), the blots are: lane 1, resistant translocation line A906001; lane 2, susceptible sugar beet line 93161p; lane 3, *B. procumbens* chromosome 1 monosomic addition line; lane 4, *B. patellaris* chromosome 1 monosomic addition line; lane 5, *B. webbiana* chromosome 1 monosomic addition line; and lane 6, *B. procumbens*. Plants were tested for nematode resistance in the greenhouse (*20*). Genomic DNA (10 μg) was restricted with Hind III and electrophoretically separated on a 0.75% agarose gel, blotted onto Hybond N$^+$ membrane (Amersham), hybridized with [^{32}P]deoxycytidine 5′-triphosphate–labeled probes (*21*), and washed with high stringency at 65°C in 0.2× standard saline citrate.

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ning the $Hs1^{pro-1}$ locus, from a yeast artificial chromosome (YAC) library of line A906001 (6).

To identify transcribed sequences from the YACs, a cDNA library made from roots of A906001 plants infected with the nematodes was screened with the three YACs, resulting in the isolation of three cDNA clones, numbers 1832, 1845, and 1859 (7). Clone 1845 crosshybridized with DNA of susceptible sugar beet, and clone 1859 gave multiple banding patterns with DNA of both susceptible and resistant beet. Therefore, we subsequently concentrated on cDNA 1832 for several reasons: (i) This cDNA gave a single-copy signal exclusively with DNA of resistant lines; no signal was visible with DNA from susceptible sugar beet, which suggested that this gene is not present in cultivated beets. All monosomic addition lines carrying the $Hs1^{pro-1}$ gene gave a signal with this probe (Fig. 1B). (ii) It cosegregated with the resistance character in F_2 populations. (iii) A 1.6-kb transcript was present mainly in roots of resistant plants as revealed by Northern (RNA) blot analysis. A stronger hybridization signal was found with RNA of roots infected with *H. schachtii* compared with RNA from noninfected roots (Fig. 2). (iv) Sequence analysis of the predicted polypeptide revealed motifs common to resistance gene products recently cloned. In summary, clone 1832 represents a wild beet gene that is expressed mainly in roots and enhanced upon nematode infection.

The hairy root cultures obtained by induction with *Agrobacterium rhizogenes* were used for genetic complementation analysis. The compatible reaction of susceptible roots, as well as the incompatible reaction of resistant roots to cyst nematodes, is maintained in hairy root cultures of sugar beet (8). A susceptible sugar beet line (number 93161p) was transformed with the 1450–base pair cDNA 1832. After inoculation with J2 juveniles, six independently transformed roots expressing the 1832 gene exhibited the same incompatible reaction as the resistant line A906001, whereas nematodes developed regularly on susceptible controls and on hairy roots lacking the gene (Fig. 3, A and B). Susceptibility was restored after transformation of one resistant root culture (number 100B) with an antisense construct of cDNA 1832 (Fig. 3B). These experimental data confirm that the resistance in hairy roots from line 93161p results from the expression of the 1832 gene. The isolated gene is referred to as the $Hs1^{pro-1}$ gene, because it confers nematode resistance to the susceptible sugar beet line in a way that is indistinguishable from resistance in line A906001.

Sequencing the full-length cDNA and the corresponding genomic clone revealed an intronless open reading frame of 846 base pairs encoding a predicted gene product of 282 amino acids, which is in agreement with the data obtained from RNA blots (9). No sequences with homology to $Hs1^{pro-1}$ were found in different protein and nucleotide databases, except for three expressed sequence tags (ESTs) from the *Arabidopsis thaliana* database with unknown function (10).

The amino acid sequence of the predicted polypeptide can be dissected into different subdomains (Fig. 4). A putative signal peptide (domain A) could be defined at the NH_2-terminus (11), which may be engaged in targeting the protein to the cytoplasmic membrane. A leucine-rich region (domain C) arranged in imperfect leucine-rich repeats (LRRs) is obvious from the NH_2-terminus of the $Hs1^{pro-1}$ polypeptide. LRRs take part in protein-protein interaction and have been found in previously cloned resistance genes from plants, for instance the *RPS2* gene from *A. thaliana* (12). Their function varies from putative recognition sites in receptor-like molecules that are located extracellularly, to catalytic domains of enzymes being active in the cytoplasm (13). Similar to other LRRs identified in plant resistance genes, the LRRs of the $Hs1^{pro-1}$ polypeptide are not well conserved compared with the consensus sequence of the LRR consensus superfamily (13). The LRRs of the $Hs1^{pro-1}$ polypeptide are characterized by a 20–amino acid consensus motif (XLXXaXXaXLXXLXXaXXXL, with L = leucine or isoleucine, a = aliphatic or aromatic amino acids, and X = any amino acid). Leucines and aliphatic residues at positions 2, 5, and 16 are located at the same position as in the consensus of the LRR superfamily. The highly conserved asparagine (N) at position 10 (13) is substituted by a leucine or isoleucine. This asparagine is also lacking from the consensus LRR of the *RPS2* polypeptide. The hydro-

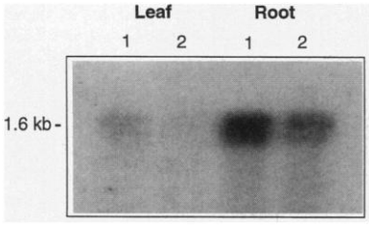

Fig. 2. RNA blot analysis of the $Hs1^{pro-1}$ gene in the resistant line A906001. Total RNA was isolated from leaves and roots of 6-week-old plants either infected (lane 1) or not infected (lane 2) with *H. schachtii* juveniles 2 weeks after germination. The cDNA clone 1832 was used as a probe. Total RNA (20 μg) was separated on a 1.3% agarose-formaldehyde gel and blotted on a nylon membrane.

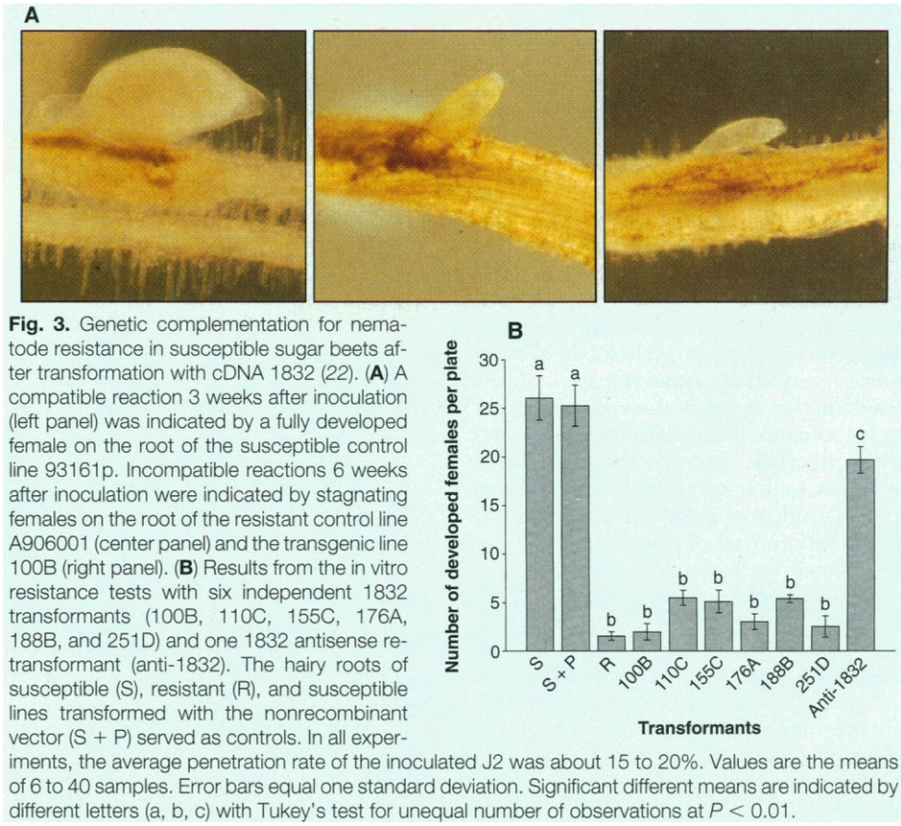

Fig. 3. Genetic complementation for nematode resistance in susceptible sugar beets after transformation with cDNA 1832 (22). (**A**) A compatible reaction 3 weeks after inoculation (left panel) was indicated by a fully developed female on the root of the susceptible control line 93161p. Incompatible reactions 6 weeks after inoculation were indicated by stagnating females on the root of the resistant control line A906001 (center panel) and the transgenic line 100B (right panel). (**B**) Results from the in vitro resistance tests with six independent 1832 transformants (100B, 110C, 155C, 176A, 188B, and 251D) and one 1832 antisense retransformant (anti-1832). The hairy roots of susceptible (S), resistant (R), and susceptible lines transformed with the nonrecombinant vector (S + P) served as controls. In all experiments, the average penetration rate of the inoculated J2 was about 15 to 20%. Values are the means of 6 to 40 samples. Error bars equal one standard deviation. Significant different means are indicated by different letters (a, b, c) with Tukey's test for unequal number of observations at $P < 0.01$.

Fig. 4. Analysis of the predicted amino acid sequence of the *Hs1*$^{pro-1}$ gene corresponding to a 33-kD (282 amino acids) protein (*23*); numbers indicate amino acid position. The polypeptide can be separated into eight domains (left margin, letters A through H): A, a putative signal peptide; B, a subdomain of no specified features; C, a LRR consisting of 24% leucines and isoleucines arranged into seven imperfect repetitive units (LRRs) of 20 amino acids, which contributes to 63% of all leucines in the predicted protein; D, a hydrophilic region; E, a subdomain of no specified features; and F, a hydrophobic region (*24*) with a predicted α-helical secondary structure (*25*) suggestive of a transmembrane span. The charged amino acids (Lys, Glu) flanking the hydrophobic domain are typical for transmembrane segments. A search for homologies between this hydrophobic region and the *Arabidopsis* protein data set showed homology to transmembrane segments of a vacuolar membrane proton pump (SwissProt accession number P31414). The last two domains are G, a subdomain with no specified features, and H, a basic COOH-terminal tail with a putative *N*-glycosylation signal (underlined). No consensus motifs indicative of nucleotide binding sites (*26*) and protein kinases (*27*) were found. The DNA sequence has been submitted to GenBank (accession number U79733).

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A   1  MRRCGYSLGLGEPNLDG
B  18  KPNLDYDAVCRPS
C  31  ELHALKKGALDYIQNSENQI
      51   LFTIHQIFESWIFSSKK L
      69   LDRISE RISKEE FTKAA  DDCW
      90  ILEKIWKL LEEIENLHL L  MDPDD
     113 FLH LKTQ LRMKT VADSE  TFCFRSK
     137 GLIEVTKLSKD LRHKVPKI
     156   LG VE VDPMGGPVIQESA  MELY
D  177 REKRRYEK
E  185 IHLLQAFQGVESAVK
F  200 GFFFNYKQLLVIMMGSL
G  217 EAKANFAVIGGSTESSDLLAQLFLEPTYYP
     247 SLDGAKTFIGDCWEHDQAVGSGLDC
H  272 RHHRKNRTAKQ
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phobic stretch of 17 amino acids of the *Hs1*$^{pro-1}$ gene (domain F) is indicative of a transmembrane segment. The COOH-terminal domain (H) contains amino acids with positively charged residues and one putative *N*-glycosylation site.

The elicitor-receptor model of plant-pathogen interaction suggests that the products of resistance genes function as a specific receptor for pathogen elicitors (*14*) according to the gene-for-gene hypothesis (*15*). The sequence analysis of *Hs1*$^{pro-1}$ indicates that it may be involved in a gene-for-gene resistance as part of a cascade of defense reactions. The predicted polypeptide consists of imperfect LRRs located at the NH_2-terminus with an additional signal peptide, a putative transmembrane spanning domain, and a positively charged COOH-terminus, thus fitting the second subclass of plant resistance genes as proposed (*16*). Similar protein structures between *Hs1*$^{pro-1}$ and the resistance gene *Cf-9* from tomato (*17*) could be predicted, even though no significant sequence homology was detected. As a possible mode of resistance reaction, the extracytoplasmic LRRs may function as a receptor recognizing putative elicitors. Nematodes produce secretions injected through the nematode's stylet, as well as compounds released from cuticular ducts of gland cells or contained in the surface coat of nematodes, that may interact with membrane-bound plant receptors (*18*). The positively charged COOH-terminus possibly interacts with cytoplasmic components for signal transduction. Alternatively, as a protein located in the cytoplasm, it may function as a receptor for elicitors injected into the cell through the nematode's stylet.

The molecular cloning of a plant gene involved in nematode resistance should lead to a better understanding of host-pathogen–specific defense against nematodes. *Arabidopsis thaliana*, which serves as a model species for many aspects of molecular plant-microbe interactions, turned out not to be useful in this respect because, except for non–host resistance, it does not show genetic variation for specific resistance against cyst nematodes (*19*). Isolation of *Hs1*$^{pro-1}$ offers the possibility to transfer resistance to species of agronomical importance that do not naturally carry a form of this gene, for example oilseed rape (*Brassica napus*).

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9. The genomic clone 1832,02 (~15 kb) has been isolated by screening a genomic λ-Dash II library of line A906001. A 5.5-kb Hind III fragment covering the cDNA 1832 has been sequenced.
10. Different versions of the BLAST algorithm [S. F. Altschul, G. Warren, W. Miller, E. W. Meyers, D. J. Lipman, *J. Mol. Biol.* **215**, 403 (1990)] were used to search DNA and protein databases. A search with tblastn on the *A. thaliana* database revealed strong similarity to three ESTs of unknown function (GenBank accession numbers N65731, N65729, and Z37599). The similarity between cDNA 1832 and EST N65731 is about 52% on the amino acid level between position 117 and 220.
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22. The full-length cDNA 1832 was cloned into the Eco RI site of the binary vector pAM194 [provided by J. Kraus, PLANTA GmbH, Einbeck, Germany] under the transcriptional control of the 35*S* promoter from cauliflower mosaic virus and was transformed into the *A. rhizogenes* strain C58C1 with the plasmid pRi15834. Hairy roots of the susceptible sugar beet line 93161p were induced after co-infection of leaf segments with the *A. rhizogenes* strain containing the recombinant pAM194 plasmid and the *A. tumefaciens* strain LBA4404. The transgenic nature of the hairy roots evolving from the infection site was verified by β-glucuronidase assay [R. A. Jefferson, T. A. Kavanagh, M. W. Bevan, *EMBO J.* **6**, 3901 (1987)] and subsequent DNA blot analysis (D. Cai *et al.*, data not shown). The hairy roots from the resistant line A906001 and from the susceptible line 93161p and the hairy roots from the susceptible line 93161p that were transformed with the nonrecombinant pAM194 vector were used as controls. After 3 weeks of cultivation at 24°C on MS medium [T. Murashige and F. Skoog, *Physiol. Plant* **15**, 473 (1962)], hairy roots were dissected from the primary transformants and transferred to new petri dishes. After 1 week of cultivation under the same conditions, 300 sterile nematode juveniles (J2) were inoculated to each culture plate. The penetration sites and the developed females were counted under a stereo microscope 3 weeks after inoculation. The in vitro testing experiments were repeated at least twice. Each time, about 6 to 40 hairy root segments of one primary transformant were examined. The transgenic root culture 100B was retransformed with a pAM194 vector carrying the full-length cDNA 1832 in antisense orientation and tested for nematode development as described.
23. Abbreviations for the amino acids are as follows: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; Q, Gln; E, Glu; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; and V, Val.
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