Potato Gene *Y-1* is an *N* Gene Homolog That Confers Cell Death Upon Infection with *potato virus Y*

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ADG2 is a DNA sequence mapped to a resistance (*R*) gene-rich region at the distal end of chromosome XI in potato (*Solanum tuberosum* subsp. *andigena*). The gene, in which ADG2 represents the predicted nucleotide-binding domain (NBS), was cloned and characterized. The coding region of the gene (designated as *Y-1*) is 6,187 bp long and structurally similar to gene *N* that confers hypersensitive resistance to Tobacco mosaic virus in *Nicotiana* spp. Both belong to the TIR-NBS-LRR class of genes and show 57% identity at the amino acid sequence level. The introns of *Y-1* were spliced as predicted from the sequence. *Y-1* cosegregated with *Ry_{adg}*, a gene for extreme resistance to Potato virus Y (PVY) on chromosome XI, as tested in a potato-mapping population and with independent potato cultivars. Leaves of the transgenic potato plants expressing *Y-1* under the control of Cauliflower mosaic virus 35S promoter developed necrotic lesions upon infection with PVY, but no significant resistance was observed, and plants were systemically infected with PVY.

Potato Y potyvirus (PVY) is an important viral pathogen in potato and other solanaceous crops, such as tomato, tobacco, and pepper (De Bokx and Huttinga 1981). The two main types of resistance to PVY in potato are extreme resistance (ER) and hypersensitive resistance (HR). ER protects potato plants against all strains of PVY. It strongly suppresses virus accumulation in infected cells, and no visible symptoms or detectable amounts of PVY are observed in inoculated plants (Barker and Harrison 1984; Cockerham 1970; Härmäläinen et al. 1998). In contrast, HR to PVY is strain specific. For example, *Ny* confers HR to the “ordinary strains” of PVY (PVY⁰) (Cockerham 1970; Jones 1990). HR is characterized by development of necrotic lesions (cell death) at the initial infection sites on inoculated leaves. HR may prevent the spread of virus within and from the inoculated leaf, but the resistance mechanism itself is unknown. In some cases, HR may be activated but fails to re-

strict virus movement in plant tissues, which results in the development of larger necrotic lesions, vein necrosis, or lethal necrosis in the inoculated leaf and also in other parts of the plant following systemic virus movement. Higher temperatures can modulate expression of HR, reduce its efficiency to restrict viral movement, and cause extensive tissue necrotization symptoms (Adams et al. 1986; Dykstra 1939; Valkonen 1997; Valkonen et al. 1998). A well-studied example is the temperature-sensitive expression of HR to Tobacco mosaic tobamovirus (TMV) conferred by the *N* gene in tobacco (*Nicotiana tabacum* L.). At 22°C, HR is activated upon infection with TMV, necrotic local lesions develop, and no systemic infection with TMV is observed; whereas, at 32°C, no HR is observed and plants are systemically infected showing mosaic symptoms (Dinesh-Kumar and Baker 2000; Ross 1961; Westeijn 1981).

The northern distal end of the potato chromosome XI contains a resistance (*R*) gene-rich region, or a “resistance hotspot” (Gebhardt and Valkonen 2001). In *Solanum tuberosum* L. subsp. *andigena* Hawkes, line 2x(V-2)7, the gene *Ry_{adg}* for ER to PVY and the gene *Na_{adg}* for HR to Potato A potyvirus (PVA) map to this resistance hotspot (Härmäläinen et al. 1997, 1998, 2000). Another gene for extreme resistance to PVY (Brigneti et al. 1997), a major quantitative trait locus (QTL) for resistance to Potato leaf roll polerovirus (Marczewski et al. 2001), dominantly inherited genes conferring resistance to potato wart and nematodes, and major QTLs for resistance to potato late blight and *Erwinia* soft rot have been mapped to this resistance hotspot in other potato lines (reviewed in Gebhardt and Valkonen 2001). Isolation and characterization of the genes have not been reported. The *R* gene-rich region of chromosome XI is syntenic in potato and tobacco. It contains the gene *N* in tobacco (Whitham et al. 1994) and genes homologous to *N* with unknown functions in potato (Härmäläinen et al. 1998; Hehl et al. 1999; Leister et al. 1996).

ADG2 (354 bp) is a nucleotide sequence isolated from the diploid potato line 2x(v-2)7. ADG2 corresponds to the nucleotide-binding domain (NBS) characteristic of the class of *R* genes also containing a C-proximal leucine rich repeat (LRR) region (Ellis et al. 2000). It is 79% similar to the corresponding region of the *N* gene (Härmäläinen et al. 1998). Cleaved amplified polymorphic sequence (CAPS) and sequence-characterized amplified region (SCAR) markers that detect single nucleotide differences unique to ADG2 distinguish potato genotypes carrying *Ry_{adg}* from other potato genotypes, irrespective of their genetic background (Kasai et al. 2000; Sorri et al. 1999). Therefore, ADG2 is tightly linked to *Ry_{adg}* and maps to the resistance hotspot on chromosome XI.

The aim of this study was to isolate and characterize the gene in which ADG2 covers the NBS region. The gene, designated as

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S. Vidal and H. Cabrera contributed equally. Nucleotide and amino acid sequences have been submitted to the EMBL/GenBank/DDJB databases under accession number AJ300266.

Isolation of full-length cDNA sequences corresponding to ADG2.1.

Full-length cDNAs containing the ADG2.1 sequence were isolated from mRNA purified from a healthy plant of line 2x(v-2)7 using rapid amplification of cDNA ends (RACE)-PCR. Two nested primers (ADG2C and ADG2E) spanning the 3' region of ADG2.1 were used together with adapter primers for amplification of 5' sequences. To obtain 3' sequences, two new nested primers (ADG2D and ADG2F) were designed to cover the 5' region of ADG2.1 and were used in combination with the adapter primers. PCR products were cloned and sequenced. Four transcripts identical in sequence but different in size were identified (Fig. 2), and several clones of each were sequenced.

The transcript designated C-38 (3,802 bp; 5' and 3' untranslated regions (UTRs) 255 nt and 169 nt, respectively), encodes a protein of 1,126 amino acids (aa) (Fig. 2) sharing significant similarities with *R* genes of the NBS-LRR class, in which the N-proximal region contains a domain (TIR) with similarity to *Drosophila* Toll and mammalian interleukin-1 receptor proteins (Ellis et al. 2000). The LRR region comprises 13 imperfect repeats of 22 to 32 aa in length. The deduced amino acid sequence encoded by C-38 shows 57% identity to the *N* gene product (Fig. 3). Additionally, it shows high amino acid sequence identity to the putative proteins of the potato genes *Nl-25* (61%) and *Nl-27* (65%) linked with the *Sen1* locus that

confers resistance to potato wart (*Synchytrium endobioticum*) (Hehl et al. 1999). Low overall amino acid sequence identities were found with RPP5 (28%), L6 (27%), M (26%), and Mi (16%) encoded by *R* genes of *Arabidopsis* spp. (*RPP5*), flax (*L6*, *M*), and tomato (*Mi*) (Anderson et al. 1997; Lawrence et al. 1995; Parker et al. 1997; Vos et al. 1998).

The other transcript (C-19; 3,727 bp) encodes for a protein of 1,101 aa. The proteins encoded by C19 and C38 are identical, except for 25 aa that are missing from the N-terminal part of the TIR region in C19 (Fig. 2). The 75-bp sequence encoding for these 25 aa in C-38 is flanked by consensus splice acceptor and donor sites, which suggests that the sequence represents an alternatively spliced intron. Hence, it is possible that C38 and C19 are transcripts derived from the same gene (see below).

Two additional transcripts were obtained in the RACE experiments. They are identical to clones C-38 and C-19, but lack the sequence encoding for the LRR region at the C-proximal part of the protein (Fig. 2). These truncated clones (T-C-38 and T-C-19) are 2,175 and 2,100 bp long, respectively.

The gene corresponding to ADG2.1 cosegregates with *Ry_{adg*}*

The gene encoding for the transcript C-38, C-19, or both, was obtained by PCR-amplification of total DNA from the PVY-resistant parental line using primers designed according

Fig. 2. Schematic representation of the genomic and cDNA sequences of *Y-1*. The genomic sequence is 6,187 bp long and contains six exons (I to VI) and six introns (1 to 6). According to the deduced amino acid sequence, a *Toll* and interleukin 1 receptor-like domain (TIR), and a nucleotide binding domain (NBS), including kinase 1 (p-loop), kinase 2, and kinase 3 domains and a hydrophobic domain, are found at the N-proximal part of the protein. The C-proximal part contains a leucine-rich repeat (LRR) domain. The structure of *Y-1* is similar to *N* (Whitham et al. 1994), shown for comparison. Four cDNAs (C-38, C-19, T-C-38, and T-C-19) identical to the *Y-1* sequence but differing in length were obtained by rapid amplification of cDNA ends polymerase chain reaction with primers that detect nucleotide substitutions unique to the NBS region of *Y-1* (ADG2.1 sequence; position depicted). C-38 and C-19 are identical except that C-19 lacks a 75-bp putative alternatively spliced intron (ASI-2) within the TIR domain. T-C-38 and T-C-19 are C-terminally truncated forms of C-38 and C-19, respectively, and lack most of the LRR domain.

Y-1	MASSSSSSSE	NSQYSCPQK	YKYDVFLSFR	GKDTRRNFTS	HLYERLDNRG	50
N	MASSSSSS..	R	WSYDVFLSFR	GEDTRKTFTS	HLYEVLNDKG	39
Y-1	IFTFLDDKRL	ENGDSLSEL	VKAIKESQVA	VIIFSKNYAT	SRWCLNEVVK	100
N	IKTFQDDKRL	EYGATIPGEL	CKAIEESQFA	IVVFSENYAT	SRWCLNELVK	89
Y-1	IMECKEENGQ	LVIPVFYDVD	PSDVRKQTKS	FAEAFAEHES	RYKDDVEGMO	150
N	IMECKTRFKQ	TVIPIFYDVD	PSHVRNQKES	FAKAFEEHET	KYKDDVEG..	137
Y-1	KVQRWRTALS	EAADLKG.YD	IRERIESECI	GELVNEISPK	LCETSLSYLT	199
N	.IQRWRIALN	EAANLKGSCD	NRDKTADDCI	RQIVDQISSK	LCKISLSYLQ	186
Y-1	DVVGIDAHLK	KVNSLLEMKI	DDVRIVWIWG	MGGVGKTTIA	RAIFDILSS.	24
N	NIVGIDTHLE	KIESLLEIGI	NGVRIMGIWG	MGGVGKTTIA	RAIFDTLLGR	236
Y-1	KFDGA	CFLPDNKENK	YEIHSLSQIL	LSKLVGEEKEN	CVHDKEDGRH	293
N	MDSSYQFDGA	CFLKDIKENK	RGMHSLQNAL	LSELLREKAN	YNNE.EDGKH	285
Y-1	LMARRLRLLK	VLVVLNDIDH	ED.QLKYLAL	DLGWFGNGTR	IIATTRDKHF	342
N	QMASRLRSKK	VLIVLDDIDN	KDHYLEYLAG	DLDWFGNGSR	IIITTRDKHL	335
Y-1	IRKNDVYVPV	TTLLEHDAVQ	LFNQYAFKNE	VPDKCFEEIT	LEVVSHAEGE	392
N	IEKNDIIYEV	TALPDHESIQ	LFKQHAFGKE	VPNENFEKLS	LEVVNAYAKG	385
Y-1	PLALKVWGSS	LHKKDIHVWR	SAVDRIKRNP	SSKVVENLKV	SYDGLEREDQ	442
N	PLALKVWGSL	LHNLRLETKW	SAIEHMKNS	YSGIIDKLKI	SYDGLEPKQQ	435
Y-1	EIFLDIACFL	RGRKQTEIKQ	ILESCDFGAD	DGLRVLIDKS	LVFISEYDTI	492
N	EMFLDIACFL	RGECKDYILQ	ILESCHIGAE	YGLRILIDKS	LVFISEYNQV	485
Y-1	QMHDLIQEMG	KYIVTMQKDR	GEVTRLWLTQ	DPEKFSNAKI	QGTKAIEAIW	542
N	QMHDLIQDMG	KYIVNFQKDP	GERSRLWLAK	EVEEVMNS.N	TGTMAEAIW	534
Y-1	IPEIQD.LSF	RKKAMKDVEK	LRILYINGFH	TPDGSNDQYL	PSNLRWFDC	59
N	VSSYSSTLRF	SNQAVKNMKR	LRVFNMGRSS	THYAI.D.YL	PNNLRFCVCT	582
Y-1	KYPWESLPAK	FDPDMLVHLD	LQQSSLFHLW	TGTFKFPFLR	RDLSSCANL	641
N	NYPWESFPST	FELKMLVHLQ	LRHNSLRHLW	TETKHLPSLR	RIDLSWSKRL	632
Y-1	MRTPDFTDMP	NLEYLGLEEC	SNLKEVHHS	RCSKKLIKLN	LRDCKNLESF	691
N	TRTPDFTGMP	NLEYVNLYQC	SNLEEVHHS	GCCSKVIGLY	LNDCKSLKRF	682
Y-1	SYVCWESLEC	LHLQGCNLE	KFPRIRGKLE	PEIEIQVQRS	GIRKLPSAII	741
N	PCVNVESLEY	LGLRSCDSLE	KLPEIYGRMK	PEIQIHMQGS	GIRELPSSIF	732
Y-1	QHSSSLTELD	LSGMKNLATL	SCSIGELKSL	VMLKVSYSCK	LKSLPEEIGD	791
N	QYKTHVTKLL	LWNMKNLVAL	PSSICRLKSL	VLSVSGCSK	LESLEPEIGD	782
Y-1	LENLEILKAG	YTLISQPPSS	IVRLNRLKFL	TFAKQKSEVG	LEDEVHVFVP	841
N	LENLRVFDAS	DTLILRPPSS	IIRLNKLIIL	MFR.....G	PKDGVHFEFP	826
Y-1	PVNQGLCSLK	TLNLSYCNLK	DEGLPDQIGS	LSSLEVLNLR	GNNFEHLPQS	891
N	PVAEGLHSLE	YLNLSYCNLI	DGGLPEEIGS	LSSLKKLDLS	RNNFEHLPSS	876
Y-1	LTRLSSSLQSL	DLLDCKSLTQ	LPEFFRQLDT	IYADWN....		927
N	IAQLGALQSL	DLKDCQRLTQ	LPPELPELNE	LHVDCHMALK	FIHYLVTKRK	926
Y-1		.NDSICN...	.SLFQNISSF	QHDICASDSL	SLRVFTNEWK	962
N	KLHRVKLDDA	HNDTMYNLFA	YTMFQNISSM	RHDISASDSL	SLTVFTGQPY	976
Y-1	..NIPRWFHH	QGKDKSVSVK	LPENWYVCDN	FLGFAVCYSG	CLIIETTAQFL	1010
N	PEKIPSWFHH	QGWDSVSVSN	LPENWYIPDK	FLGFAVCYSR	SLIDTTAHLI	1026
Y-1	..CDEGMPCI	TQKLALPKHS	EE...FPESA	IHFFLVPSAG	LLDTSKANGK	1055
N	PVCDDKMSRM	TQKLALSECD	TESSNYSEWD	IHFFVFPFAG	LWDTSKANGK	1076
Y-1	TPNDYRHIML	SFSEELKEFG	LRLLYRDESK	LKALFKMTEN	NDEPTEYCVV	1105
N	TPNDYGIIRL	SFSGEEKMYG	LRLLYKEGPE	VNALLQMREN	SNEPTEHSTG	1126
Y-1	KRRGQYDEAR	CSSSKKQRSQ	L			1126
N	IRRTQYNN.R	TSFYELING.	.			114

to the 5' and 3' terminal 25 nt of these cDNAs (primers 5'-38 and 3'-38, respectively). Using high-stringency PCR conditions, a single 6.2-kb product was obtained from the resistant parental line 2x(v-2)7 but not from the susceptible parental line 84.194.30. The amplified gene was designated as *Y-1*. The sequences of the cDNAs obtained by RACE-PCR (C-38, C-19, T-C-38, and T-C-19) were identical with *Y-1*.

Cosegregation of *Y-1* with ER to PVY (i.e., the resistance phenotype conferred by *Ry_{adg}*) was tested on potato lines characterized for their PVY resistance phenotypes (Hämäläinen et al. 1998). First, DNA from the 77 F1 progeny lines of the diploid mapping population was extracted, and amplification of *Y-1* was tested with primers 5'-38 and 3'-38 using high-stringency PCR conditions (Fig. 4A). Amplification of *Y-1* was observed in all progeny expressing ER to PVY but was not observed in any other progeny line. Subsequently, 50 potato cultivars and breeding lines were tested, selected from those previously tested with the CAPS or SCAR primers (Kasai et al. 2000; Sorri et al. 1999). Again, *Y-1* could be amplified only from those genotypes that express ER to PVY and carry *Ry_{adg}* derived from *S. tuberosum* subsp. *andigena* (Fig. 4B), which was fully consistent with results obtained with the CAPS and SCAR primers in the previous studies. Cosegregation of *Y-1* with *Ry_{adg}* indicated that the 6.2-kb *Y-1* gene is applicable as a novel SCAR marker for selection of *Ry_{adg}* in breeding programs. Also, the data indicated a high likelihood that *Y-1* belongs to the same gene cluster as *Ry_{adg}*.

Structure and expression of *Y-1*.

A schematic representation of the structure of *Y-1* and the transcripts obtained by RACE-PCR are shown in Figure 2. *Y-1* (6,187 bp) corresponds to the genomic transcribed region lacking the promoter region. Six introns are predicted from the sequence. Intron 1 and intron 6 are spliced from the 5' and 3' UTR, respectively. Three other introns (3, 4, and 5) are predicted to be spliced to form an open reading frame (ORF) of 3,378 bp (corresponding to C-38) that encodes for a protein of 1,126 aa. The predicted intron-splicing pattern of *Y-1* was examined using the transcripts produced from *Y-1* under the control of the *Cauliflower mosaic caulimovirus* (CaMV) 35S promoter in transfected tobacco protoplasts and in transgenic potato plants. RNA samples extracted from protoplast were treated with DNase to remove possible traces of plasmid DNA, and transcripts were analyzed with reverse transcriptase (RT)-PCR using primers that did not amplify sequences from tobacco and the nontransformed potato. Size and sequence analyses of the RT-PCR products confirmed that introns 3, 4, and 5 were spliced as predicted (Fredriksson 2000).

The transcript C-19 may be produced via alternative splicing of a 75-bp intron (intron 2, further called ASI-2), which results in an ORF of 3,303 bp encoding for a protein of 1,101 aa. Alternatively, this transcript might be produced by a homolog of *Y-1* lacking the 75-bp intron, but this possibility seems less likely because no *Y-1* homolog lacking ASI-2 was detected by PCR in the genomic DNA of the parental line 2x(v-2)7. The two transcripts appeared to be expressed at a 1:1 ratio in non-inoculated plants of the parental line 2x(v-2)7: of the 15 cDNAs analyzed, 8 contained ASI-2 whereas 7 did not. The additional cDNAs T-C-38 and T-C-19 obtained by RACE-PCR might have resulted from prematurely terminated transcription of *Y-1* and would encode for truncated polypeptides lacking the LRR region. The generation of multiple transcripts via alternative splicing of a single mRNA, and the production of C-terminally truncated proteins, are characteristics of the TIR-NBS-LRR class of *R* genes, for example *N* (Dinesh-Kumar and Baker 2000).

Generation of transgenic plants expressing *Y-1* sequences.

The genomic clone (*Y-1*), C-38 (with ASI-2), and C-19 (without ASI-2) were placed under the constitutive CaMV 35S promoter (Fig. 5) and transformed into a potato line (v2-134) chosen from the progeny of our mapping population for its efficient regeneration capacity in vitro. Line v2-134 is a PVY-susceptible progeny line obtained from a cross between the parental lines 2x(V-2)7 (ER to PVY conferred by *Ry_{adg}*) and 84.194.30 (susceptible to PVY) (Valkonen et al. 1994b). It lacks the *Ry_{adg}*-containing region of chromosome XI, as indicated by the marker data (Hämäläinen et al. 1998; Sorri et al. 1999). Tobacco cv. Samsoun nn was transformed with the same constructs used for potato transformation. As a control, potato and tobacco plants were transformed only with the *NPTII* marker gene that confers resistance to kanamycin.

Potato regenerants (56 to 100 shoots per construct) were excised from calli, transferred to rooting media, and grown for analysis. Regenerants were screened by PCR using the SCAR primers specific to ADG2.1 (Kasai et al. 2000) to detect at least 10 putative transgenic lines, which then were tested with additional primers designed according to sequences flanking the T-DNA in the binary transformation plasmid. This screening was done in order to discard at least the majority of lines that were internally contaminated with *Agrobacterium* spp. Fi-

Fig. 4. The use of *Y-1* as a sequence-characterized amplified region (SCAR) marker for extreme resistance (E) to *Potato virus Y* (PVY) conferred by *Ry_{adg}*. Examples of marker data from F1 progeny of **A**, a diploid potato mapping population (total of 77 lines; Hämäläinen et al. 1998) and **B**, other potato genotypes (total of 50 genotypes tested) are shown. High-stringency polymerase chain reaction conditions and primers directed to the ends of the *Y-1* sequence (primers 5'38 and 3'38) were used. Amplification of *Y-1* (6.2 kb) was observed only from potatoes that express E to PVY and carry *Ry_{adg}* according to the pedigree data. The 6.2-kb product was not amplified from potato genotypes that express E to PVY conferred by genes (E^a, Sorri et al. 1999) other than *Ry_{adg}* or from plants that express hypersensitive resistance (H) or susceptibility (S) to PVY. Some progeny lines (S*) of the diploid mapping population develop diffuse brown lesions on leaves inoculated with PVY^O but are systemically infected and develop mosaic symptoms in the upper leaves, in contrast to the H genotypes that develop vein necrosis or top necrosis following systemic infection.

nally, integration of the transgenes to the genome was confirmed in the selected lines by Southern blot analysis using the 35S sequence as a probe (the *Y-1* sequence could not be used as a probe due to other highly homologous genes in potato genomes). One to three integrated copies of the transgene were detected, depending on the transgenic line. Control lines were tested only with the *NPTII* probes. The transgenes seemed to cause no abnormal morphology compared with the nontransgenic wild-type plants of line v2-134.

Expression of *Y-1* was tested in the transgenic potato lines by RT-PCR using the ADG2.1-specific SCAR primers. *Y-1* was expressed in all transgenic lines grown in the greenhouse and tested after various periods of growth (4 to 10 weeks). This was similar to the PVY-resistant parental line 2x(v-2)7, and in contrast to the nontransgenic line v2-134 from which no amplification product was obtained (Fig. 6).

Tobacco leaf explants transformed with the three *Y-1*-based constructs had numerous tiny, necrotic pinpoint lesions at the cut edges 2 days post inoculation (dpi) with *Agrobacterium* spp. Regeneration of shoots was unexpectedly poor compared with the controls transformed with the *NPTII* gene. Finally, 31 to 35 regenerants were obtained per construct but none was transgenic as tested by PCR, in contrast to the control regenerants, most of which contained and expressed the *NPTII* gene.

Resistance to PVY in the transgenic potato lines expressing *Y-1*.

Transgenic lines were multiplied in vitro, transferred to soil in a greenhouse, and sap inoculated with PVY⁰ in four experiments. In two and three experiments, a similar set of the same lines was inoculated with PVA and *Potato V potyvirus* (PVV), respectively. Each transgenic line was tested in at least two independent experiments during October to February in 2 years. Temperatures were recorded continuously during all experiments. Dark-brown local lesions were observed in the PVY-inoculated leaves of plants transformed with *Y-1* (18 transgenic lines tested) at 9 to 10 dpi. In contrast, diffuse chlorotic or brownish lesions were observed in the inoculated leaves of plants transformed with C-19 (12 lines), C-38 (13 lines), or the "empty vector" (*NPTII* gene only) (15 lines) at 10 to 12 dpi. No lesions developed in leaves inoculated with PVV or PVA. All PVY-inoculated plants of all transgenic lines were systemically infected with PVY, as determined by enzyme-linked immunosorbent assay (ELISA), and systemic infection was associated with mosaic symptoms in leaves. The systemically infected

leaves contained PVY antigen at a concentration of approximately 5.5 µg/g as determined by ELISA using known amounts of purified PVY virions for comparison. No significant differences in virus concentrations were observed between the transgenic lines (data not shown). However, in the experiment during which temperatures were the lowest in the greenhouse (18 to 20°C, compared with 20 to 25°C in the three other experiments), systemic infection with PVY was delayed in the *Y-1* transgenic lines. Three plants of only one line out of eight were systemically infected at 30 dpi, whereas all plants of the seven C-19 lines and eight C-38 lines included in this experiment were systemically infected, as indicated by mosaic symptoms and positive ELISA readings. At 44 dpi, all plants of all *Y-1* transgenic lines were systemically infected. Taken together, the results obtained in the greenhouse experiments indicated that *Y-1* induced a cell death response upon infection with PVY, but no significant resistance to PVY was observed.

The temperatures in the greenhouse could not be strictly controlled. Therefore, two additional experiments were carried out in a growth chamber at 17 to 19°C using three *Y-1* transgenic lines and three control (*NPTII*) lines. Two to four plants per line (8 weeks old), and three to four full-grown leaves per plant were inoculated with PVY⁰. No symptoms were observed in inoculated leaves at 8 dpi. At 10 dpi, the inoculated leaves of *Y-1* transgenic lines contained dark-brown to black, sharp-edged local lesions (Fig. 7B). In some leaves, the edges of a few lesions were somewhat diffuse (Fig. 7A), but no differences were observed in this respect between the three *Y-1* transgenic lines tested. In contrast, the leaves of control plants contained chlorotic, diffuse lesions, and in the middle of some of these lesions a few brown, necrotic cells were observed (Fig. 7C). The numbers of the sharp-edged lesions (Fig. 7B) on inoculated leaves were counted in the *Y-1* transgenic lines at 11 dpi. They were higher in the line s1g-4 (20.7 ± 9.8) than in the two other lines, s1g-23 (9.6 ± 3.0) and s1g-26 (8.0 ± 3.7) ($n = 12$; analysis of variance, $p < 0.05$). Comparison with the control plants was not done because it was difficult to count the chlorotic, diffuse lesions (Fig. 7C) on them. At 2 to 3 weeks later, some plants of the *Y-1* transgenic lines had vein necrosis symptoms in the upper noninoculated leaves, which was not observed in the control plants showing mosaic symptoms.

Expression levels of the *Y-1* transcript were studied in the leaves of two *Y-1* transgenic lines at two different temperatures (17 to 19°C and 20 to 25°C) and in the leaves of these plants at 0, 4, 8, and 24 h post inoculation with PVY. The ADG2.1-specific SCAR primers (Kasai et al. 2000) and an RT-PCR based method were used as described (Timmusk and Wagner 1999). Expression of *Y-1* was detected in both transgenic lines at both temperatures and all time points, but no differences in the expression levels were observed (data not shown).

DISCUSSION

The northern, distal end of chromosome XI in potato is rich in *R* genes (Gebhardt and Valkonen 2001). Many RGL sequences with unknown function have been isolated from this resistance hot spot (Hehl et al. 1999; Leister et al. 1996). ADG2 is one of them (Hämäläinen et al. 1998; Sorri et al. 1999). Therefore, the aim of this study was to clone and characterize the gene that includes ADG2. First, cDNAs corresponding to the transcripts of the gene (designated as *Y-1*) were cloned using ADG2.1-specific SCAR primers in RACE-PCR. Subsequently, the genomic clone of *Y-1* was obtained by PCR. Screening of our potato mapping population and 50 independent potato cultivars showed that *Y-1* was PCR amplified reliably and exclusively from potato genotypes carrying *Ry_{adg}*,

Fig. 5. Constructs for transformation of *Y-1* and the corresponding cDNAs C-38 and C-19 to potato (line v2-134) and tobacco cv. Samsoun nn using *Agrobacterium tumefaciens*. The CaMV 35S promoter and the termination sequence of the T-DNA gene *g7* including its polyadenylation signal were used. Only the coding regions were inserted into the transformation vector.

indicating that *Y-1* is tightly linked to *Ry_{adg}*. *Y-1* has the highest similarity to the gene *N* that confers HR to TMV in *Nicotiana* spp. (Whitham et al. 1994). This is consistent with previous studies indicating that genes homologous to *N* reside in the resistance hotspot on the northern arm of chromosome XI (Gebhardt and Valkonen 2001; Hämäläinen et al. 1998; Hehl et al. 1999; Leister et al. 1996).

The results of our study show that expression of *Y-1* in a PVY-susceptible potato line induces a cell death response in leaves upon infection with PVY but not with PVA or PVV. The cell death response to PVY infection conferred by *Y-1* closely resembles the HR activated in potato plants expressing PVY strain-specific resistance genes, such as *Ny* and *Nc* (De Bokx and Huttinga 1981; Jones 1990; Valkonen 1997; Valkonen et al. 1994a). However, *Y-1* conferred no effective resistance against the movement of PVY. Some of the PVY-specific HR genes also may fail to restrict the movement of PVY, depending on environmental conditions and other genes of the potato genotype (Dykstra 1939; Ross 1952; Valkonen 1997; Valkonen et al. 1998). The cell death phenotype expressed in the *Y-1* transgenic potatoes was not observed in the plants containing *Y-1* in our potato mapping population, probably due to ER to PVY conferred by the tightly linked gene *Ry_{adg}* that also is present in those plants. Previous studies have indicated, based on genetic evidence, that expression of ER is epistatic to the expression of HR to the same virus (Cockerham 1970; Valkonen et al. 1994b). Moreover, comparison of the resistance responses triggered by the gene *N* and the gene *Rx* for ER to *Potato X potexvirus* (PVX) in transgenic tobacco plants shows that *Rx* induces resistance earlier than *N* (Bendahmane et al. 1999). Therefore, the strong resistance against virus multiplication conferred by the genes for ER (Barker and Harrison 1984; Köhm et al. 1993) probably prevents accumulation of virus to the concentrations required for activation of HR, or the cell death response induced by *Y-1*.

Y-1 is the first gene isolated from the resistance hotspot of potato chromosome XI and for which a phenotype is now described. Two other genes (*Nl-25* and *Nl-27*) with 61 and 67% amino acid sequence similarity to *Y-1*, respectively, have been isolated from another potato genotype, but their phenotypes are unreported and it is uncertain whether *Nl-25* resides in the same chromosomal region with *Y-1* (Hehl et al. 1999). *Y-1* is very tightly linked to genes conferring resistance to PVY (*Ry_{adg}*) and PVA (*Na_{adg}*) (Hämäläinen et al. 1998, 2000), and *Nl-27* is linked to a major QTL for resistance to *Potato leaf roll polerovirus* located at the resistance hotspot of chromosome XI (Marczewski et al. 2001). All aforementioned isolated potato genes are homologous to the gene *N* that confers HR to TMV in tobacco. It is therefore interesting that *Y-1* is associated with a cell death response to another virus, PVY, in potato. Future studies will show whether *Ry_{adg}* and *Na_{adg}* are homologous to the *N* gene and *Y-1*. Our data do not formally exclude the possibility that *Y-1* could be *Ry_{adg}*. Both genes are specific to PVY, and the lack of putative natural *cis*-regulatory regions for transcription or translation up- and downstream of the coding sequence of *Y-1* could have affected resistance expression in transgenic plants. For example, expression of *N* under the CaMV 35S promoter does not result in resistance to TMV (Dinesh-Kumar and Baker 2000). However, there are many examples of *R* genes that confer the expected type of resistance when expressed under the CaMV 35S promoter in transgenic plants (Bendahmane et al. 1999, 2000; Tai et al. 1999). We therefore consider *Y-1* and *Ry_{adg}* to be different, tightly linked genes until otherwise shown.

Genetic analyses indicate a tight linkage between *Y-1* and *Ry_{adg}*, as shown by this study and our previous studies (Kasai et al. 2000; Sorri et al. 1999), but a question that remains to

be solved in future is whether or not *Y-1* is required for the resistance conferred by *Ry_{adg}*. In common bean (*Phaseolus vulgaris*), expression of race-specific resistance to the bean rust fungus *Uromyces appendiculatus* is determined by genes at the *Ur-3* locus. Several of these contain an NBS region typical of *R* genes and they, along with other genes, act in a complementary manner to determine pathogen race specificity (Kalavacharla et al. 2000; Rivkin et al. 1999). Also, the gene *Prf* encoding for a TIR-NBS-LRR class protein is necessary for specific expression of HR to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* in tomato (Salmeron et al. 1996), whereas the recognition of this pathogen (i.e., the avirulence factor AvrPto) is determined by another gene, *Pto*, that encodes for a protein kinase (Martin et al. 1993). Interaction of another serine/threonine kinase, Pti1, with Pto enhances the HR (Zhou et al. 1995). The gene *HRT* confers HR to *Turnip crinkle carmovirus* (TCV) in *Arabidopsis thaliana*, but another gene, *RRT*, regulates the resistance to systemic movement of TCV (Kachroo et al. 2000). These examples of other *R* gene systems requiring more than a single gene for an effective resistance response also suggest that *Y-1* might have some regulatory role required for expression of the ER conferred by *Ry_{adg}*.

Over-expression of *Y-1* in tobacco cells infected with *Agrobacterium* spp. resulted in visible necrotic pinpoint lesions and no transgenic tobacco plant expressing *Y-1* was obtained, which contrasted with the successful generation of transgenic tobacco lines expressing the *NPTII* gene. These data suggest that *Y-1* can activate signaling for cell death, which seems to occur more readily in tobacco than potato, because the latter developed no detectable necrosis following infection with *Agrobacterium* spp. These data are consistent with previous studies showing that *R* genes are functional in related species (Whitham et al. 1996) but may induce a different resistance phenotype (Bendahmane et al. 1999; Hammond-Kosack et al. 1998), perhaps due to differences in interactions between the *R* protein and downstream signaling components (Ellis et al. 2000; Gebhardt and Valkonen 2001; Young 2000). Also, the over-expression of *Pto* under the transcriptional control of the CaMV 35S promoter in transgenic tomato plants results in de-

Fig. 6. Expression of *Y-1* in transgenic potato plants transformed with the genomic clone of *Y-1* placed under the CaMV 35S promoter. Reverse-transcriptase polymerase chain reaction was carried out on DNase-treated total RNA from transgenic lines using the sequence-characterized amplified region-marker primer pair RYSC3 developed by Kasai and associates (2000). All transgenic lines examined expressed *Y-1*. The *Potato virus Y*-resistant parental line 2x(v-2)7 and the susceptible progeny line v2-134 used for transformations were included as a positive and a negative control, respectively.

Fig. 7. Local lesions on **A** and **B**, leaves of the *Y-1* transgenic lines and **C**, in a leaf of a control line transformed with *NPTII* marker gene only, 11 days post inoculation with *Potato virus Y* ordinary strain in a growth chamber (17 and 19°C).

velopment of numerous tiny, necrotic lesions in leaves (Tang et al. 1999).

The *R* genes are required for pathogen recognition and initiation of signal transduction, which leads to activation of the defense genes conditioning the cells and tissues to resist invasion of the pathogen (Ellis et al. 2000; Young 2000). The *R* gene product interacts either directly or indirectly, though very specifically, with a product produced by action of a pathogen avirulence gene (*Avr*) (gene-for-gene interaction) (Ellis et al. 2000; Frederick et al. 1998; White et al. 2000; Young 2000). The results of the current study suggest that *Y-1*, a gene encoding for a TIR-NBS-LRR class protein, is involved in recognition of PVY and subsequent activation of cell death. *Y-1* did not confer significant resistance to PVY but merely a PVY-specific cell death response; therefore, we have chosen not to name *Y-1* according to the standard nomenclature of virus resistance genes in potato (Valkonen et al. 1996). Recently, the gene *Rih* was discovered and mapped to linkage group B in *Avena* spp. *Rih* resembles *Y-1* in that it induces a pathogen-specific cell death response upon infection with the crown rust fungus *Puccinia coronata* f. sp. *avenae* but does not confer resistance (Yu et al. 2001). Isolation and characterization of *Rih* will show whether it is structurally similar to *Y-1*. The gene *HRT* of *A. thaliana* belongs to the NBS-LRR class of *R* genes, in which the N-proximal region contains a leucine zipper (LZ) (Cooley et al. 2000). It recognizes the coat protein of TCV, which activates a putative signaling pathway for a cell death response. In TCV-susceptible *A. thaliana* plants transformed with *HRT* under its own promoter, *HRT* induces variable phenotypic responses, ranging from cell death and no resistance to no cell death and high levels of resistance, upon infection with TCV. However, in transgenic plants expressing *HRT* under the CaMV 35S promoter, infection with TCV induces a cell death response but plants are not resistant to systemic infection with the virus (Cooley et al. 2000). Thus, in many respects, the phenotypic responses conferred by *HRT* resemble our results on *Y-1*.

Five virus resistance genes functional on the gene-for-gene basis have previously been cloned and described from plants: *N* from tobacco (Whitham et al. 1994), two nearly identical *Rx* genes from potato (Bendahmane et al. 1999, 2000), *Sw-5* conferring resistance against systemic infection with tospoviruses in tomato (Brommonschenkel et al. 2000), and *HRT* (Cooley et al. 2000). The four last-mentioned genes belong to the LZ-NBS-LRR class of *R* genes, in contrast to *N* and *Y-1*, which belong to the TIR-NBS-LRR class. The four first-mentioned genes and *Y-1* confer four different types of virus-specific responses in related plant species (family *Solanaceae*). It will be an interesting subject for future studies to determine how the similar genes recognize different viruses and activate different responses. Transfer of the different *R* genes to the same species by genetic transformation and comparison of the signaling pathways and defense gene activation using the novel approaches of genome research should prove useful to this end.

MATERIALS AND METHODS

Plant material, plasmids, and bacterial strains.

The diploid potato lines 2x(v-2)7 and 84.194.30, the F1 progeny lines resulting from the cross of these (Hämäläinen et al. 1997, 1998; Valkonen et al. 1994b; Watanabe et al. 1994) and the potato cultivars used in this study have been previously described (Sorri et al. 1999). Plasmid vectors used for cloning were pCR 2.1 (Invitrogen, San Diego, CA, U.S.A.) and pUC119 (Clontech, Palo Alto, CA, U.S.A.). The *Escherichia coli* strains used were DH5 α (Hanahan 1983) and TOP10F' (Invitrogen,

Groningen, The Netherlands). Plasmid DNA and plant DNA and RNA were prepared using Qiagen DNA/RNA kits (Qiagen, Hilden, Germany) or standard techniques.

PCR, RACE-PCR, RT-PCR, and DNA sequencing.

PCR to generate ADG2 fragments was carried out as described by Sorri and associates (1999). PCR for detection of the SCAR markers associated with *Ry^{adg}* was carried out using the primers and conditions described by Kasai and associates (2000). When these primers were used to study gene expression, cDNA was synthesized using the primer SCAR-RT (5'-ATATT GGTTG AACAA CTGAA CAGCA-3'). To obtain the full-length cDNA sequences containing the ADG2 region, RACE-PCR was carried out using the Marathon cDNA amplification kit (Clontech) according to manufacturer's instructions, and gene-specific primers. The nested primers ADG2C (5'-TTTCC GAATG AAATG CTTGT CTCTC-3') and ADG2E (5'-CTGGT GCCAT TGCCA AACCA ACCAA G-3') spanning the 3' region of ADG2.1 were used together with adapter primers for amplification of 5' sequences. To obtain 3' sequences, the nested primers ADG2D (5'-AATTT GATGG TGCTT GTTTC CTTCC-3') and ADG2F (5'-GTGTG CATGA TAAGG AGGAC GGGAG GC-3') were designed to cover the 5' region of ADG2.1 and were used in combination with the adapter primers. The 5' and 3' sequences were cloned into plasmid vectors and 5 to 10 clones of each were analyzed by sequencing. To study splicing of intron 2 (ASI-2), we used PCR primers 5RyNot (5'-AATAA GCGGC CGCTT GATGG CATCA TCATC TTCTT CTTCT GAG-3') and ADG2E to amplify the cDNA synthesized using primer SCAR-RT. To study splicing of introns 4 and 5, we used primers 3prim-2 (5'-GGAGC TGATG ACGGA TTGAG GGTGC TG-3') and 3RyFse (5'-ATA TTT GGC CGG CCT TAG AGC TGT GAC CTT TGT TTC TTA G-3') to amplify cDNA synthesized using primer 3RyFse. cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, U.S.A.) and either 0.5 µg of total RNA or 50 ng of mRNA. Before cDNA synthesis, RNA was treated with RNase free DNaseI (Roche, Mannheim, Germany) for 2 h at room temperature. As a control for DNA contamination, PCR was carried out using approximately 100 ng of total RNA as a template. Samples that did not give rise to any amplification product using appropriate primers were considered DNA free. RT-PCR then was carried out using 1/20 of the volume from the first strand cDNA reaction as template. DNA sequencing was done with the cycle sequencing method using the DyeNamic DNA sequencing kit (AmershamPharmacia Biotech, Buckinghamshire, UK) and an ABI377 Sequencer (Perkin-Elmer, Boston, MA, U.S.A.).

Expression levels of *Y-1* mRNA were compared in the leaves of two transgenic potato lines (s1g-26 and s1g-30) using the RT-PCR based method of Timmusk and Wagner (1999). Samples were taken from full-grown leaves of 8-week-old plants (discussed above). For each RT reaction, 0.5 µg of total RNA and an oligo dT²⁵ cDNA synthesis primer were used. RT reactions were diluted 1:1, 1:10, and 1:100 and equal volumes of the dilutions were used as a template for PCR amplification with the SCAR primers described by Kasai and associates (2000).

Cloning of *Y-1* and full-length cDNAs, and construction and analysis of transgenic plants.

The full-length genomic and cDNA sequences were cloned using PCR primers directed to the ends of the transcripts identified by RACE-PCR. These primers, 5'38 (5'-CTCGT CGAGG TACAT GTGGC ATG-3') and 3'38 (5'-GCTCA CATTG TCAAC ATCTT CAGTC TCTTG-3'), also were used to screen potato lines for the presence of *Y-1*. PCR was carried out in a re-

action mixture containing 1× PCR buffer, 0.3 mM of each dNTP, 0.2 µM of each primer, 100 ng of template DNA, and Expand Long Template PCR System (Roche). Amplification was done in the PTC-100 Programmable Thermal Controller (MJ Research, Inc. Watertown, MA, U.S.A.) programmed at 94°C for 30 s followed by five cycles of: denaturation at 94°C for 30 s, annealing at 62°C for 45 s, and extension at 68°C for 10 min. Then, an additional 25 cycles were run as before, except that annealing was at 66°C. Finally, extension was done at 72°C for 10 min. Samples were analyzed on agarose gels to check for amplified products. Southern and dot blot analyses were carried out as described by Sambrook and associates (1989).

Construction of chimeric genes, generation of transgenic plants, and transfection of protoplasts.

The constructs for plant transformation (Fig. 5) were first engineered into the cloning vector pKOH122 by PCR amplification and insertion into a polylinker containing cleavage sequences for different restriction endonucleases recognizing eight nucleotides. Promoter and coding regions of the different constructs were PCR-amplified using synthetic oligonucleotides with specific restriction sites. A 955-bp fragment corresponding to bases -947 to +8 of the *CaMV* 35S promoter (Odell et al. 1985) was obtained by PCR amplification from the vector pHTT294 (provided by T. Teeri, University of Helsinki, Finland) (Koivu et al. 1995) and cloned into *AscI*-*NotI* sites in the cloning vector. Coding regions for *Y-1*, C-38, and C-19 were PCR amplified from plasmids containing these sequences and inserted into *NotI*-*FseI* restriction sites. The termination sequence used in all constructs was the 3' end of the T-DNA gene *g7*, including its polyadenylation signal. This region was introduced by cleavage with *FseI* and *Sse8387I* from the construct pKOH55 and insertion into the pKOH122 cloning vector into the same restriction sites. Fragments containing the promoter region, coding region, and termination sequence in each case were then cleaved with *PacI* and *Sse8387I* restriction enzymes from the cloning vector and introduced into the same sites from the transformation vector pKOH200 that contains the chimeric kanamycin resistance gene *pnos-nptII-3-ocs* as a selective marker. The pKOH vector plasmids (Holmström 1997) are derived from the plasmid pDE1001 (Denecke et al. 1992) and were kindly provided by K.-O. Holmström (University of Skövde, Sweden).

The final binary vectors were transferred by electroporation into *Agrobacterium tumefaciens* C58C1 containing the noncogenic Ti plasmid pGV3850 (Deblaere et al. 1985). The empty vector pKOH200 and the different vectors containing the chimeric genes described above were transferred to potato line v2-134 by infection of stem and leaf explants excised from 4-week-old in vitro shoots, and to the leaf explants of tobacco cv. Samsoun nn (Dunigan et al. 1987) excised from in vitro shoots grown from sterilized seeds. Transformation procedures and tissue culture conditions were essentially as described by Beaujean and associates (1998). Tobacco protoplasts were generated as described by Denecke and Vitale (1995). Transformants then were checked for *Agrobacterium* contamination by PCR using primers directed to a sequence outside the left and right border of the transformation vector pKOH200. Although this approach may have led to elimination of some stable transformants (Kononov et al. 1997), it was helpful for detection of lines contaminated with the binary vector-containing *Agrobacterium* spp. and their elimination from further experiments.

Virus inoculation and detection.

Potato plantlets were transferred from tissue culture to soil in an aphid-proof greenhouse or a growth chamber. In the

greenhouse, daylight was extended to 18 h using illumination from fluorescent lamps. Night and day temperatures were 20 to 18°C and 25 to 20°C, respectively. In the growth chamber (8.8 m², Weiss Umwelttechnik, Reiskirchen, Germany), plants were grown under highly constant conditions (photoperiod 18 h, light intensity 250 µEs⁻¹ m²; temperature 19 and 17°C during day and night, respectively; relative humidity 40%).

The virus isolates (PVY⁰-UK, PVA-U, and PVV-Dv42) have been previously described (Oruetebarria et al. 2000; Valkonen 1997). Viruses were maintained in potato plants. Infected potato leaves were ground in sterile distilled water (1 g per 5 ml) and the sap was rubbed on to two Carborundum-dusted lower leaves of two 3-week-old potato plantlets of each transgenic and control potato line. Symptoms were observed until 45 dpi. The uppermost fully expanded leaf of each plant was tested for virus by double-antibody sandwich ELISA using previously described reagents and procedures (Oruetebarria et al. 2000; Rajamäki et al. 1998).

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