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Note

A Bacterial Artificial Chromosome (BAC) Library for Potato and Identification of Clones Related to the Potato Y Potyvirus Resistance Gene Ry_{adg}

Hangning Zhang[1,2], Jari P. T. Valkonen[3,4] and Kazuo N. Watanabe*[1,5]

[1] *Laboratory of Plant Molecular Biology, School of Biology-Oriented Science and Technology*, Uchita, Naga, Wakayama 649-6493, Japan

[2] *Present address: Department of Agronomy, Nanjing Agriculture University*, 1 Weigang, Nanjing, 210095, China

[3] *Genetics Centre, Swedish University of Agriculture Science (SLU)*, P.O.Box 7025, S-75007 Uppsala, Sweden

[4] *Present address: Department of Applied Biology, University of Helsinki*, P.O. Box 27, FIN-00014, Helsinki, Finland

[5] *Present address: Gene Research Center, University of Tsukuba*, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8572, Japan

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Potato Y virus (PVY) is one of the most important viral pathogens globally in potato as well as in other species of *Solanaceae* including tomato, tobacco and pepper (Matthews 1991). The infected plants may suffer from severe foliage necrosis or even die. Yield reduction of up to 80% was brought by PVY in most potato-growing areas, especially in Northern Europe, Russia and North America. Moreover, PVY infection occurs elsewhere, e.g., in Africa, China and Latin America, the seed-potato harbors the virus causes inherent problems. A major resistance gene, namely Ry_{adg}, was identified from tetraploid cultivated species *Solanum tuberosum* subsp. *andigena* Hawkes, which confers extreme resistance (E) to PVY (Muñoz *et al.* 1975). Plants expressing E usually remain symptomless, except for the limited necrosis that may develop in the systemically infected leaves in a few genotypes following graft-inoculation, and the PVY titers are undetectable by ELISA in inoculated plants (Valkonen *et al.* 1996). Ry_{adg} has been genetically localized on chromosome XI and mapped in a 2.7 cM region flanked by RFLP markers *GP125/TG508* and *TG651* (Hämäläinen *et al.* 1997, 1998). In this region, other RFLP markers (Hämäläinen *et al.* 1997, 1998) as well as a cleaved amplified polymorphic sequence (CAPS) marker (Sorri *et al.* 1999) and a sequence characterized amplified region (SCAR) marker (Kasai *et al.* 2000) have found to be tightly linked to the resistance gene Ry_{adg}. Therefore, a map-based cloning of the gene Ry_{adg} will be possible if a genomic library covering the gene is available.

Since its introduction as a cloning system (Shizuya *et al.* 1992), bacterial artificial chromosome (BAC) has been extensively applied in plant genome analysis, e.g., physical mapping (Lapitan *et al.* 1997, Song *et al.* 2000), integration of physical and genetic maps (Tao *et al.* 2001), cytogenetic analysis based on BAC clone sequences (Dong *et al.* 2000, Hudakova *et al.* 2001, Lagudah *et al.* 2001) and positional gene cloning (Yang *et al.* 1998), because BAC has distinct advantages over other systems including: easy handling of the *E. coli* host, high transformation efficiency, easy recovery of the insert, and low frequency of rearrangement (Shizuya *et al.* 1992, Woo *et al.* 1994, Wang *et al.* 1995). To facilitate the map-based cloning of the PVY resistance gene Ry_{adg}, we constructed a BAC library using high molecular weight (HMW) DNA from a diploid potato breeding line 2x(V-2)7 which carries the gene Ry_{adg} conferring extreme resistance against PVY (Watanabe *et al.* 1994). Characterization of the BAC library and screening of the library with DNA markers linked to Ry_{adg} suggested that the library was useful for the positional cloning of the target resistance gene.

Illustrative protocol

Plant material: The diploid potato breeding line 2x(V-2)7 carrying PVY resistance gene Ry_{adg}, was provided by the International Potato Center (CIP), Lima, Peru (Watanabe *et al.* 1994). Plants were maintained and propagated by tissue culture. Whole plantlets after 3 weeks culture in MS (Murashige and Skoog 1962) liquid medium with 15 gL^{-1} sucrose (hormone-free) were harvested, frozen in liquid nitrogen and stored at −80°C.

Probes: Eight RFLP probes which have markers linked to the gene Ry_{adg}, i.e., GP125, TG508, CT182, CP58a, TG523, CD17, ADG2 and TG651 (Hämäläinen *et al.* 1997, 1998), were used to screen the BAC library for map-based cloning. PCR-based markers also have been employed for the verification of the RFLP result (Kasai *et al.* 2000, Sorri *et al.* 1999). The genetic distance between each marker and Ry_{adg} is shown in Fig. 1. Three tobacco chloroplast probes, pTBa4, pTS6 and pTBa2 were kindly provided by Dr. Y. Ogihara, Kihara Institute of Biological Research, Yokohama City University, Japan. The three probes were spaced almost equally apart in the tobacco chloroplast genome, ca. 50 kb

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*Corresponding author (e-mail: nabechan@gene.tsukuba.ac.jp)

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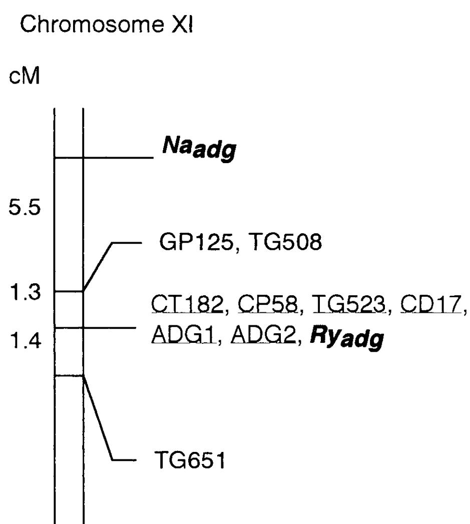

Fig. 1. Markers linked to the PVY resistance gene Ry_{adg} on chromosome XI of potato breeding line 2x(V-2)7. The numbers on the left indicate the genetic distance in cM. Tightly linked markers to Ry_{adg} are underlined. Another disease resistance gene Na_{adg} conferring hypersensitive resistance to potato A potyvirus (PVA) is mapped 5.5 cM from marker GP125/TG508 (Refer to Hämäläinen *et al.* 1997, 1998)

apart. In all cases, the inserts of the clones were purified on an agarose gel and then used as probes in the hybridization experiment.

BAC constructions: Preparation and cloning of HMW DNA fragments were conducted as described by Wang *et al.* (1995). The restriction enzyme, *Hind* III (Roche Diagnostics, Switzerland) was used for the partial digestion of genomic DNA and the digest was separated by PFGE (CHEF DRIII System, Bio-Rad, USA). After electrophoresis, the LMP agarose containing DNA fragments in size of 200–350 kb was excised from the gel and the BAC library was constructed using the vector, pBeloBAC11 (Research Genetics Inc., USA) in *E.coli* ElectroMAX DH10B cells (GIBCO BRL, USA) by electroporation using the Gene Pulser (Bio-Rad, USA). White colonies containing potato DNA inserts were picked up and transferred to 384-well microtiter plates (Nalge Nunc International, USA) containing 70 μl of LB freezing buffer (36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, 4.4% v/v glycerol, 12.5 μg/ml chloramphenicol, LB) each well. The microtiter plates were then incubated at 37°C for 24 h, in replicate and stored in −80°C freezers.

Characterization of the insert DNA: Randomly selected individual BAC clones were digested with *Not* I to release the DNA insert from the cloning vector (Sambrook *et al.* 1989, Song *et al.* 2000). The digested BAC DNA was analyzed by PFGE as described in Wang *et al.* (1995). The same gel was then subjected to Southern hybridization using the 2x(V-2)7 total DNA as a probe.

Screening of BAC library: Hybond-N+ filters (115 × 75 mm) (Amersham Life Science, USA) were inoculated with the bacterial clones in 384-well microtiter plates using the Nunc replication system. The inoculated filters were then placed on Nunc OmniTrays (Nalge Nunc International, USA) containing LB medium with 12.5 μg/ml chloramphenicol and incubated at 37°C until the colonies were ca, 1 mm in diameter (14–16 h). The filters were then used for colony hybridization using the eight probes related to Ry_{adg} as well as three chloroplast probes. We used the Gene Images AlkPhos Direct Kit (Amersham Life Science, USA) and followed the manufacturer's instruction for probe-labeling and hybridization. For hybridization, the filters were incubated at 55°C over-night. After hybridization, the filters were washed with 0.5 M phosphate containing 0.1% SDS at 60°C for 10 min twice and 1 M Tris, pH 10.0 at room temperature twice. The chemiluminescent hybridization signals were finally detected with CDP-star (Amersham Life Science, USA) and viewed on chemiluminescence film (Amersham Pharmacia Biotech, USA) after 0.5–3 h exposure depending on the signal intensity.

Positive candidate BAC clones identified by library screening with Ry_{adg} linked markers were isolated from the library, digested with restriction enzymes, run on an agarose gel, and blotted to a Hybond-N+ membrane (Amersham Life Science, USA), and probed with corresponding RFLP clones. The restriction enzymes were those originally used in the mapping of the Ry_{adg} with RFLPs (Hämäläinen *et al.* 1997, 1998). The membrane blotting was performed according to the manufacturer's instruction and hybridization conditions were the same as described for the colony hybridization.

CAPS and SCAR markers associated with Ry_{adg} were used according to the procedures reported by Sorri *et al.* (1999) and Kasai *et al.* (2000), respectively.

Quality and value of the potato BAC library

Genomic libraries with large cloned fragments are essential for genome analysis such as physical mapping, generation of chromosome contigs and isolation of genes through map-based cloning (Lapitan *et al.* 1997, Martin *et al.* 1992, 1993, Song *et al.* 2000, Wang *et al.* 1995).

We have constructed a BAC library for the potato breeding line 2X(V-2)7. The partially digested HMW DNA was cloned into the *Hind* III site of the pBeloBAC11 vector. The library consists of 28,800 clones stored in seventy five 384-well microtiter plates. All of the clones were derived from two ligation mixtures (100 μl each). Furthermore, after the first set of the work with the 28,800 clones, in addition to this, another set of 28,800 clones is being evaluated to increase the chance of selecting specific genomic regions. However, hereafter, the statements shall be focused on the first set of the 28,800 clones.

One electroporation could yield approximately 500 recombinant clones giving a transformation efficiency of 3.6×10^5 cfu per microgram insert DNA. To estimate the insert size of BAC clones, plasmids from 140 randomly selected individual clones were isolated, digested with *Not* I, and an-

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alyzed on a CHEF gel (Fig. 2A). The results showed that all of the clones contained inserts ranging in size from 40 kb to 320 kb with an average of 125 kb. More than 60% of the BAC clones have cloned fragments larger than 100 kb. The distribution of insert size is shown in Figure 3. To further confirm that the insert DNA in BACs was from potato and to estimate the extent of repetitive DNA sequences in the library, the BAC DNA on the gels shown in Figure 2A was Southern-blotted and probed with total genomic DNA of 2x(V-2)7 (Fig. 2B). All BAC clones gave hybridization signals, which suggests that all cloned fragments are from potato and contained repetitive DNA sequences.

We also tried to use larger fractioned HMW DNA in size of 350–600 kb for ligation and succeeding transformation. The transformation efficiency, however, turned out to be much lower with only a few recombinants generated by one electroporation. This is consistent with the observations in other reports of BAC construction (Song *et al.* 2000, Wang *et al.* 1995). However, the protocols described in this study were effective for potato BAC library construction. The cloned potato DNA fragments with an average of 125 kb in length were comparable to the majority of plant BAC libraries (Danesh *et al.* 1998, Tomkings *et al.* 1999, Wang *et al.* 1995).

To test the stability of BAC clones, we grew twelve randomly selected BAC clones and sub-cultured them serially for 5 days. The bacteria should have undergone 100 generations by the 5th day of subculture. Plasmid DNA isolated from the day 1 and day 5 cultures of bacteria were digested with *Hind* III and *Xho* I and separated by PFGE. Comparison of the generated DNA fingerprints did not reveal any differences, indicating the stability of the potato DNA in the BAC clones (data not shown).

One of the other major concerns is the occurrence of organellar DNA originated clones, especially from chloroplasts (Martin *et al.* 1992, Wang *et al.* 1995, Woo *et al.* 1994). Chloroplast DNA clones may mislead chromosome walking toward a target gene when a seriously contaminated library is used for map-based cloning due to homology between some organellar and nuclear DNA sequence (Timmis and Scott 1983, Zhang *et al.* 1996). Additionally, contamination of a chloroplast sequence could contribute to the overestimation of the genome content of the library. Use of the HMW DNA from isolated nuclei could reduce the probability of occurrence of chloroplast clones rather than use the protoplast in the BAC construction.

The BAC library was investigated in terms of contamination with clones originating from chloroplast DNA. A total of 4608 BAC clones from the 2x(V-2)7 potato library were hybridized with three tobacco probes, pTBa4, pTS6 and pTBa2 simultaneously. These three probe clones are spaced approximately equally apart in the chloroplast genome (ca. 50 kb) such that any BAC insert larger than 50 kb should hybridize to one of the probes, indicating that the insert is probably of chloroplast DNA origin. The results showed that 0.54% (25 clones) of the 2x(V-2)7 potato BAC clones originated from chloroplast DNA.

It is comparable to some reported BAC libraries, i.e., 0.3% in the rice cultivar IR-BB21 BACs (Wang *et al.* 1995), 2.9% in a *indica* rice BACs and 2.9% in a *japonica* rice BACs (Zhang *et al.* 1996), but rather lower than the 14% in the *Sorghum* BACs developed from the protoplast (Woo *et al.* 1994). Therefore, 99.45% of the clones in our constructed library were of nuclear DNA origin and covered at least 2834 megabase of the nuclear genome. Based on an estimated genome size of 860 Mbp/1C for diploid potato (Arumuganathan and Earle 1991), this library represents approximately 3.3 haploid genome equivalents. Theoretically, this gives us a probability of 0.97 for finding a specific sequence from this library (calculated after Clarke and Carbon 1976).

The cloned fragments from 140 randomly selected BACs were characterized. The fact that each BAC clone contained different inserts and different hybridization sig-

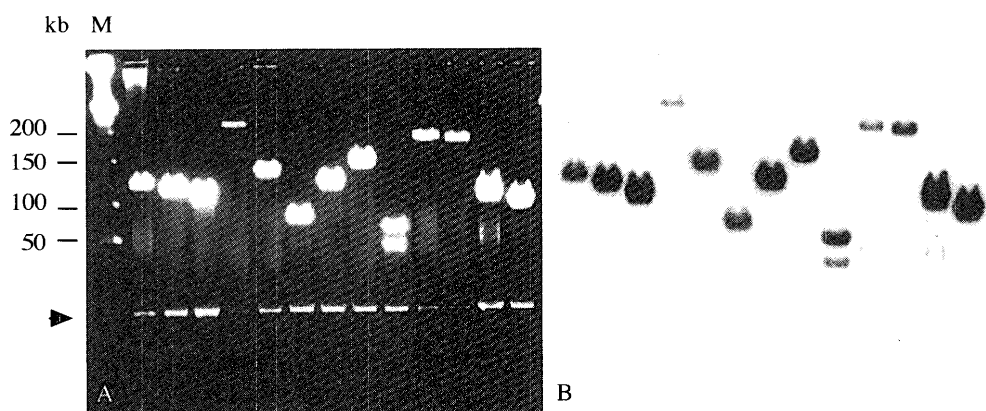

Fig. 2. Insert analysis of the BAC library. A) Ethidium bromide stained agarose gel showing randomly selected BAC clones digested with *Not* I and separated by PFGE. The arrow indicates the 7.4 kb pBeloBAC 11 vector viewed in all clones. M, molecular size marker (sizes are given in kb). B) Southern analysis of gel A which was transferred to a nylon membrane and probed with total DNA of 2x(V-2)7.

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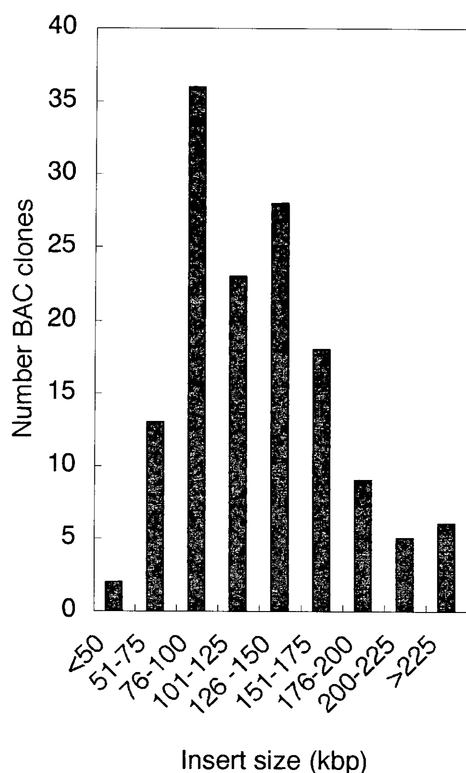

Fig. 3. Insert size distribution of the clones in the potato library. To estimate the insert size range, plasmid DNA from 140 randomly selected BAC clones was analyzed as shown in Figure 2A. Results indicate that the average insert size is 125 kbp with over 60% clones larger than 100 kbp.

nals suggests that these clones have different origin and that the potato library may be representative and suitable for physical mapping and the map-based cloning. The representation of the BAC library was also testified by screening the library with RFLP probes with each probe detecting an average of 3.6 clones.

BAC clones associated with Ry_{adg}

To facilitate positional cloning of the Ry_{adg}, gene in potato line 2x(V-2)7, eight RFLP markers linked to Ry_{adg}, GP125, TG508, CT182, CP58a, TG523, CD17, ADG2 and TG651 (Fig. 1) (Hämäläinen *et al.* 1997, 1998) were used to screen the BAC library. At least one positive clone could be identified for each probe. The probe ADG2, which is a resistance gene-like (RGL) fragment (Hämäläinen *et al.* 1998), detected 52 positive clones out of 19,200 BACs, suggesting that there are more than 20 copies of this fragment in one haploid genome. Seven other probes detected totally 25 positive clones from the whole library with an average of 3.6 per probe. The probes and number of detected clones are shown in Table 1.

All of the RFLP markers showed the presence of multiple restriction fragments in the 2x(v-2)7 (Table 1). These fragments may represent different loci in the genome. To determine whether the positive BAC clones identified by library screening corresponded to the RFLPs linked to Ry_{adg}, we digested the candidate BAC clones by the restriction enzyme originally used to map the RFLPs, blotted to a nylon membrane and hybridized by corresponding probes. If the BAC clone showed a restriction fragment in the fingerprint with the same size as the RFLPs which had been mapped near Ry_{adg} (Hämäläinen *et al.* 1997, 1998), we inferred that this clone originated from the RFLP locus linked to Ry_{adg}. All the 9 RFLPs detected by the ADG2 probe in 2x(V-2)7 (Hämäläinen *et al.* 1998) were identified from 54 ADG2 candidate BAC clones, while two potentially overlapped BAC clones, i.e., clone 46B17 and 49D15, contained the 3.5 kb target restriction fragment which was reported to co-segregate with Ry_{adg} (Hämäläinen *et al.* 1998) (Fig. 4). Clone 46B17 and 49D15 had two and three restriction fragments detected by the ADG2 probe, respectively (Fig. 4). These clones were confirmed by CAPS (Sorri *et al.* 1999) (data not shown) and SCAR (Kasai *et al.* 2000) (Fig. 5) markers. Using the probes GP125, TG508, TG523 and TG651, we identified one BAC clone containing the corresponding RFLP locus related to Ry_{adg} (Table 1). From the candidate BAC clones of three other probes CT182, CP58 and CD17, we found no RFLPs related to Ry_{adg} but detected other restriction fragments with different sizes representing other loci of these probes (Table 1).

Towards positional cloning of plant resistance genes

For the purpose of facilitating the map-based cloning of

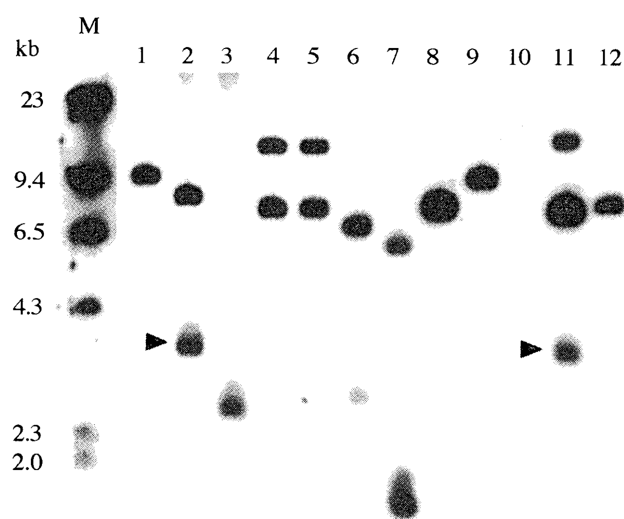

Fig. 4. Identification of the BAC clones containing the ADG2 fragment related to Ry_{adg}. Plasmid DNA of candidate ADG2-positive BAC clones were digested with *Eco* RI, run on an agarose gel, blotted to Hybond N+ membrane, and probed with ADG2. The arrows indicate the clone 46B17 and 49D15 (lanes 2 and 11, respectively) comprising the 3.5 kb fragment which was co-segregated with Ry_{adg} in a segregation analysis (Hämäläinen *et al.* 1998). Lane 10 was a false clone detected in colony hybridization but could not give any hybridization signals here. The gel slice containing the molecular size marker was cut and subjected to Southern hybridization separately.

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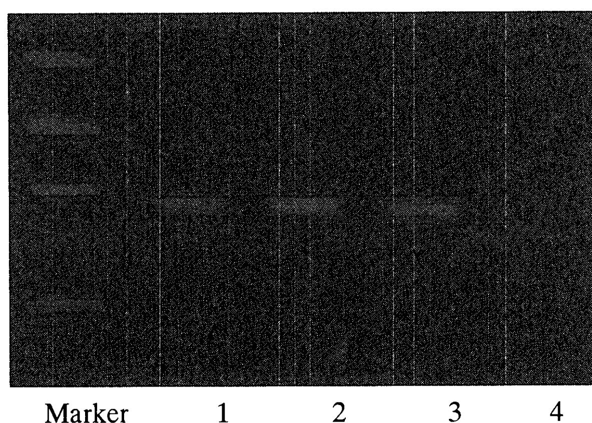

Fig. 5. Representative result on sequence characterized amplified region (SCAR) marker identification using the primer set RYSC3 for Ry_{adg} of Kasai *et al.* (2000). Marker: 500, 400, 300 and 200 bp, respectively.
1.2x(V-2)7, 2. BAC clone 46B17, 3. BAC clone 49D15, 4. pBeloBAC11.
Positive clones with Ry_{adg} show about 270 bp PCR product.

PVY resistance gene Ry_{adg}, we identified totally six BAC clones to be related to the target gene by screening the library and succeeding Southern hybridization of the positive clones with eight RFLP markers linked to Ry_{adg} (Table 1, Fig. 4). Ry_{adg} was mapped within a 2.7 cM region between the RFLP markers *GP125/TG508* and *TG651* on chromosome XI (Hämäläinen *et al.* 1997, 1998) (Fig. 1). The BAC clones identified to contain the corresponding RFLPs of these three markers can be used to initiate chromosome walks toward the gene Ry_{adg}. Three other clones corresponding to the marker *TG523* and *ADG2*, which are tightly linked to Ry_{adg}, can be used as anchors in the construction of a BAC contig containing this gene. However, if we assume that a 1 cM genetic distance equals approximately 750 kb in potato genome (Tanksley *et al.* 1992), 2.7 cM is about 2025 kb in physical distance. Besides the high amounts of repetitive sequences in the BAC clones, the mapping individuals with 54 individuals for Ry_{adg}, was relatively smaller (Hämäläinen *et al.* 1997, 1998). These may bring difficulties in the map-based cloning of Ry_{adg}.

Plant disease resistance genes are usually arranged in clustered multigene families in the genome (Dixon *et al.* 1996, Jones *et al.* 1994, Martin *et al.* 1993, van der Vossen *et al.* 2000) and share some structure similarity and conserved motives (Dangl and Jones 2001, Leister *et al.* 1996, Staskawicz *et al.* 2001). These characters have led to PCR-amplification of resistance-gene-like fragments (RGL) from different plant species (Kanazin *et al.* 1996, Leister *et al.* 1996, Shen *et al.* 1998, Speulman *et al.* 1998, Yu *et al.* 1996). The ADG2 probe used in this study is one such RGL fragment derived from purified mRNA pools of 2x(V-2)7 and was found to co-segregate with Ry_{adg} (Hämäläinen *et al.* 1998, Sorri *et al.* 1999). Sorri *et al.* (1999) even postulated that the ADG2 fragment was part of the gene Ry_{adg}. Here we identified two BAC clones containing ADG2 fragment and the locus has been verified by RFLP fingerprinting (see results). Further sequence analysis of these two BAC clones will reveal if they have open reading frames with similarity to cloned plant disease resistance genes. Nowadays, vectors which can harbor HMW DNA and suitable for direct plant transformation are available (Hamilton *et al.* 1996, Liu *et al.* 1999). Complementation studies by transferring the inserts from the two BAC clones identified in this study to susceptible potato line will provide direct evidence for the successful positional cloning of Ry_{adg}.

The diploid potato breeding line 2x(V-2)7 carries another disease resistance gene, i.e., Na_{adg} conferring hypersensitive resistance against potato A potyvirus (PVA). It was also mapped on chromosome XI with 5.5 cM to the locus of marker *GP125/TG508* (Fig. 1) (Hämäläinen *et al.* 1998, 2000). Thus the positive BAC clones to marker GP125 and TG508 can also be used as a starting point for chromosome walking toward Na_{adg}. Actually, as proposed by Vinatzer *et al.* (1998), use of the BAC library constructed from 2x(V-2)7 is not restricted to the cloning of genes present in this potato line. For example, a DNA marker linked to a gene absent in 2x(V-2)7 can be used as a probe to screen the BAC library and to perform chromosome walking. The BAC clone containing the locus of the gene can then be employed as a probe to screen a cDNA library of a variety containing the gene allele of interest and then the gene can be isolated. Thus, the BAC library for 2X(V-2)7 can be applied to map-based cloning of genes from other potato varieties.

In summary, we have constructed a BAC library for potato line 2x(V-2)7. The library has a proper insert size, well

Table 1. Identification of the BAC clones related to the genomic region associated with Ry_{adg} that confers the resistance to PVY

RFLP Probe	# BAC clones screened	# positive BAC clones in colony hybridization	# RFLP signals detected in 2x (V-2)7	Size of restriction fragment linked to Ry_{adg} (kbp)	BAC clones with corresponding RFLPs to Ry_{adg}
ADG2	19200	54	9	3.5	46B17, 49D15
CD17	28416	2	2	6.0	0
CP58	28416	4	4	2.8	0
CT182	28416	2	5	2.0	0
GP125	28416	4	4	6.5	48I4
TG508	28416	3	4	3.5	15O18
TG523	28416	9	3	3.5	9B14
TG651	28416	1	1	6.5	11B14

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representing the genome with a low ratio of chloroplast and chimeric clones. It should be competent for genome physical mapping, map-based cloning of gene or other possible uses. Six BAC clones related to the PVY resistance gene Ry_{adg} were identified with two containing the RGL fragment ADG2. Physical cloning of the gene Ry_{adg} is underway.

Simplification of tetraploid genetics by diploid genetics lines

Final objects of marker development, gene cloning and genomics using the diploid potato clone, 2x(V-2)7 is to simplify the tetraploid potato genetics at the diploid level (Peloquin *et al.* 1989). A BAC library with a diploid potato wild species, *S. bulbocastanum* was reported by Song *et al.* (2000), claiming that it is helpful for understanding tetraploid potato genetics. However, this library has weaknesses (Watanabe *et al.* 1995) : 1) this species has been used merely for breeding, 2) this species has been recognized as far distantly related genetically to cultivated potatoes, and 3) interspecific hybridization with diploid and cultivated potatoes was difficult because of high cross incompatibility. The present BAC library shall be far more effective for studying the cultivated potato genomes. Indeed, the number of BAC clones in our library would be sufficient for gene cloning at the diploid level, but we have obtained an additional set of 28,800 BAC clones that we are evaluating for further screening by increasing the statistical chance of selecting candidate clones of interest.

On the other hand, if one were to work at the tetraploid level, a two or three times larger library size would be needed. Also a comparative genomics study between diploids and tetraploids can be done by a constructing a library at the tetraploid parental counterpart clone, for examining the constitution of the tetrasomic polyploid genome.

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