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Genomic distribution and characterization of EST-derived resistance gene analogs (RGAs) in sugarcane

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Abstract A large sugarcane EST (expressed sequence tag) project recently gave us access to 261,609 EST sequences from sugarcane, assembled into 81,223 clusters. Among these, we identified 88 resistance gene analogs (RGAs) based on their homology to typical pathogen resistance genes, using a stringent BLAST search with a threshold e-value of e^{-50}. They included representatives of the three major groups of resistance genes with NBS/LRR, LRR or S/T KINASE domains. Fifty RGAs showed a total of 148 single-dose polymorphic RFLP markers, which could be located on the sugarcane reference genetic map (constructed in cultivar R570, $2n = \sim 115$). Fifty-five SSR loci corresponding to 134 markers in R570 were also mapped to enable the classification of the various haplotypes into homology groups. Several RGA clusters were found. One cluster of two LRR-like loci mapped close to the only disease resistance gene known so far in sugarcane, which confers resistance to common rust. Detailed sequence comparison between two NBS/LRR RGA clusters in relation to their orthologs in rice and maize suggests their polyphyletic origins, and indicates that the degree of divergence between paralogous RGAs in sugarcane can be larger than that from an ortholog in a distant species.

Keywords Sugarcane · Polyploid · Genetic mapping · Resistance gene analogs (RGAs) · Nucleotide binding site/leucine rich repeat (NBS/LRR)

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Introduction

Pathogen recognition is the first step in the process that triggers plant resistance responses, and is usually mediated by single dominant resistance genes (*R* genes). Each of these gene products interacts directly or indirectly with the product of a corresponding avirulence (*Avr*) gene in a pathogen (Flor 1971; Keen 1990). Many R proteins from different dicot and monocot plant species, which confer resistance to a wide variety of pathogens, share several conserved motifs (Hammond-Kosack and Jones 1997). Based on the deduced structure of their products, *R* genes can be classified into three main groups. Members of the major group encode proteins with a nucleotide binding site (NBS) domain followed by a leucine rich repeat (LRR) region. This group can be further sub-divided into two classes based on the nature of its N terminal region. Proteins in the first class show homology to Drosophila Toll or the human interleukin receptor (TIR); whereas proteins in the second class have a coiled coil (CC) domain (Hammond-Kosak and Jones 1997; Pan et al. 2000). The second group of *R* genes encodes proteins with only LRRs (Dixon et al. 1998). The third group, represented exclusively by *Pto* (Martin et al. 1993), displays a serine-threonine kinase (S/T KINASE) domain. In addition, one example of an R protein with an LRR followed by a S/T KINASE has been reported (Song et al. 1995). As new genes with novel motifs are cloned, new classes of *R* genes are emerging; this is the case for the genes *RPW8* (Xiao et al. 2001) and *Rpg1* (Brueggeman et al. 2002).

Isolation of *R* genes has historically involved map-based cloning or transposon tagging, both of which are very labor-intensive and expensive strategies. The common features shared by R proteins have led to new cloning strategies. Since the late 90s, PCR primers corresponding to highly conserved amino acid sequences of the NBS domain have been used to amplify resistance gene analog (RGA) fragments from various plant species (Wang et al. 2001). Many of these RGAs appear to be linked to previously described resistance loci or QTLs. However, *R* genes are often members of multigene families, frequently organized in clusters, and this PCR strategy generally fails to identify the functional *R* genes within a given cluster (Graham et al. 2000).

Sugarcane is an economically important crop. However, analysis of its genome has lagged behind, compared to other important grass species, mostly due to its genetic complexity. Modern sugarcane cultivars are highly polyploid (2n = 100–130), derived from interspecific hybridizations between the domesticated sugar-producing species *Saccharum officinarum* L. (2n = 80) and the wild species *S. spontaneum* L. (2n = 40–128). They thus represent a particular challenge for breeding, genetics and gene cloning purposes (Butterfield et al. 2001; D'Hont and Glaszmann 2001; Grivet and Arruda 2001).

The Brazilian Sugarcane EST Sequencing Project (SUCEST) database, with 291,689 EST sequences, provides an invaluable source of information (Vettore et al. 2001). In this paper, we report the results of a search for resistance gene analogs (RGAs) using the SUCEST database. We have mapped these RGAs on the sugarcane reference genetic map (Grivet et al. 1996; Hoarau et al. 2001 and unpublished data), in order to investigate their genomic distribution and their relationship with disease resistance loci in sugarcane. Fifty-five single-sequence-repeat (SSR) loci were also mapped to allow the classification of the different haplotypes into homology groups. In addition, we compared the sequences of various members of two NBS/LRR resistance gene clusters to those of their orthologs in rice and maize.

Materials and methods

SUCEST database and sequence analysis

The SUCEST database encompasses 291,689 EST sequences derived from 37 different sugarcane cDNA libraries constructed from total RNAs isolated from various tissues, developmental stages and stress conditions including pathogen inoculated seedlings (Vettore et al. 2001). A total of 261,609 sequences have been grouped into 81,223 clusters based on an analysis with the phrap fragment assembly program. Results of comparisons between cluster consensus sequences and GenBank data were available for homology searches (Telles et al. 2001).

The 81,223 clusters were screened to identify RGAs. "NBS-LRR" and "disease resistance" were used as keywords, and *Mi-1.2* (gi3449380), *Rpm1* (gi963017), *RPS2* (gi549979), *Xa21* (gi1122443), *Prf* (gi1513144), *Pto* (gi430992), *Cf-2.1* (gi1184075), *N* (gi558887), *L6* (gi862905), *M* (gi1842251), *Pti1* (gi3668069), *RPR1* (gi4519936), *I2* (gi4689223), *Hcr2–5D* (gi7488988), *Hs1* $^{pro-1}$ (gi1850968), *b5* (gi2792210) and *Rp1-D* (gi5702196) coding sequences as key genes. The genes were chosen to represent a broad range of plants, pathogen specificities, and R protein structures known at the time the searches were carried out. To avoid spurious hits due to the enormous amount of data, a very stringent expectation value of e^{-50} or better was used.

Plant material

The progeny analyzed in this study consisted of 112 individuals obtained by the self-fertilization of cultivar R570; this is a subset of the population used to build an AFLP genetic map by Hoarau et al. (2001). R570 is a rust-resistant cultivar developed by CERF (Center d'Essai de Recherche et de Formation, Réunion). Rust resistance phenotypes were determined in the field on the island of Réunion, using natural infection as described in Daugrois et al. (1996).

Restriction Fragment Length Polymorphism (RFLP) analysis

The 55 selected clone sequences were amplified by PCR with universal primers (T7, T3, SP6). The PCR products were purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) and radioactive random priming labeling was carried out with the Megaprime DNA Labeling System (Amersham Pharmacia Biotech). Genomic DNA extraction, Southern blotting, and hybridizations were performed as previously described by Grivet et al. (1996). The enzymes used for DNA digestion were *Hin*dIII, *Sst*I, *Dra*I and *Eco*RV.

Simple Sequence Repeat (SSR) analysis

The progeny was analyzed with 76 SSRs developed at CIRAD in collaboration with Génoscope (Evry, France) from an enriched library made with DNA from the cultivar R570, and these markers were localized on a reference RFLP map (in preparation). The primers were end-labeled with [γ-^{33}P]ATP, and amplification was performed in an MJ Research PTC 100 Thermal Cycler in 20-µl reaction mixtures containing 50 ng of sugarcane DNA, 0.2 mM dNTP mix, 2 mM $MgCl_2$, 50 mM KCl, 10 mM TRIS-HCl (pH 8.3), each primer at 0.2 µM, and 1 U of *Taq* polymerase (Eurobio). The samples were denatured at 94°C for 5 min and subjected to 35 cycles of 94°C for 1 min, 46°C–55°C (depending on the SSR primer sequence) for 45 s, and 72°C for 30 s, followed by an extension step for 10 min at 72°C. After the addition of 20 µl of loading buffer (98% formamide, 10 mM EDTA, bromophenol blue, xylene cyanol), the amplified products were denatured at 92°C for 3 min, and 4 µl of each sample was loaded onto a 5% polyacrylamide gel with 7.5 M urea and electrophoresed in 0.5% TBE buffer at 55 W for 1 h 40 min. The gel was dried for 30 min at 80°C and exposed for 4 days to X-ray film (Fuji RX).

Marker scoring, analysis and map construction

Each segregating RFLP and SSR band was scored independently as a dominant marker (presence vs. absence) and the following nomenclature was adopted; for RGAs: RGA, followed by three digits indicating the EST clone number, then three letters indicating the enzyme used to reveal the marker and a letter indicating the marker; for SSRs: mSSCIR (microsatellite, *Saccharum* Spp, CIRAD), followed by the number of the SSR, and then the letter 'm' followed by a number indicating the marker. Since sugarcane is highly polyploid, only single-dose markers (Wu et al. 1992) were used for map construction. Such markers show a segregation ratio that is not significantly different (by the $\chi 2$ test) from 3:1 (presence:absence) at $P = 0.05$ (Grivet et al. 1996).

The single-dose markers were added to the AFLP matrix (883 markers × 112 individuals) developed by Hoarau et al. (2001). The new map was built using MAPMAKER 3.0 (Lander et al. 1987). Marker grouping was performed by two-point analysis at a LOD score threshold of 5 and a recombination fraction threshold of 0.35. Co-segregation groups (CGs) were then ordered by multipoint analysis and the distances calculated using the Haldane function. For homology group VII, we had additional data and thus the map distances were calculated with data from 316 individuals. CGs were assembled into homology groups (HGs) based on (1) common RGA or SSR markers between CGs; and (2) common SSR and AFLP markers with a R570 map encompassing mainly RFLP markers (Grivet et al. 1996, and unpublished results). A minimum of two common markers was necessary for assembly of two CGs into the same HG. When a correspondence between HG and CG could be established between the two maps, we assigned the same name to them, a Roman numeral from I to VIII for the HG, and a number for the CG. Assigned CGs with no correspondence between the two maps were named with the number of the HG followed by a letter. CGs not assigned to a HG were named as U (unassigned) followed by a number.

Analysis of clusters of NBS/LRR-like RGAs

The full length sequences of eight NBS/LRR-like EST clones (RGA118, RGA281, RGA326, RGA185, RGA267, RGA162, RGA152 and RGA087) were obtained by primer walking. Nucleotide sequences were aligned using the program Sequence Navigator 1.0.1 for Macintosh. Sequence variability was estimated using Nei's measure of nucleotide diversity (π) and calculated with the program DnaSP (Rozas and Rozas 1997).

Results

Identifying RGAs in the SUCEST database

Key gene and keyword searches in the SUCEST database identified 88 clusters homologous to known pathogen resistance genes with an expectation cut-off value, for the best matching query, of e^{-50} or better. Twenty-two ESTs presented homology to genes encoding NBS-LRR resistance proteins, 13 showed homology to LRR-coding genes and 53 were S/T KINASE homologs. No TIR/NBS/LRR-like RGAs were identified, even though genes encoding these three domains (like *N, L6* or *M*) were used as key genes (Table 1). Matches to the NBS or LRR regions of these genes had poorer e-values than did CC/NBS/LRR genes.

A single clone per cluster was selected for further analysis. To increase the likelihood of obtaining full length mRNAs, we chose the most 5′ clone. After identity confirmation by sequencing, 55 of the 88 clones analyzed were selected for mapping. We excluded clones that were wrongly addressed, showed evidence of rearrangement or represented redundant information. Table 1 indicates, for the 55 ESTs, the corresponding cluster-consensus homology and the relevant protein domain (Genbank accession numbers: BQ803996 to BQ804049). Only the best hits against a known *R* gene or RGA are included in Table 1. Hence, not all clones listed show an e value of e^{-50} or better. A number of clusters in Table 1 are indicated as *Pti1* homologs. *Pti1* is not a resistance gene, but is a *Pto* interactor which shares 36.4% overall protein identity with it (Zhou et al. 1995).

The distribution of RGAs in the sugarcane genome

Fifty-five ESTs were tested on the self-progeny of cultivar R570; no polymorphisms were detected for three of them (RGA251, RGA231 and RGA176) with any of the four enzymes assayed. The other 52 ESTs produced 272 polymorphic markers (an average of 5.23 markers/probe) and, of these, 177 segregated as single-dose markers (3:1 ratio, average of 3.40 markers/probe) and could be used for mapping. Out of these 177 markers, 148 markers corresponding to 50 RGA clones, were localized on the AFLP map (Hoarau et al. 2001) while the others remained unlinked (Fig. 1). Seventy-six SSRs tested on the same progeny produced 170 single-dose markers, of which 134, corresponding to 55 SSRs, were localized on the map. SSR and RGA markers were used to assemble co-segregation groups (CGs) into homology groups (HGs) as described in Materials and methods. The map encompasses 128 CGs, of which 66 could be assigned to seven of the eight HGs in the reference RFLP maps (Grivet et al. 1996, and unpublished results). The RGA markers map on 59 of the 128 CGs. They are present in all seven identified HGs. Six RGAs map on HG I, seven on HG II, two on HG III, six on HG IV, seven on HG VI, two on HG VII and 16 on HG VIII. Alleles of the same RGA map mainly onto the same HG, with four exceptions: RGA142 and RGA526 map on HG IV and HG VIII, RGA258 maps on HG II and HG VI, and RGA149 maps on HG III and HG VI.

RGAs are not equally distributed along the chromosomes. RGAs that were not more than 5 cM apart were defined as members of a cluster. On this basis, we determined all cluster loci, referred to the basic genome complement, that contain different RGAs (Table 2). We identified four cluster loci with three to six different RGAs, and six cluster loci with two different RGAs. Clusters 1, 7 and 8, contain four, six and three RGAs, respectively, that map on several homologous chromosome segments of HG I and VIII. In these three cases, not all RGAs were mapped on all the homologous CGs. The distance between RGAs on each CG is variable, but in at least one CG the distance between each pair of consecutive RGAs is ≤ 5 cM.

Sixteen RGAs produced more than one marker on the same CG. The majority are clustered and have been identified with an asterisk in the CG column in Table 1. Some of these markers may be redundant, identifying the same allele due to the presence of a restriction site in the RGA sequence. However, since a few of them are separated by recombination events, we retained all of them on the map.

To date, the only pathogen resistance locus mapped in sugarcane is the common rust resistance gene located

Table 1 Characteristics and map location of the 55 EST-RGA studied

RGA	EST clone	Phrap cluster	Cluster consensus homology				Map position[c]		
			Gene[a]	Species[b]	e-value	Domain	HG	CG (bands mapped)	Copies
482	SCSFSB1102C02.g	SCJLRT1020F05.g	*Cf-2.1*	Lp	2e-78	LRR	VI	VI2, U5	9
183	SCEQRZ3024A05.g	SCEQRZ3024A05.g	*Xa21*	Ol	1e-08	LRR	I	I6, I5	~25
131	SCCCST1C06H04.g	SCSGLR1045B05.g	*Xa21*	Ol	2e-75	LRR	VIII	VIII2	6
386	SCRFLV1037B07.g	SCVPRT2081F09.g	*Xa21*	Ol	1e-67	LRR	II	II8, II6	7
327	SCQGAM2108A06.g	SCQGAM2108A06.g	*Xa21*	Os	4e-65	LRR	–	–	–
366	SCRFAD1117H03.g	SCMCRT2102E02.g	*Xa21*	Ol	5e-62	LRR	II	II9, II10, II12	10
173	SCEQRT2028B11.g	SCEQRT2028B11.g	*Xa21*	Os	5e-53	LRR	VIII	VIII5(3)*, VIII15(2)*, VIII16, VIII2	11
024	SCAGFL3024G01.g	SCSGRT2064H06.g	*Hcr2-5D*	Le	2e-34	LRR	I	I7, Ia, I6, I5(2)*	7
149	SCEPRZ3048F07.g	SCCCLR1001A03.g	*Xa21*	Ol	1e-84	LRR	III, VI	III4, VI3, VI5	11
137	SCEPAM1051A02.g	SCEPAM1051A02.g	*Hcr2-2A*	Lp	1e-31	LRR	VII	VII1a, VIIa(3)*	~16
019	SCCCLR1066C04.g	SCCCLR1066C04.g	*Hcr2-0A*	Le	2e-16	LRR	VII	VII14, VIIa	~24
441	SCSBAD1126D07.g	SCSBAD1126D07.g	*I2C-2*	Le	2e-43	NBS/LRR	IV	IV1(2)*, IV2(2)*	11
251	SCJFSB1010F08.g	SCEQSB1C01F10.g	*I2C-2*	Le	5e-22	NBS/LRR	–	–	–
088	SCCCCL3080E03.g	SCCCCL3080E03.g	*Xa1*	Os	9e-32	NBS/LRR	–	U7	~13
118	SCCCNR2002A04.g	SCJLRZ1019B10.g	*RPR1*	Os	0.0	NBS/LRR	VIII	VIIIa, VIII5, VIII15(2)*, VIII2	~16
016	SCAGAM2124A05.g	SCAGAM2124A05.g	*Pib*	Os	5e-25	NBS/LRR	–	U4	~15
057	SCBFSB1045F08.g	SCCCAM2002B02.g	*I2*	Le	2e-46	NBS/LRR	I	Ia, I9(2)*, Ib	12
039	SCBFAD1089A09.g	SCBFAD1089A09.g	*PIC17*	Zm	3e-42	NBS/LRR	VIII	VIII2, VIII9, VIII11	9
272	SCJLLB2079H05.g	SCSGAD1006H08.g	*RPP1-WsA*	At	3e-18	NBS/LRR	VI	VI3, VI2, VIb, VIa, U36	7
542	SCUTST3130D10.g	SCCCFL2002E03.g	*Hv1LRR2*	Hv	3e-35	NBS/LRR	VIII	VIIId, VIIIe	6
196	SCEZAM2031E03.g	SCEZAM2031E03.g	*Gpa2*	St	2e-35	NBS/LRR	VI	VI2, VI1a(2)*, VIa, VIb	~12
145	SCEPLB1044F11.g	SCEPLB1044E11.g	*TMV*	At	8e-72	NBS/LRR	II	IIa	6
162	SCEQAD1016E12.g	SCEQAD1016E12.g	*Rp1-D*	Zm	9e-80	NBS/LRR	–	U11(3)*	~12
326	SCQGAM2029B12.g	SCMCST1052G12.g	*RPR1*	Os	7e-73	NBS/LRR	VIII	VIII1, VIII2, VIIIa(2)*, VIIIb, VIII5	8
267	SCJLFL3019A12.g	SCJLFL3019A12.g	*RPR1*	Os	5e-63	NBS/LRR	VIII	VIIIb, VIII5	~17
185	SCEQRZ3090E04.g	SCRLRZ3043E10.g	*RPR1*	Os	1e-61	NBS/LRR	VIII	VIII1, VIII2, VIIIa, VIIIb, VIII5	8
281	SCJLRZ1018G01.g	SCJLRZ1018G01.g	*RPR1*	Os	2e-59	NBS/LRR	VIII	VIII1, VIII2, VIIIa(2)*, VIIIb, VIII5	10
087	SCCCCL3080B03.g	SCCCCL3080B03.g	*Rp1-D*	Zm	1e-99	NBS/LRR	–	U11	4
152	SCEPRZ3130D02.g	SCEPRZ3130D02.g	*Rp1-D*	Zm	2e-83	NBS/LRR	–	U11, U51	13
313	SCMCSD1063E01.g	SCCCLR1072H06.g	*Pto*	Le	5e-99	S/T KINASE	I	I6(2)*, I9(2)*	11
488	SCSFSD1066C01.g	SCEQRT2096A03.g	*Pto*	Le	1e-98	S/T KINASE	VIII	VIII11, VIIIg, U58	8
231	SCJFLR1071E05.g	SCJFLR1071E05.g	*Pti1*	Le	4e-59	S/T KINASE	–	–	–
258	SCJFST1047F02.g	SCRFLB1056D11.g	*Pti1*	Le	8e-75	S/T KINASE	II, VI	II9, II10, VI2	8
184	SCEQRZ3089A06.g	SCJFRZ1007B02.g	*Pti1*	Le	2e-61	S/T KINASE	IV	IV1(2)	~13
526	SCUTFL1064A02.	SCVPLB1015A04.g	*Pto*	Le	8e-58	S/T KINASE	IV, VIII	IV1, VIII3, VIII4	9
083	SCCCAM2097B01.g	SCMCCL6052C09.g	*Pto*	Le	3e-55	S/T KINASE	I	I7, I4, Ic	~11
406	SCRLLV1051H06.g	SCRULB1062B02.g	*Pto*	Le	3e-55	S/T KINASE	VIII	VIII2	6
142	SCEPFL3088D07.g	SCQGST1032C12.g	*Pto*	Le	2e-53	S/T KINASE	IV, VIII	IV1, VIII3, VIII4	~11
169	SCEQHR1079G02.g	SCBFLR1046A11.g	*Pti1*	Le	1e-159	S/T KINASE	II	II9, II10, II8	11
342	SCQGSB1080F03.g	SCJFST1012D01.g	*Pto*	Le	4e-56	S/T KINASE	VIII	VIII2, VIII11(2)	10
396	SCRLFL1010A02.g	SCMCLR1053B12.g	*Pto*	Le	5e-50	S/T KINASE	VI	VIc, VId, U51	6
335	SCQGFL8014H09.g	SCQSLR1061E06.g	*Pto*	Le	6e-50	S/T KINASE	VIII	VIII2, VIII11(2)*	14
372	SCRFFL1030E07.g	SCCCRZ2C03F07.g	*Pti1*	Le	1e-131	S/T KINASE	IV	IV1(2)*	8
012	SCACRZ3109D03.g	SCJFRT1059H08.g	*Pti1*	Le	1e-96	S/T KINASE	II	IIb, IIa, II6, U27	10
031	SCAGFL8043B10.g	SCJFRT2057D03.g	*Pti1*	Le	2e-71	S/T KINASE	–	U43	~12
275	SCJLRT1013B11.b	SCCCRZ3002D04.g	*Pti1*	Le	2e-69	S/T KINASE	II	II9, IIb, IIa, II7	~10
082	SCCCAM2003A02.g	SCCCAM2003A02.g	*Pti1*	Le	2e-66	S/T KINASE	VIII	VIII1(2)*	~12
125	SCCCSB1002G04.g	SCRFLB1053B04.g	*Pti1*	Le	2e-64	S/T KINASE	I	I4, I9, I8	8
533	SCUTSB1075A03.g	SCCCCL4007D06.g	*Pti1*	Le	3e-58	S/T KINASE	IV	IV1, IV2	~20
116	SCCCLR1C06B05.g	SCRFLR1012B04.g	*Pti1*	Le	8e-57	S/T KINASE	–	–	–
523	SCUTFL1058C03.g	SCEZRZ3016C11.g	*Pti1*	Le	2e-53	S/T KINASE	III	IIIb, IIIa	7
371	SCRFAM2127C10.g	SCEZLB1009D01.g	*Pti1*	Le	1e-51	S/T KINASE	–	U3, U46	8
176	SCEQRT2096C09.g	SCJFRT1007C05.g	*Pti1*	Le	1e-51	S/T KINASE	–	–	–
367	SCRFAD1118B11.g	SCRLRZ3039D01.g	*Pti1*	Le	6e-51	S/T KINASE	VI	VId, VI11	13
129	SCCCST1C02D03.g	SCJFST1015D08.g	*Pti1*	Le	3e-50	S/T KINASE	–	U55, U46	8

[a]Accession No.: *Cf-2.1*, gi7489083; *Xa21* (O.l.), gi7434424; *Xa21* (O.s.), gi2130082; *Hcr2-5D*, gi7488988; *Hcr2-2A*, gi3894389; *Hcr2-0A*, gi3894385; *I2C-2*, gi7489066; *Xa1*, gi7489454; *RPR1*, gi4519936; *Pib*, gi6172381; *I2*, gi4689223; *PIC17*, gi3982626; *RPP1-WsA*, gi3860163; *Hv1LRR2*, gi5669782; *Gpa2*, gi5911745; *TMV*, gi9757959; *Rp1-D*, gi5702196; *RPR1*, gi4519936; *Pto*, gi626010; *Pti1*, gi:3668069

[b]Species of origin: At, *Arabidopsis thaliana*; Hv,*Hordeum vulgare*; Le,*Lycopersicon esculentum*; Lp, *Lycopersicon pimpinellifolium*; Ol, *Oryza longistaminata*; Os,*Oryza sativa*; St,*Solanum tuberosum*; Zm, *Zea mays*

[c]HG, homology group; CG, co-segregation group; Copies, number of bands detected with the enzyme used for mapping. When there are several markers derived from the same RGA on the same CG, the number of markers is indicated in *parentheses*; if they are clustered, this is indicated by an *asterisk*

in CG VII1a (Asnaghi et al. 2000). Two LRR RGAs (RGA137 and RGA019) map on HG VII. Alleles of RGA019 map on CGs VIIa and VII14. Alleles of RGA137 map on CG VIIa, clustered with RGA019, and on CG VII1a some 5.2 cM from the rust resistance gene.

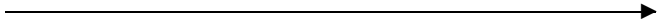


Fig. 1 Locations of the 148 RGA markers (*shaded*) on the genetic map of the sugarcane cultivar R570. The map encompasses 1123 markers, including AFLP and SSR (mSSCIR) markers, assembled into 128 Cosegregation Groups and seven Homology Groups (*numbered boxes*). Genetic distances in centiMorgans are indicated on the *left*. The rust resistance gene is indicated on CG VII.1

Characterization of the NBS/LRR RGA clusters

Two NBS/LRR RGA cluster loci were identified. Cluster 10 is located on CG U11 and contains three RGAs (RGA162, RGA152 and RGA087) with homology to the maize rust resistance gene *Rp1-D* (Collins et al. 1999). Cluster 7 is located on HG VIII on six homologous CGs (VIII1, VIII2, VIIIa, VIIIb, VIII.5 and VIII.15) and includes five NBS/LRR RGAs (RGA118, RGA281, RGA326, RGA185 and RGA267) with homology to the rice gene *RPR1*, which is responsible for probenazole-induced resistance to rice blast disease (Sakamoto et al. 1999).

Analysis of the full-length sequences of eight RGA clones revealed that almost all cDNAs seem to be incomplete at the 5′ end when compared to rice *RPR1* and maize *Rp1-D*, due possibly to an incomplete reverse transcriptase reaction. There were two exceptions: RGA118 from the *RPR1*-like cluster and RGA162 from the *Rp1-D* -like cluster. Figures 2 and 3 show the derived protein sequence alignments for *RPR1*-like and *Rp1-D* -like clusters, respectively, and indicate the NBS and LRR domains as well as their conserved motifs. Clones RGA162 and RGA152, from the *Rp1-D* -like cluster, appear to be pseudogenes, as they have stop codons in amino acid positions 655 and 233, respectively. RGA267, from the *RPR1*-like cluster, also has stop codons at positions 330 and 335 (Table 3). Although there is no difference between RGA162 and RGA152 at the amino acid level, the cDNAs are not derived from the same gene because they have sequence differences in the 3′ non coding region (data not shown) and they map 1.7 cM apart (Fig. 1).

With the aim of evaluating the divergence between and within these two NBS/LRR cluster loci, we calculated the sequence variability. For inter-cluster comparison, we aligned the part of the nucleotide sequence encoding the NBS domain of RPR1, RGA118, Rp1-D and RGA162 which were the only full length clones (amino acids 223 to 624 of RPR1 with amino acids 248 to 454 of Rp1-D). We chose this domain because it is the domain most conserved between *R* genes and outside of this region there is no significant alignment between RPR1 and Rp1-D. Since some RGA clones are incomplete, and do not include the NBS domain, it was impossible to align this region for intra-cluster analysis. Thus, we aligned part of the LRR nucleotide sequence (amino acids 557 to 901 of RPR1 for the *RPR1*-like cluster with amino acids 1008 to 1292 of Rp1-D for the *Rp1-D*-like cluster). This region corresponds to the most variable region in the *R* genes. Despite the fact that the comparison involved a variable region for intra-cluster analysis and a conserved region for inter-cluster analysis, the intra-cluster diversity at the nucleotide level (0.10 ± 0.04 for the *Rp1-D*-like cluster and 0.22 ± 0.03 for the *RPR1*-like cluster) appeared lower than the inter-cluster value (0.42 ± 0.1). This allowed the separation of these sugarcane RGAs into two clearly distinct groups: the *RPR1*-like group and the *Rp1-D*-like group.

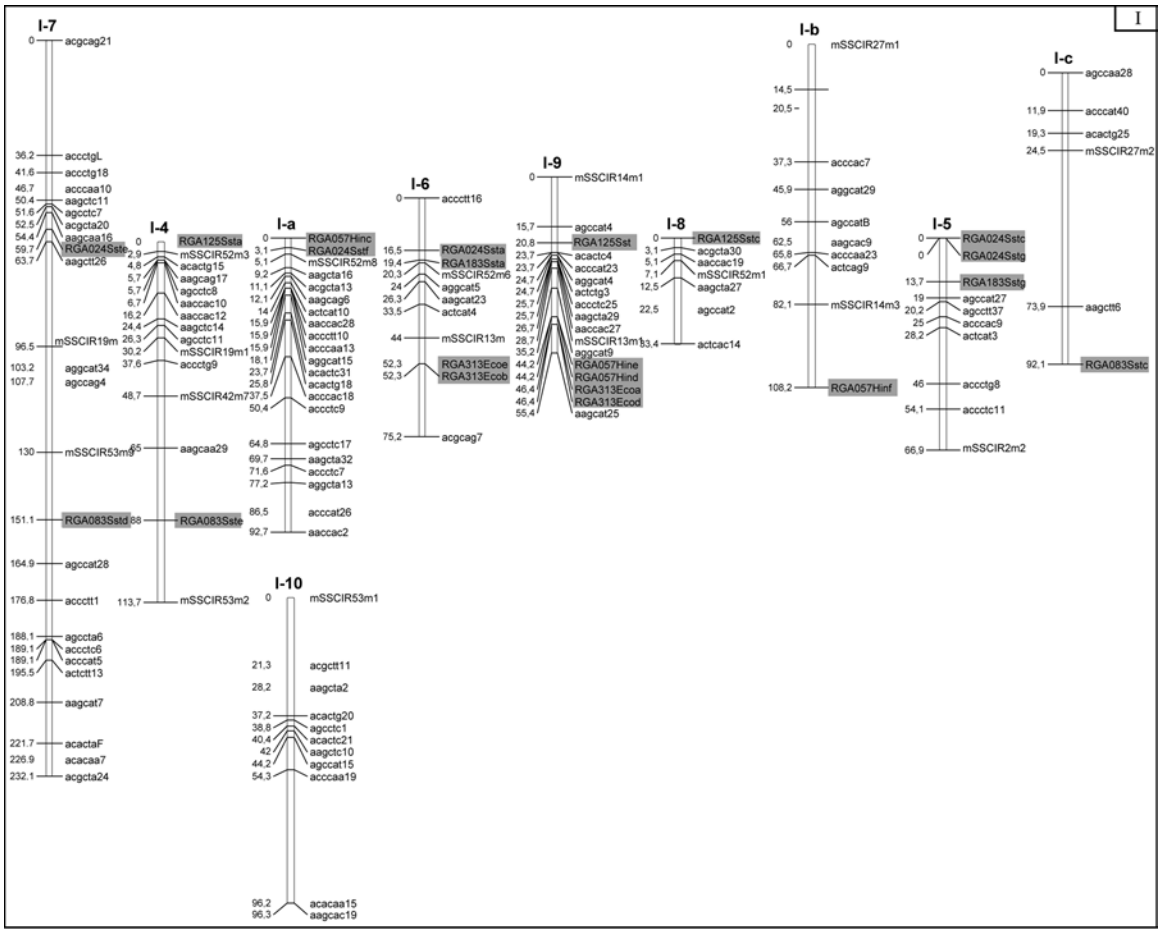
Discussion

The discovery of common sequence motifs between plant resistance genes has led to their use to develop candidate gene approaches for identifying resistance genes and analyzing their distribution in plant genomes. In this study, we have exploited the sugarcane EST database assembled in the course of the SUCEST project for both purposes.

Among the 81,223 phrap clusters comprising the 261,609 EST sequences, we have identified 88 clusters that are highly similar to *R* genes, using stringent screening procedures. Examples of RGAs encoding proteins with the three classical domains present in *R* genes (NBS/LRR, LRR and S/T KINASE) were found. No TIR/NBS/LRR-like RGAs were identified, supporting the hypothesis that this class of *R* genes has undergone divergent evolution in grasses and dicots (Pan et al. 2000, Goff et al. 2002).

We have mapped 148 markers representing 50 RGAs on the AFLP genetic map of the sugarcane cultivar R570 (Hoarau et al. 2001). Since sugarcane cultivars are highly polyploid and heterozygous, these RGAs were mapped simultaneously on several haplotypes. The SSR markers enabled us to relate the RGA mapping data to the RFLP map of R570 (consisting of approximately 1000 RFLP markers; Grivet et al. 1996, and unpublished results) and thus to organize the different haplotypes into homology groups. This will also allow the comparison of the distribution of RGAs in sugarcane to that in other species of Gramineae (Glaszmann et al. 1997, Dufour et al. 1997).

R genes are frequently reported to occur in clusters (Michelmore and Meyers 1998). In the *Arabidopsis* genome, 33% of the *R* genes are organized in pairs and 36% in clusters of three to nine members (The *Arabidopsis* Genome Initiative 2000). In sugarcane, 16 of the 50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75,2 acccac13
82,2 RGA386Draa
86 RGA169Hina
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119, aaccac24
120, mSSCIR69m2
122, aaccac5
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II-a
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39,7 acgcag19
58,7 acactg21
63,3 aggctc5
66,4 accctc5
82,1 acgctt10
87,9 agccaa21
92,5 agccaa1
115, accctc10
116, acccaa29
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120, agccat12
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143, RGA145Ecoe
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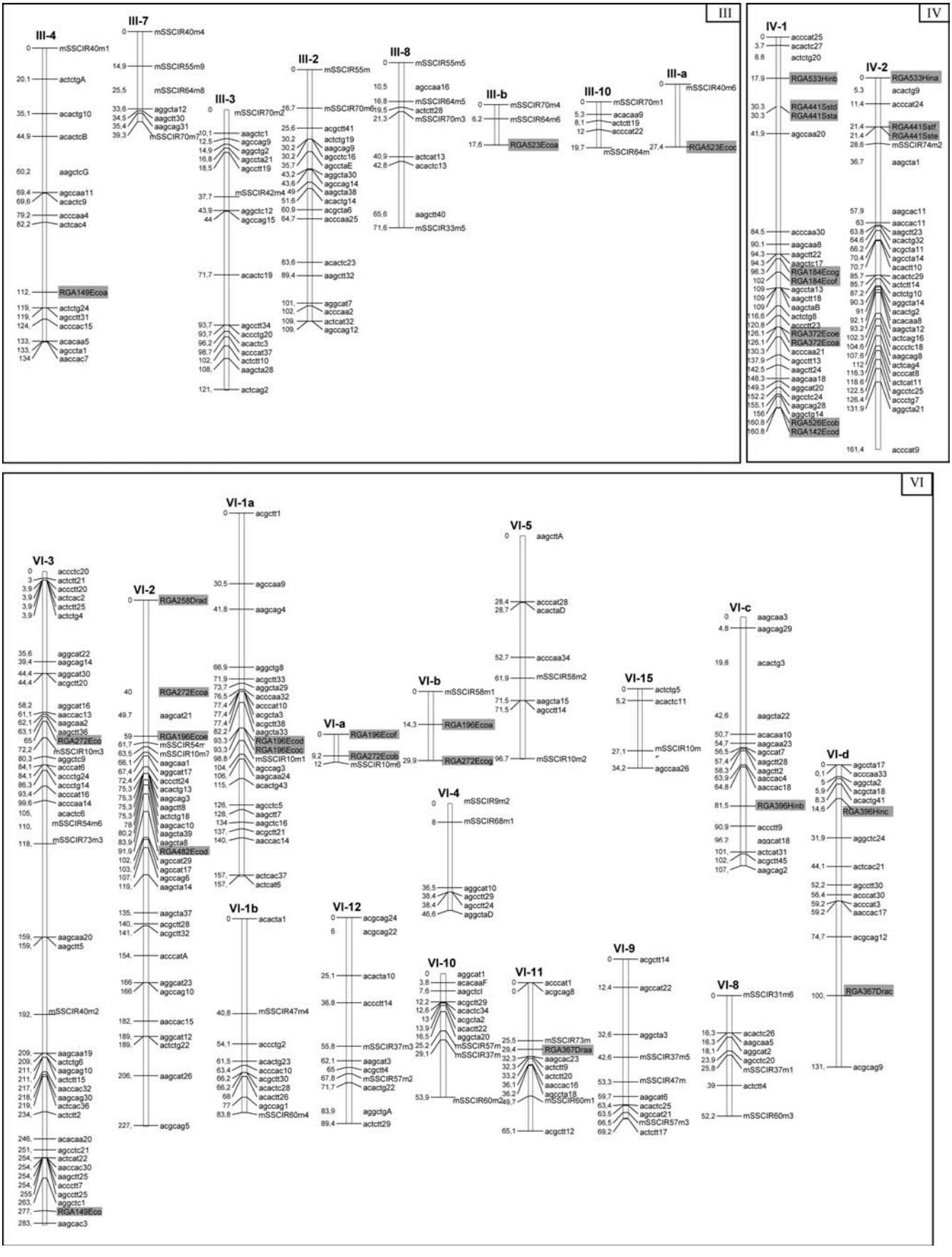
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96,3 RGA184Ecog
102 RGA184Ecof
109 agccta13
109 aagctt18
109 aagctaB
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Fig. 1 (Contd.)

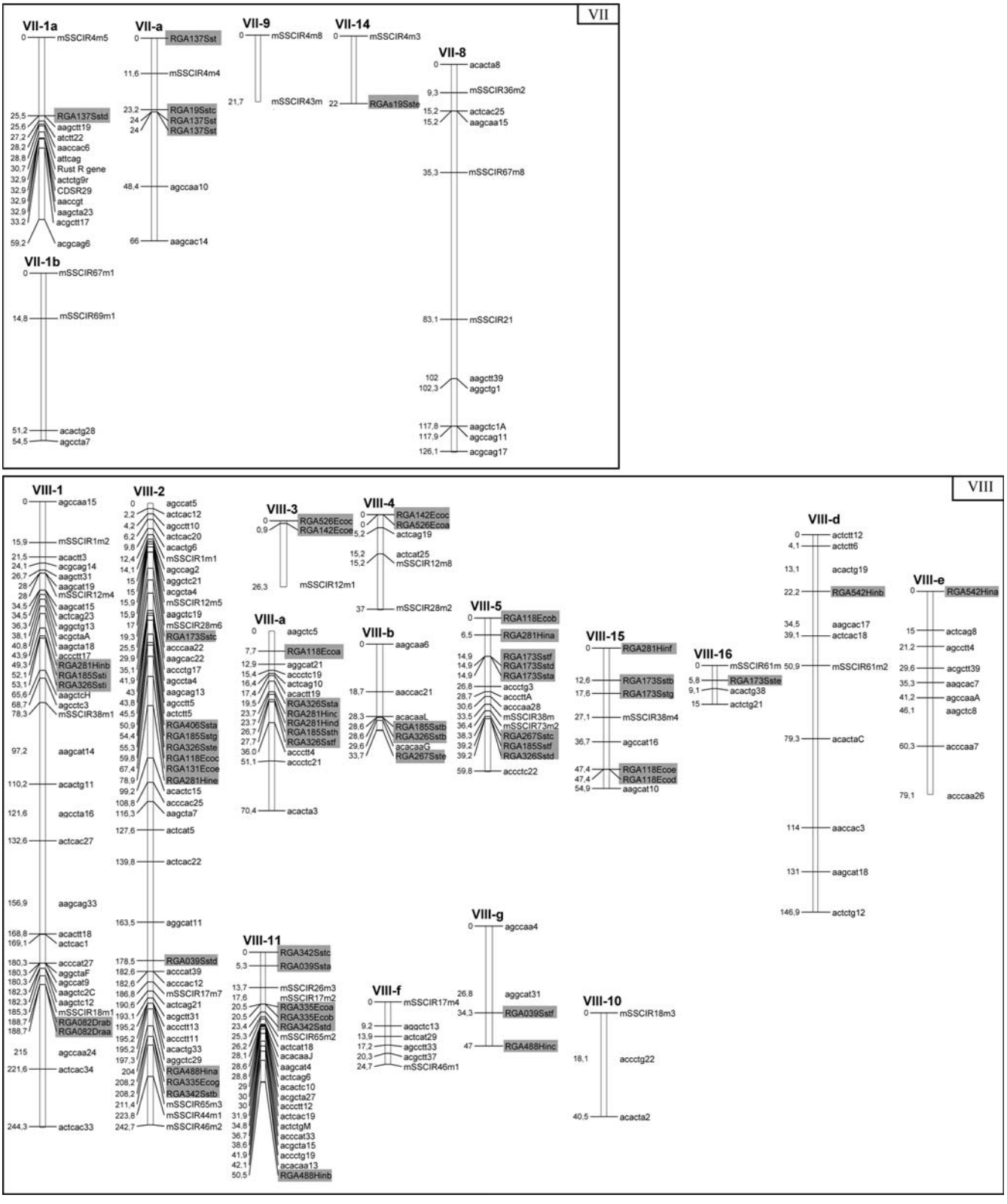

Fig. 1 (Contd.)

mapped RGA loci are organized in four clusters containing three to six different RGAs, while 12 are in pairs (Table 2). The RGAs that belong to the same cluster were not all mapped on every homologous CG. This is probably due to the constraints of mapping in polyploids (only single dose markers can be mapped) but could also be a consequence of gene losses that may have been part of the rapid and extensive genome changes

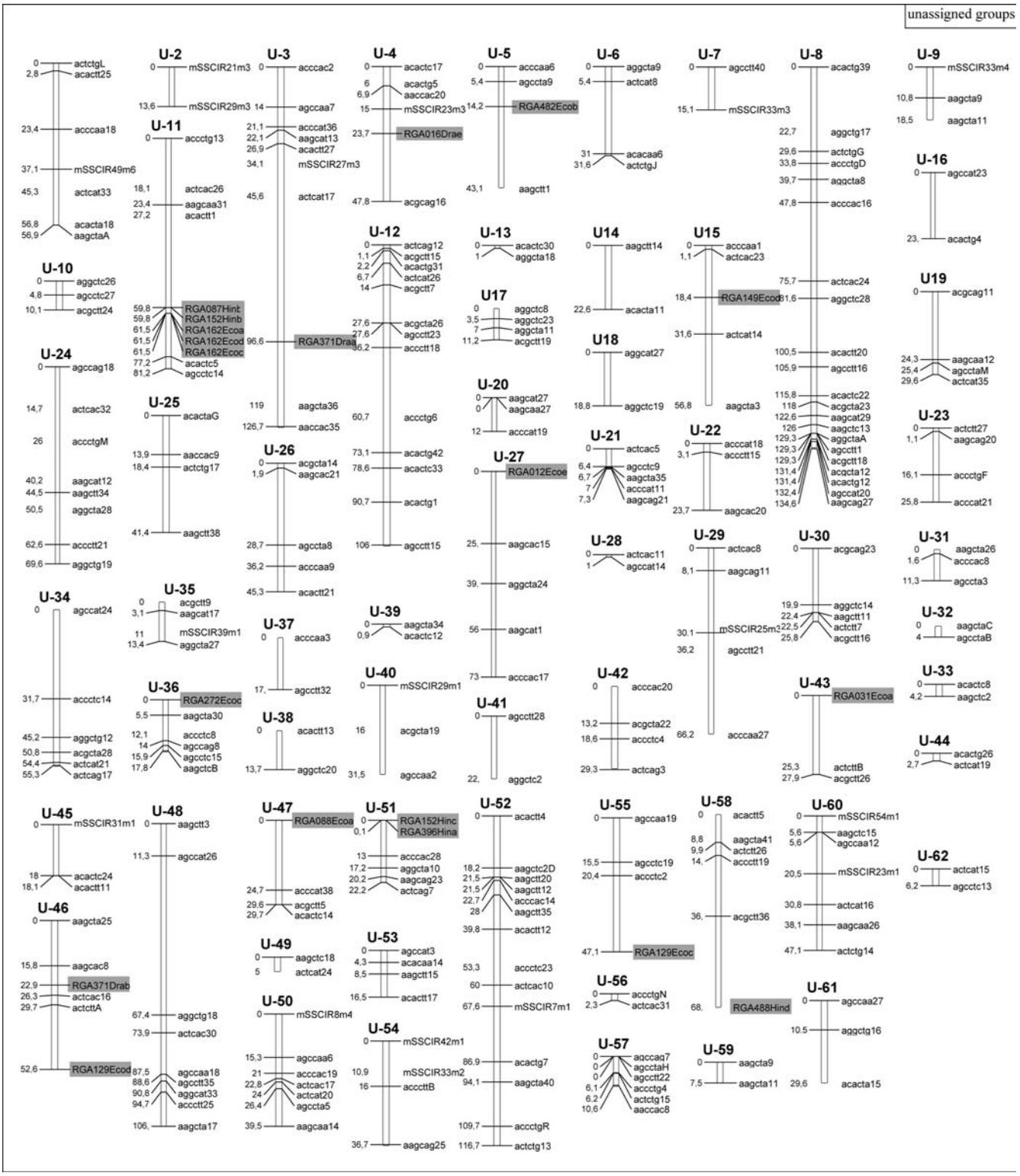

Fig. 1 (Contd.)

after polyploidization (Wendel 2000; Feuillet et al. 2001).

There is evidence that *R* gene clusters may contain functionally related genes that are not necessarily similar at the sequence level. This is the case for the tomato *Pto* cluster, which encodes five related kinases and a NBS/LRR (*Prf*) protein also required for resistance to *Pseudomonas syringae* (Salmeron et al. 1996). In this study, we also observed a few clusters containing RGAs with different protein domains (Table 2).

Table 2 Clusters containing different RGAs

Cluster No.[a]	RGA clones RGA domains	Cluster size (cM)	Map position	
			HG	CG
1*	057 (NBS/LRR), 024 (LRR), 183 (LRR), 313 (S/T KINASE)	–	I	Ia; Ib; I5; I6; I7;I9
2	275 (S/T KINASE), 012 (S/T KINASE)	4.7; 4.4	II	IIa; IIb
3	386 (LRR), 169 (S/T KINASE)	3.8	II	II8
4	526 (S/T KINASE), 142 (S/T KINASE)	0	IV	IV1
5	526 (S/T KINASE), 142 (S/T KINASE)	0.9; 0	VIII	VIII3; VIII4
6	019 (LRR), 137 (LRR)	0.8	VII	VIIa
7*	118 (NBS/LRR), 185 (NBS/LRR), 267 (NBS/LRR), 281 (NBS/LRR) 326 (NBS/LRR), 406 (S/T KINASE)	–	VIII	VIII1; VIII2; VIIIa; VIIIb; VIII5; VIII15
8*	335 (S/T KINASE), 342 (S/T KINASE), 488 (S/T KINASE)	–	VIII	VIII2; VIII11; VIIIg
9	152 (NBS/LRR), 396 (S/T KINASE)	0.1	–	U51
10	087 (NBS/LRR), 152 (NBS/LRR), 162 (NBS/LRR)	1.7	–	U11

[a]This number refers to the basic genome. Clusters 1, 7 and 8 (indicated by the *asterisks*) consist of several RGAs that map on several homologous chromosome segments, the distance between RGAs on each CG is variable but at least in one CG the distance between each pair of consecutive RGAs is less 5 cM or less. Not all RGAs were mapped on each CG (see Fig. 1 and text for details)

It is noteworthy that all the RGAs homologous to rice RPR1 map together in cluster 7, and all the NBS/LRR RGAs homologous to maize Rp1-D map together in cluster 10. Sequence comparison of these NBS/LRR RGAs with the respective references in rice or maize reveals that the members of a given sugarcane cluster are more similar to the alien reference (RPR1 or Rp1-D) than to members of the other sugarcane NBS/LRR locus. This observation suggests the existence of a common ancestral gene for rice *RPR1*and the sugarcane *RPR1*-like cluster, and for maize *Rp1-D* and the sugarcane *Rp1-D*-like cluster.

In addition, protein sequence alignments of the RPR1-like group (Fig. 2), as well as nucleotide sequence analysis (data not shown), show that sugarcane RGAs are not always more similar to each other than to the corresponding rice ortholog. This phenomenon of greater distance between paralogous than between orthologous sequences has already been highlighted by others (Michelmore and Meyers 1998; Feuillet et al. 2001), and led Michelmore and Meyers (1998) to propose a model for resistance cluster evolution called ''birth and death''.

Many authors have reported linkages between RGAs and disease resistance loci or QTLs (Wang et al. 2001, Graham et al. 2000). This is particularly interesting for sugarcane since, due to its particularly complex genome, only one resistance gene has been localized so far (Daugrois et al 1996; Asnaghi et al. 2000). This major resistance gene, which confers resistance to common rust, has been located on R570 maps and is the focus of a map-based cloning approach (Asnaghi et al. 2000, and unpublished results). The present work identified an LRR cluster near the rust resistance locus, thus indicating the presence of RGAs in this genome region. In addition, the data generated in this study on the distribution of RGAs in the sugarcane genome will provide extremely valuable information for current efforts aimed at mapping resistance genes for other sugarcane diseases including leaf scald (Offmann, personal communication) and smut (Raboin et al. 2001).

Despite the success of the RGA approach to the identification of disease resistance loci, the challenge often remains in recognizing the functional gene within clusters. *R* gene clusters typically contain several related sequences, and even in the best studied cases, only for half of them has any specificity been demonstrated (Michelmore and Meyers 1998). With regard to this aspect, EST-RGA resources may have advantages, compared to PCR amplification of conserved motifs or ''candidate genes'' from genome sequencing data. The EST approach considers only expressed genes, thus eliminating many pseudogenes that cannot be transcribed. However, cDNAs with internal stop codons, indicative of non-functional protein, were also found in this study (RGA162, RGA152 and RGA267), and already reported by Vicente and King 2001. In polyploids, the formation of pseudogenes through accumulation of mutations may be a consequence of the reduction in selection pressure on genes that are present in several copies (Wendel 2000).

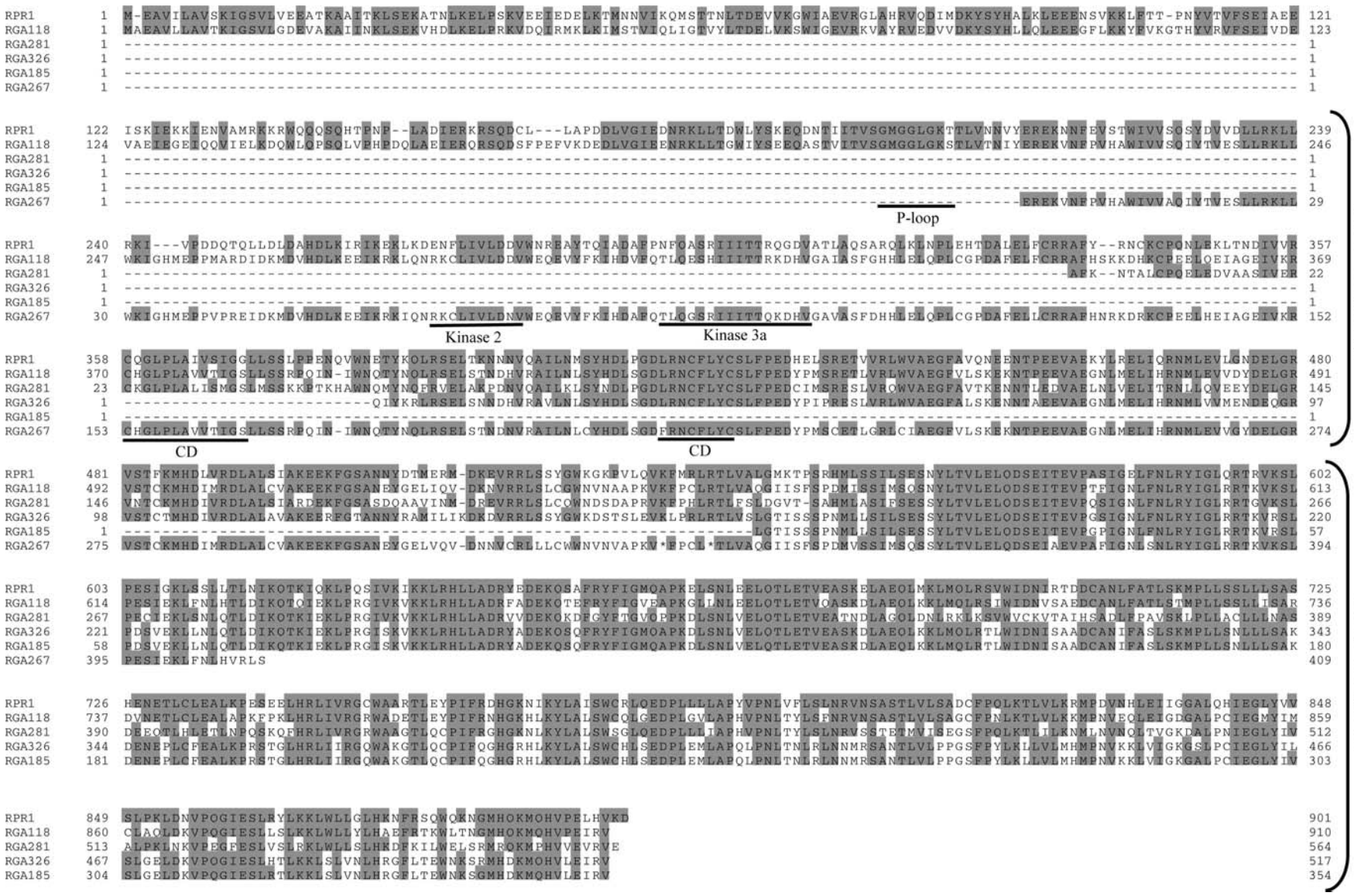

Fig. 2 Alignment of derived protein sequences encoded in the *RPR1*-like NBS/LRR-like RGA cluster on HG VIII. The *shaded* amino acids indicate sequence identity to the RPR1 protein of rice. NBS motifs (P-loop, Kinase 2 and Kinase 3a) and regions conserved between resistance gene products (CD) are *underlined*. Protein domains are indicated on the *right*

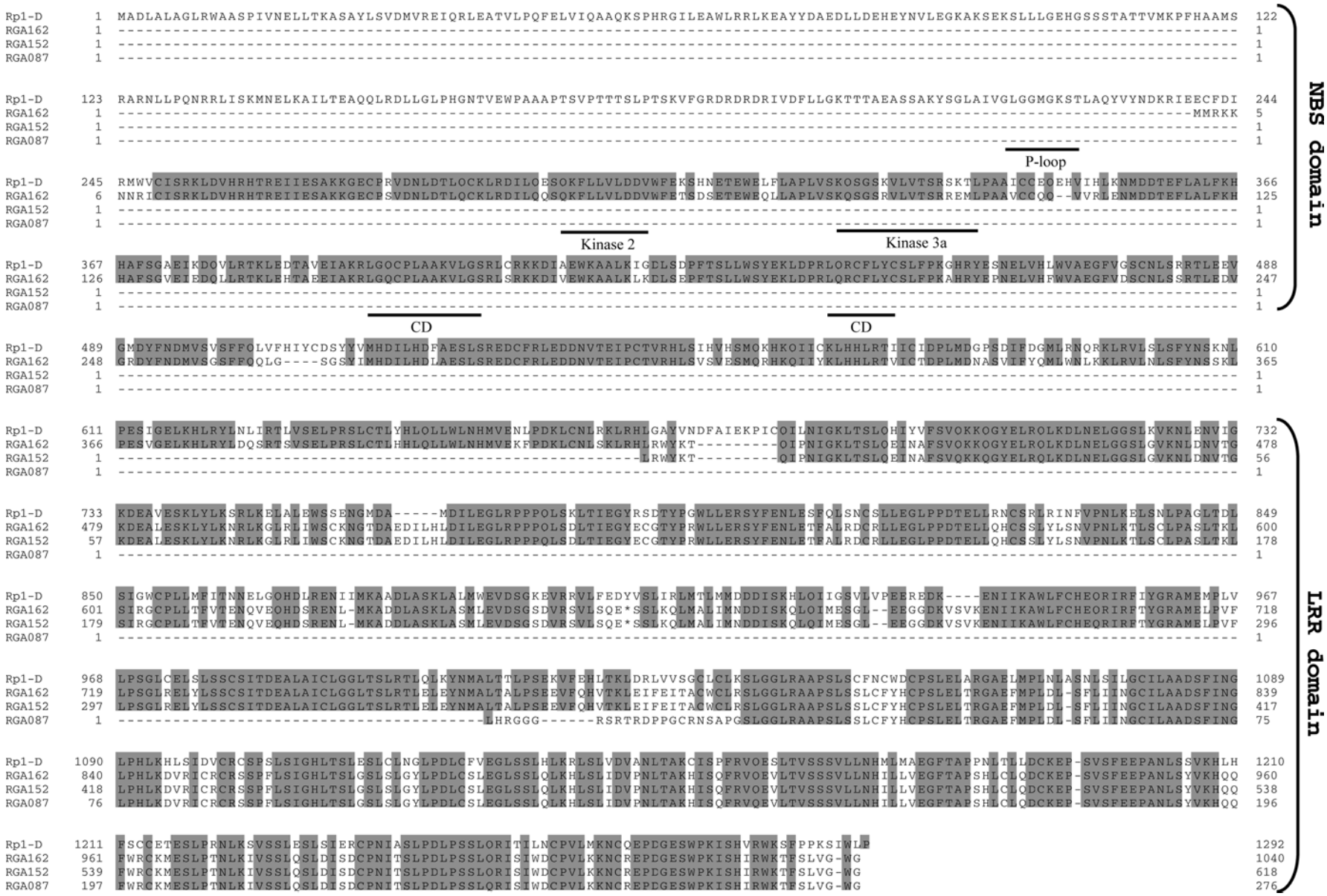

Fig. 3 Alignment of derived protein sequences encoded in the *Rp1-D* -like NBS/LRR-like RGA cluster on CG U11. The *shaded* amino acids indicate sequence identity to the Rp1-D protein from maize. NBS motifs (P-loop, Kinase 2 and Kinase 3a) and regions conserved between resistance gene products (CD) are *underlined*. Protein domains are indicated on the *right*

Table 3 Characteristics of *RPR1*- and *Rp1-D* -like RGAs

Clone name	Closest homolog	Size (nt)	Poly(A) tail	Size of putative protein product (aa)	Met Start Codon	Internal stop codon
RGA118	*RPR1* (901 aa)	3182 bp	+	910	+	–
RGA281	*RPR1* (901 aa)	1992 bp	+	564	–	–
RGA326	*RPR1* (901 aa)	1840 bp	+	517	–	–
RGA185	*RPR1* (901 aa)	1359 bp	+	354	–	–
RGA267	*RPR1* (901 aa)	1436 bp	+	409	–	+
RGA162	*Rp1-D* (1292 aa)	4012 bp	+	1040	+	+
RGA152	*Rp1-D* (1292 aa)	2462 bp	+	618	–	+
RGA087	*Rp1-D* (1292 aa)	1392 bp	+	276	–	–

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