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Inter-simple-sequence-repeat (ISSR) polymorphisms are useful for finding markers associated with disease resistance gene clusters

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Abstract We describe a simple and new approach, based on inter-simple sequence repeats (ISSRs), for finding markers linked to clusters of disease resistance genes. In this approach, simple sequence repeats (SSR) are used directly in PCR reactions, and markers found to be linked to disease resistance genes provide important information for the selection of other sequences which can be used with PCR to find other linked markers. Based on an ISSR marker linked to a gene of interest, many new markers can be identified in the same region. We previously demonstrated that ISSR markers are useful in gene tagging and identified a marker, UBC-855_{500}, linked to the gene for resistance to fusarium wilt race 4 in chickpea. This ISSR marker provided the information used in the present study for selecting other primers which amplified a region linked to the gene for resistance to fusarium wilt race 4. The primers were based on homology with the $(AC)_n$ sequence and were used for PCR amplifications. Changes in the sequence were at the anchor region of the primers. The repeat $(AC)_8T$ amplified a marker, UBC-825_{1200}, which was located 5.0 cM from the gene for resistance to fusarium wilt race 4 and was closer than other markers. These results indicated that ISSR markers can provide important information for the design of other primers and that by making changes at the 3′ and 5′ anchors close linkage to the desired gene can be found. The approach allows rapid scanning of the targeted region and may provide important information for genome analysis of plant species.

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Introduction

Inter-simple sequence repeats (ISSRs) are a new type of DNA marker which involves the use of microsatellite sequences directly in the polymerase chain reaction (PCR) for DNA amplifications (Gupta et al. 1994; Zietkiewicz et al. 1994; Sanchez et al. 1996). This technique enables amplification of genomic DNA and provides information about many loci simultaneously. Recent reports have shown Mendelian inheritance of simple-sequence-repeat (SSR) fragments scored as dominant markers (Tsumura et al. 1996) or as codominant markers (Wu et al. 1994). Simple sequence repeats (SSRs) are short tandem repetitive DNA sequences with a repeat length of few base pairs (1-5) (Litt and Luty 1989). The sequences are abundant, dispersed throughout the genome and highly polymorphic in comparison with other molecular markers (Akkaya et al. 1992; Morgante and Olivieri 1993; Wang et al. 1994). SSRs have been used successfully in the genome mapping of a variety of crop species including maize, rice, barley and wheat (Senior and Heun 1993; Wu and Tanksley 1993; Saghai-Maroof et al. 1994; Roder et al. 1995). However, due to the technical difficulties in developing SSRs, these markers have not been commonly used for gene tagging in plants. ISSR has been proposed as a new source of genetic markers which overcomes the technical limitations of restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD).

Ratnaparkhe et al. (1998) were the first to demonstrate that ISSR markers are useful in gene tagging and can be used for finding markers linked to the gene of interest. Previous reports indicated that SSRs are not randomly distributed in the genome but are often

clustered (Arens et al. 1995; Broun and Tanksley 1996). The clustering of SSRs has also been observed in humans (Erickson et al. 1988) and on sex chromosomes of different organisms (Epplan 1988; Nanda et al. 1990). Similarly, disease resistance genes have also been found to form clusters (Sheperd and Mayo 1972; Islam et al. 1993; Kesseli et al. 1993). Studies on disease resistance genes have indicated a high level of polymorphism and the presence of SSRs at certain loci (Yu et al. 1996). In the study presented here we exploited the combination of ISSR markers to find markers at the fusarium wilt disease resistance gene cluster. The approach is based on the association of a cluster of SSRs with the disease resistance gene cluster. We also investigated the potential of an ISSR-directed approach for selecting other sequences which can be used to find linked markers and for marker enrichment in the desired region. The technique is based on the use of SSR primers with variations at 5′ and 3′ anchors, which provides markers linked to the desired gene. We studied the inheritance of ISSR polymorphisms using 96 recombinant inbred lines (RILs) derived from the cross of *C. arietinum* (ICC-4958), a cultivated chickpea germplasm line with resistance to fusarium wilt, and *C. reticulatum* (PI 489777), the closest wild relative of the cultivated species, and identified markers linked to the gene for resistance to fusarium wilt races 4 and 5.

Materials and methods

Plant material

A set of 96 F_6-derived F_7 recombinant inbred lines (RILs) obtained from a cross of *C. arietinum* (ICC-4958) and *C. reticulatum* (PI 489777) was used in this study. The RILs were developed by the single-seed descent procedure. Scoring for resistance to fusarium wilt in the two parents and 96 RILs was done in the greenhouse.

DNA extraction

DNA was isolated from vegetative buds and leaf tissues of the parents and RILs using the microprep method of Doyle and Doyle (1987). One gram of each sample was submerged in liquid nitrogen and then ground to a fine powder. The powder was quickly transferred to a tube containing 7.5 ml of ice cold extraction buffer (0.35 *M* sorbitol, 0.1 *M* TRIS, 5 m*M* EDTA, pH 7.5). The tube was briefly shaken, and 7.5 ml of nuclei lysis buffer (2 *M* NaCl, 0.2 *M* TRIS, 50 m*M* EDTA, 2% CTAB, pH 7.5) was then quickly added, followed by 3 ml of 5% sarkosyl solution. Sample sets were incubated in a 65°C waterbath for 20 min. After incubation, the tubes were allowed to cool for a few minutes and then 18 ml of chloroform/isoamyl alchohol (24 : 1) was added to each tube. The tubes were then centrifuged at 500 *g* for 15 min. The aqueous layer was removed and extracted again with 15 ml chloroform mixture. Finally DNA was precipitated with chilled ethanol and suspended in 1 ml of TE buffer.

Culture preparation and inoculation procedures

Inoculum was prepared from a single-spored fungal isolate grown on sterile filter paper placed on potato-dextrose-agar (PDA) as described by Tullu (1996). When the filter paper was completely colonized by fungus, only those colonies representative of the wild type were aseptically removed and placed in a fresh petri dish to dry for 5 days in a laminar flow hood. The dried filter paper was aseptically cut into pieces using a pair of sterile scissors, and these pieces were then used to prepare the primary inoculum. The conidia concentration was adjusted to 1×10^6 spores per milliliter with a hemacytometer. Twelve to twenty seeds of each RIL were grown in the greenhouse (21°–26°C) in single rows in plastic trays filled with sterile coarse perlite. When the seedlings reached the three- to four-nodal stage, they were carefully removed from the perlite, pruned while submerged in the spore suspension and, after about 5 min in the spore suspension, replanted into the perlite. Plants were then scored as susceptible or resistant over the next 2 months.

Oligonucleotide primers

One hundred primers of 15–23 nucleotides in length (UBC set #9) were obtained from the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada and used for polymerase chain reaction (PCR) amplifications.

PCR amplification and electrophoresis

PCR amplification was performed in 10 m*M* TRIS-HCl pH 8.3, 50 m*M* KCl, 0.1% Triton ×100, 2.5 m*M* $MgCl_2$, 0.2 μ*M* dNTP, 0.24 μ× of primer, 30 ng of genomic DNA per 25 μl of reaction volume and 1 unit of *Taq* polymerase. The amplifications were carried out on a Perkin Elmer Cetus 9600 programmed for 35 cycles of de-naturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 2 min, with a 10-min final extension at 72°C. PCR products were separated on 2% agarose gels, then stained with ethidium bromide and scored for presence or absence of bands. Since ISSR markers are dominant, a locus was considered to be polymorphic if the band was present in one parent and not in the other. Linkage analysis was performed using the MAPMAKER program (Lander et al. 1987)

Results

We previously demonstrated that when the simple sequence repeat $(AC)_8YT$ is used directly in a PCR reaction it amplifies a marker, UBC-855_{500}, which is linked to the gene for resistance to fusarium wilt race 4 (Ratnaparkhe et al. 1988). The aim of our present study was to determine the suitability of the $(AC)_n$ sequences as guidelines for selecting new primers for use in marker enrichment at the desired region. For this we studied the inheritance of ISSR polymorphism in a cross of cultivated chickpea (*C. arietinum*) and the closely related wild species (*C. reticulatum*). Based on the $(AC)_8YT$ sequence which amplifies a marker linked to the disease resistance gene, we selected other sequences containing AC repeats, but ones that varied at the 3′ and 5′ anchors. We found that other SSRs with AC repeats also amplified fragments that were linked to the fusarium wilt resistance genes. The repeat $(AC)_8T$ amplified a marker UBC-825_{1200} which was located 5.0 cM from the gene for resistance to fusarium wilt race 4 and was closer than the UBC-855_{500} and

CS-27$_{700}$ markers (Mayer et al. 1997; Ratnaparkhe et al. 1998). The electrophoretic pattern of the PCR-amplified DNA fragment using primer UBC-825 is shown in Fig. 1. Repeat (AC)$_8$YG amplified fragment UBC-857$_{800}$ which was located 5.5 cM from UBC-855$_{500}$ and was associated with the gene for resistance to fusarium wilt trace 4. A one or two nucleotide change at the 3′ end in the sequence resulted in the shifting of the marker. To study the inheritance of the complimentary sequences, we selected (TG)$_n$ repeats with different anchors. The repeat (TG)$_8$RG amplified fragment UBC-860$_{600}$ from the resistant lines, and not the UBC-857$_{800}$ of the (AC)$_n$ repeats, and flanked the gene for resistance to race 4.

Genes for resistance to fusarium wilt races 4 and 5 are present in same linkage group

Recent study indicates that host resistance genes are often clustered as a multiallelic series at locus or as multiple linked loci. The best examples are rust resistance genes clustered in the L group of flax (Sheperd and Mayo 1972; Islam et al. 1993) and downy mildew resistance gene in lettuce (Hulbert and Michelmore 1985). We studied the inheritance and segregation of the gene for resistance to fusarium wilt race 5, which was found to be in the same linkage group with race 4. The genes for resistance to fusarium wilt race 1, 2, 4 and 5 were also found to be clustered in another cross, WR-315 × C-104 (Tullu 1996).

We observed an abundance of dinucleotides and trinucleotide repeats at the fusarium wilt disease resistance gene cluster. In addition to (AC)$_n$ and (TG)$_n$ repeats the trinucleotides (ATG)$_6$, (CTC)$_6$ and (GAA)$_6$ were also present at the fusarium wilt resistant gene cluster. The markers amplified by trinucleotide repeats, UBC-864$_{425}$, UBC-866$_{900}$, and UBC-868$_{700}$, were associated to the genes for resistance to fusarium wilt and were in the same linkage group. The sequence of the repeats and the size of the amplified markers are shown in Table 1. The position of ISSR markers with respect to the gene for resistance to race 4 (Foc 4) and 5 (Foc 5) are shown in Fig. 2. The size of the amplified bands and the position of various markers indicate that they were amplified from the independent loci and are not the same fragment. Our study shows that markers linked to various genes can be rapidly identified using the ISSR-directed approach. The presence of dinucleotide and

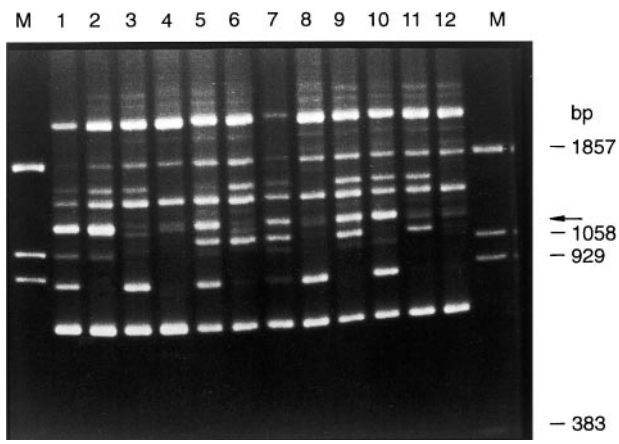

Fig. 1 PCR-amplified inter-simple-sequence-repeat patterns on 2% agarose gels. *M* Marker pBR 322 BstN1 digest, *lanes 1–12* chickpea RILs amplified with primer UBC-825. Marker UBC-825$_{1200}$ is 5.0 cM from the gene for resistance to fusarium wilt race 4 and is indicated by an *arrow*

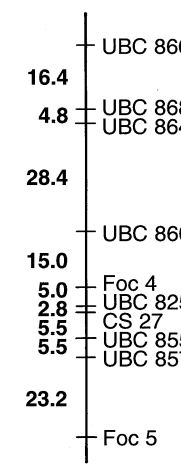

Fig. 2 Position of the genes for resistance to race 4, 5 and ISSR markers. The distances among the markers are given in centiMorgans

Table 1 Segregation of ISSR fragments in chickpea RILs developed from a cross of ICC-4958 and PI 489777

Primer no.	Sequence[a]	Fragment size (bp)	χ^2 (1 : 1)	*P*
UBC-825	ACACACACACACACACT	1200	1.36	0.25–0.50
UBC-855	ACACACACACACACACYT	500	4.0	0.02–0.05
UBC-857	ACACACACACACACACYG	800	3.3	0.05–0.10
UBC-860	TGTGTGTGTGTGTGTGRA	600	4.6	0.02–0.05
UBC-864	ATGATGATGATGATGATG	425	2.0	0.10–0.25
UBC-866	CTCCTCCTCCTCCTCCTC	900	0.52	0.25–0.50
UBC-868	GAAGAAGAAGAAGAAGAA	700	1.3	0.25–0.50

[a] R indicates purine; Y indicates pyrimidine

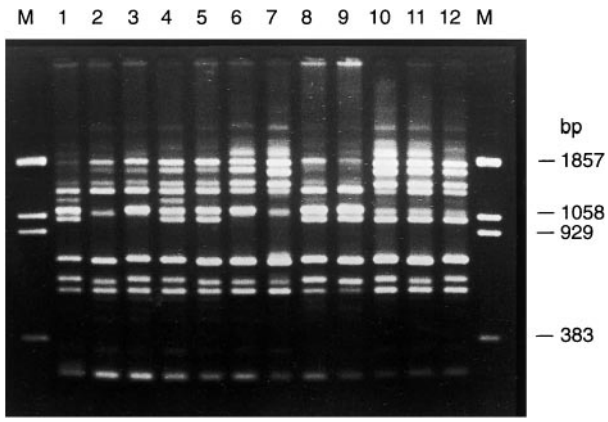

Fig. 3 Electrophoretic patterns of PCR-amplified inter-simple sequence repeats on 2% agarose gels. *M* Marker pBR 322 *Bst*N1 digest, *lanes 1–12* chickpea RILs amplified with primer UBC-880

tetranucleotide repeats can be exploited for fine mapping of fusarium wilt gene cluster.

Our study indicated that markers amplified with simple sequence repeats co-segregated and were often present in the same linkage group. The clustering of markers was noted in several cases. For example, fragments UBC-880_{1500} and UBC-880_{1700} co-segregated at a distance of 5.8 cM and were in the same linkage group (data not shown). The segregation of markers amplified by primer UBC-880 is shown in Fig. 3. Markers UBC-856_{1800} and UBC-859_{1900} amplified by the complementary repeats $(AC)_8YA$ and $(TG)_8RC$, respectively, co-segregated and were clustered.

Discussion

There are many reports of microsatellite markers linked to disease resistance genes (Yu et al. 1996; Blair and McCouch 1997). However, finding SSR markers linked to disease resistance genes is very expensive and involves screening the library, sequencing the clone and synthesizing the primers. This has prevented the broad use of microsatellites in plants. Our study demonstrated that microsatellite sequences can be used directly in the PCR reaction and can be used to find markers linked to disease resistance genes. This approach is quicker and less expensive than southern hybridization and oligonucleotide fingerprinting. The polymorphism is detected within the region spanned by the microsatellite primer and also within the amplified region between the primer binding sites.

The clustering of host resistance genes conditioning resistance to pathogenic fungi has been documented in many plant species. In soybeans, Lohnes et al. (1993) reported that two resistance genes (*Rmd* and *Rps2*) are closely linked to each other, and to a non-nodulation gene (*Rj2*). In lettuce (*Lactuca sativa* L.) 13 resistance genes for downy mildew (*Bremia lactucae* Regel) have been mapped in four clusters (Hulbert and Michelmore 1985). In the present study we found that the genes for resistance to fusarium wilt races 4 and 5 are linked. The clustering of resistance genes at a specific chromosomal region is advantageous in a breeding program, as this block of genes can be transferred to an adapted background via backcrossing, and the desired trait can be selected using the ISSR markers. Our results also indicated the presence of SSRs at the disease resistance gene cluster and the fragments amplified by SSRs often co-segregated, indicating a cluster of the microsatellite repeat. The clustering of microsatellites has also been reported in tomato where mapping of GATA- and GACA-containing microsatellite loci showed that they were not randomly distributed throughout the genome but often clustered in the same chromosomal region (Arens et al. 1995; Broun and Tanksley 1996). The remarkably high level of polymorphism around the disease resistance gene cluster indicates an association between the molecular mechanism of disease resistance and rapid sequence divergence in plants (Sudupak et al. 1993; Yu et al. 1996). The multiple genes for disease resistance are thought to be due to the duplication of the ancestral gene. It might be possible that the region surrounding the gene containing SSR repeats was also duplicated and thus provided amplifications using SSR sequences. The shift in the position in the marker varied according to the anchors present at the 3′ end and 5′ end of the SSR sequence. The anchors at the 5′ end had a small change in the position of the marker. By changing the sequence of the short nucleotide residues at the ends, many primers with different anchors can be synthesized which may amplify markers more closely linked to the disease resistance gene. Additional experiments with 'in situ' hybridization would provide detailed information on the association of various SSRs with disease resistance gene clusters.

Applications of the ISSR-directed approach

In summary, our results indicate that SSRs can be used as highly informative markers for genome mapping and gene tagging. The ISSR-directed approach in combination with bulked segregant analysis (BSA) has a wide application in plant and animal genome mapping. It can be extremely useful in (1) identifying the markers at clusters of disease resistance genes (2) filling large gaps in linkage maps (3) developing the sequence-tagged microsatellite sites and (4) providing marker enrichment at desired regions.

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References

Akkaya MS, Bhagwat AA, Cregan PB (1992) Length polymorphisms of simple-sequence repeat DNA in soybean. Genetics 132 : 1131–1139

Arens P, Odinot P, van Heusden AW, Lindhout P, Vosman B (1995) GATA and GACA repeats are not evenly distributed throughout the tomato genome. Genome 38 : 84–90

Blair MW, McCough SR (1997) Microsatellite and sequence-tagged site markers diagnostic for rice bacterial leaf blight resistance gene *xa*-5. Theor Appl Genet 95 : 174–184

Broun P, Tanksley SD (1996) Characterization and genetic mapping of simple repeat sequences in tomato genome. Mol Gen Genet 250 : 39–49

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small amount of fresh leaf tissue. Phytochem Bull 19 : 11–15

Epplen JT (1988) On simple repeated GATA/GACA sequences in animal genomes: a critical reappraisal. J Herad 79 : 409–417

Erickson RP, Ross CE, Gorski JL, Stalvey JRD, Drumm MM (1988) BKM sequences from the human X chromosomes contain large clusters of GATA/GACA repeats. Ann Hum Genet 52 : 167–176

Gupta M, Chyi Y-S, Romero-Severson J, Owen JL (1994) Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. Theor Appl Genet 89 : 998–1006

Hulbert SH, Michelmore R (1985) Linkage analysis of genes for resistance to downy mildew (*Bremia lactucae* Regal) in lettuce (*Lactuca sativa* L.) Theor Appl Genet 70 : 520–528

Islam MR, Sheperd KW, Mayo GME (1993) Recombination among genes at the *L* group in flax conferring resistance to rust. Theor Appl Genet 77 : 540–546

Kesseli R, Witsenboer H, Stanghellini M, Vandermark G, Michlmore R (1993) Recessive resistance to *Plasmopara lactucae-radicis* maps by bulked segregant analysis to cluster of dominant disease resistance genes in lettuce. MPMI 6 : 722–728

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1 : 174–181

Litt M, Luty JA (1989) A hypervariable microsatellite revealed by in vitro amplification of dinucleotide repeat within the cardiac muscle actin gene. Am J Hum Genet 44 : 397–401

Lohnes DG, Wagner RE, Bernard RL (1993) Soybean genes *Rj2*, *Rmd*, and *Rsp2* in linkage group 19. J Hered 84 : 109–111

Mayer MS, Tullu A, Simon CJ, Kumar JS, Kraft JM, Kaiser WJ, Muehlbauer FJ (1997) Development of DNA marker for fusarium wilt resistance in chickpea. Crop Sci 37 : 1625–1629

Morgante M, Olivieri AM (1993) PCR-amplified microsatellites as markers in plant genetics. Plant J 3 : 175–182

Nandal I, Deubelbeiss C, Guttenbach M, Epplan JT, Schmid M (1990) Heterogeneities in the distribution of $(GACA)_n$ simple repeats in the karyotypes of primates and mouse. Hum Genet 85 : 187–194

Ratnaparkhe MB, Santra DK, Tullu A, Muehlbauer FJ (1998) Inheritance of inter-simple-sequence-repeat polymorphisms and linkage with fusarium wilt resistance gene in chickpea. Theor Appl Genet 96 : 348–353

Roder MS, Plaschke J, Konig SU, Borner A, Sorrells ME, Tanksley SD, Ganal MW (1995) Characterization of PCR-amplified microsatellite loci in wheat. Mol Gen Genet 246 : 327–333

Saghai-Maroof MA, Biyashev RB, Yang GP, Zhang Q, Allard RW (1994) Extraordinarily polymorphic microsatellite DNA in barley: species diversity, chromosomal locations and population dynamics. Proc Natl Acad Sci USA 91 : 5466–5470

Sanchez de la Hoz MP, Davila JA, Loarce Y, Ferrer E (1996) Simple sequence repeat primers used in polymerase chain reaction amplification to study genetic diversity in barley. Genome 39 : 112–117

Senior ML, Heun M (1993) Mapping maize microsatellites and polymerase chain reaction confirmation of the targeted repeats using a CT primer. Genome 36 : 884–889

Sheperd KW, Mayo GME (1972) Genes conferring specific plant disease resistance Science 175 : 375–380

Sudupak MA, Bennetzen JL, Hulbert SH (1993) Unequal exchange and meiotic instability of disease resistance genes in the Rp1 region of maize. Genetics 133 : 119–125

Tsumura Y, Ohba K, Strauss SH (1996) Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas-fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*). Theor Appl Genet 92 : 40–45

Tullu A (1996) Genetics of fusarium wilt resistance in chickpea. Ph.D thesis, Crop and Soil Sciences Department, Washington State University Pullman, Wash. USA

Wang Z, Weber JL, Zhong G, Tanksley SD (1994) Survey of plant short tandem repeats. Theor Appl Genet 88 : 1–6

Wu K-S, Jones R, Danneberger L, Scolnik PA (1994) Detection of microsatellite polymorphisms without cloning. Nucleic Acids Res 22 : 3257–3258

Wu K-S, Tanksley SD (1993) Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol Gen Genet 241 : 225–235

Yu YG, Saghai Maroof MA, Buss GR (1996) Divergence and allelomorphic relationship of a soybean virus resistance gene based on tightly linked DNA microsatellite and RFLP markers. Theor Appl Genet (1996) 92 : 64–69

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20 : 176–183