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Discovery and genotyping of high-throughput SNP markers for crown rust resistance gene *Pc94* in cultivated oat

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Abstract

Crown rust caused by *Puccinia coronata* f. sp. *avenae* Eriks is a serious problem for oat production worldwide and pyramiding multiple resistance genes into new cultivars is a key objective of breeders. Many race specific resistance genes have been mapped and markers that are closely linked to them have been identified. However, the use of these markers in oat breeding practice has been limited due to the economics of marker assisted selection (MAS) deployment. Single nucleotide polymorphism (SNP) markers have been demonstrated to have a high-throughput capability with relatively low cost and numerous semi-automated SNP scoring platforms exist. Gene *Pc94* has remained highly effective since it was first tested on the Canadian crown rust populations in 1993 and is one of the few effective genes available in Western Canada. In the present study, PCR products were amplified using primers derived from sequences of amplified fragment length polymorphism bands which have been shown to be linked to *Pc94*. Genomic DNA from genotypes, with and without the *Pc94* gene, were used as the PCR templates. By comparative sequence alignment amongst the PCR fragments, many putative SNP sites were identified. From these sites, four SNP sites were selected and validated by the single base extension method. One SNP site, *Pc94*-SNP1a, was tested on two $F_{2:3}$ populations segregating for the resistance gene. The map distances between the SNP marker and *Pc94* were 2.1 and 5.4 cM in the two different populations. Various oat cultivars and germplasm lines were also tested for a wider application of the SNP marker. Fluorescent technology and capillary electrophoresis allowed for the semi-automated, fairly high-throughput scoring of the SNP markers.

Key words: *Avena sativa* — *Puccinia coronata* — gene *Pc94* — SNP marker — single base extension

There is a great interest in developing an automated, robust, inexpensive and high-throughput detection platform for the scoring of molecular markers. The high-throughput expectation includes both high sample processing and the multiplexing capability for numerous marker sites. For many cultivated crops, the most commonly used marker detection methods (AFLPs, amplified fragment length polymorphisms and SSRs, simple sequence repeats or microsatellites) have generated high-density genetic maps for regions containing economically important genes (Meksem et al. 1995), quantitative trait loci (QTL) sites (Jahoor et al. 2004) and fingerprints between cultivars (Ghislain et al. 2004). However, these markers employ a tedious, gel-based scoring platform and have a limited multiplexing capability. Although there are examples of marker assisted selection (MAS) for single genes (http://maswheat.ucdavis.edu), few examples exist for multigenic traits in any actual crop breeding programme (Koebner 2004). The difficulties of MAS for large breeding programmes include scaling up the number of assays, simultaneous scoring of multiple markers (multiplexing) in pyramiding schemes and the cost per assay.

Single nucleotide polymorphisms (SNPs) are becoming the next generation of molecular markers due to their abundant frequency and suitability for genotyping by high-throughput and automated scoring platforms. SNPs have been discovered for a number of economically important plant species including maize (Ching et al. 2002), wheat (http://wheat.pw.usda.gov/SNP), barley (Rostoks et al. 2005), sugar beet (Schneider et al. 2001), soybean (Zhu et al. 2003) and rice (Nasu et al. 2002). SNPs are highly stable and often found in the gene coding region which allows for the discovery of gene-associated markers for phenotypic traits (Yanagisawa et al. 2003). There exists a plethora of SNP-scoring platforms each with its unique advantages and a detailed description of these is given by Kwok (2001). In this paper, we have used the single base extension (SBE) method to determine base-calls between genotypes. SBE also known as mini-sequencing is based on the high accuracy of nucleotide incorporation by DNA polymerase. The substrate for SBE reaction is the SNP-bearing PCR product obtained from genomic DNA. The assay requires the design of an extension primer with 3′ end immediately adjacent to the SNP site, the extension reaction uses dideoxynucleotide triphosphates (ddNTP) to ensure only a single nucleotide is extended, so the amplified base will be the targeting SNP base. The four ddNTPs are labelled with different fluorescent dyes. The labelled oligonucleotides are separated by capillary electrophoresis and the single base read by a fluorescence reader. This technique has multiplexing capacity and has been found to be extremely accurate (Brazill and Kuhr 2002).

Crown rust can cause significant yield losses of oat (*Avena sativa* L.) in many regions of the world. Breeding for disease resistance is the most effective and economical method to control this disease. Presently, a large number of race-specific resistance genes have been identified and various DNA molecular markers have been found to be linked to *Pc38*, *Pc39*, *Pc48*, *Pc68*, *Pc71*, *Pc91*, *Pc92* and *Pc94* (Wight et al. 2003). Race-specific resistance can be defeated due to selection pressure on the pathogen as a result of large-scale and long-term cultivation practices. Pyramiding multiple resistance genes into oat cultivars can extend the durability since the probability that the pathogen will simultaneously mutate at two or more sites for virulence (*vr*) is much lower than a single

mutation for virulence. Gene pyramiding is difficult to accomplish because of the dominance and epistasis effect of multiple resistance genes. In addition, two or more resistance genes may have similar rust reactions to numerous races making it difficult to identify specific resistance genes. Thus, molecular markers tagged to specific rust resistance genes facilitates gene pyramiding. Successful pyramiding of three bacterial blight resistance genes with molecular markers has been demonstrated in rice (Singh et al. 2001).

Pc94 is currently the most effective gene for resistance to *P. coronata* f. sp. *avenae* Eriks, and has been incorporated into 'Leggett', a cultivar released for the rust-areas of Canada in 2005. In a previous study, two AFLP fragments (AF94a and AF94b) associated with gene *Pc*94 were identified and the AF94a fragment was converted into two SCAR markers (Chong et al. 2004). In the present study, we have identified SNP markers linked to *Pc94* using the AFLP sequence (AF94a) and tested the usefulness of the markers by applying them to genetic populations, oat cultivars and lines with and without *Pc94*.

Materials and Methods

Plant materials and genomic DNA extraction: Two genetic populations of oat, *A. sativa*, were used to validate the SNP markers. One population consisted of 129 F_2-derived F_3 ($F_{2:3}$) lines originating from a single F_1 plant of an AC Assiniboia/S42 (Asb/S42) cross. The other population consisted of 130 $F_{2:3}$ lines derived from a single F_1 plant of a Calibre/S42 (Cal/S42) cross. S42 is a near-isogenic line carrying the *Pc*94 gene in the rust-susceptible cultivar 'Sun II' background (Aung et al. 1996). The *Pc94* originated from an *Avena strigosa* accession. 'AC Assiniboia' and 'Calibre' are agronomically important parents and lack the *Pc*94 gene. Crown rust races MCCB and NQBB (nomenclature according to Chong et al. 2000) were used to test Asb/S42 and Cal/S42 population, respectively. Race MCCB was virulent on 'AC Assiniboia' and race NQBB on 'Calibre'. Both races were avirulent on lines with the gene *Pc*94. About 25 seedlings from each $F_{2:3}$ lines were inoculated with urediniospores of each race at the one leaf stage. The inoculation, incubation and rust reading procedures were described in a previous study (Chong et al. 2004).

For DNA extraction, seedlings were sampled up to the fourth-leaf stage and the leaf samples were lyophilised in a freeze-dryer for 24 h. Total genomic DNA was extracted from dried leaf samples using a modified CTAB method (Silva and Procunier 1994).

PCR primer selection and amplification: Previous studies have identified two AFLP fragments, AF94a (577 bp) and AF94b (814 bp), to be linked to the resistance gene *Pc94* (Chong et al. 2004). These band sequences were used to design PCR primers to amplify oat genomic DNA. From each of the two AFLP-band sequences, four primer pairs were designed using DNAMAN (Lynnon Corp., Vaudreuil, QC, Canada) software. These primer pairs were tested for single band amplification for each of five resistant and five susceptible genotypes. These were the two parents and four resistant and four susceptible lines from the segregating Asb/S42 population.

The PCR reaction volume was 50 µl, containing 80 ng of genomic DNA, 1x PCR buffer (10 mM Tris–HCl, 50 mmol KCl, pH 8.3), 200 µM of dATP, dCTP, dGTP and dTTP, 150 ng of each of forward and reverse primers, 1.5 mM $MgCl_2$, and five units of Taq DNA polymerase (Promega Corp., Madison, WI, USA). PCR reactions were carried out in a PE9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with a touchdown programme as follows: samples were initially denatured at 94°C for 5 min followed by 20 cycles of 1 min denaturation at 94°C, 1 min annealing at 62°C (dropping 0.5°C each cycle) and extension for 2 min at 72°C; and another 20 cycles of 1 min at 94°C, 1 min at 52°C and 2 min at 72°C with a final extension for 10 min at 72°C. PCR products were separated in 1.4% (w/v) agarose gels containing ethidium bromide at 3.75 V/cm for 3 h.

Putative SNP identification by comparative DNA sequence alignment: The touchdown PCR products that showed a single band on agarose gels from the five resistant/five susceptible genotypes were purified using the QIAquick PCR purification kit (QIAGEN Inc., Mississauga, ON, Canada) and the samples were directly sequenced on the ABI 3100 Prism® Genetic Analyser (BigDye Terminator chemistry; Applied Biosystems). No cloning step was necessary. The sequences were aligned using the Multiple Alignment program of the DNAMAN sequence analysis software. By comparative DNA sequence alignment, putative SNPs between the resistant and susceptible genotypes were identified. These base variations were checked with the DNA sequence chromatograms for confirmation.

SBE for SNP validation: The SBE assay (Brazill and Kuhr 2002) was used for validating putative SNPs discovered by the comparative sequence alignment method. The SBE primers were designed with their 3′-ends immediately before the SNP position and had at least 18 bases in length with melting temperature ranging from 42°C to 52°C. The touchdown PCR product(s) containing the putative SNP(s) was used as the template for the SBE assay. The SBE primers were tested on a panel of 16 genotypes from the Asb/S42 population consisting of the two parents and seven resistant and seven susceptible $F_{2:3}$ progeny lines. The SBE test was carried out according to suppliers specifications (Applied Biosystems) using the ABI Prism SNaPshot Multiplex kit. The SBE fragments were monitored by capillary electrophoresis in the ABI3100 Genetic Analyzer. Different fluorescent dyes were assigned to the individual bases such as ddATP (dRGG, green), ddCTP (dTAMRA, black), ddGTP (dR110, blue) and ddTTP (dROX, red). The resulting data were analyzed for peak colour and fragment size using the ABI Prism® GeneScan™ Analysis software (version 3.7, 2001; Applied Biosystems, Foster City, CA, USA).

Linkage analysis and genotyping oat cultivars and lines: After validating the SNP sites by SBE on the panel of 16 genotypes, one of the SNP sites (*Pc94-SNP1a*) was chosen to test on the two *Pc94* segregating populations (Asb/S42; Cal/S42). Linkage between *Pc94* and the SNP markers was analyzed with Mapmaker version 3.1 (Lander et al. 1987). SCAR marker primer pairs (CR24f/CR477r and CR127f/CR612r), developed in a previous study (Chong et al. 2004) were also applied to the Asb/S42 and Cal/S42 populations to determine the relationship between the SCAR and the SNP markers. Twenty-two oat cultivars/lines lacking the *Pc94* gene, the resistance donor parent *A. strigosa* accession RL1697 (Cereal Research Centre designated) and 'Leggett', the only cultivar with *Pc94* currently exists, were genotyped using the same SNP marker *Pc94-SNP1a*.

Results

Disease evaluation

The $F_{2:3}$ lines were classified as homozygous resistant, segregating and homozygous susceptible when tested with rust race NQBB for Cal/S42 and race MCCB for Asb/S42 populations. The segregation ratio for *Pc94* resistance in the two populations fit the 1 : 2 : 1 ratio indicating a single dominant gene (Table 1).

Putative SNP identification

Out of the eight PCR primer pairs tested, only the primer pair *Pc94*-577-500 (F: AACGAATCCGGCAGATGATA; R: GCGTAGGTGCAGAGGGTTAC), designed from the DNA sequence of the 577 bp AFLP band (AF94a) yielded a single, clean amplification band. This single band was present

Table 1: Segregation of rust resistance in the two $F_{2:3}$ populations

Populations	Rust races	No. of $F_{2:3}$ lines			Expected ratio	X^2	P-value
		Resistant	Segregating	Susceptible			
Calibre/S42	NQBB	30	70	30	1 : 2 : 1	0.769	0.6807
Assiniboia/S42	MCCB	30	67	32	1 : 2 : 1	0.256	0.8799

Table 2: Single base extension (SBE) primer sequences (5′ to 3′) and extended bases for detecting Single nucleotide polymorphisms (SNP) in a panel of eight resistance and eight susceptible oat genotypes

SNP site	Sequence of SBE primer	Sequence alignment derived SNP base (s)[1]	SBE base (s)	Position within 519 bp amplicon
Pc94-SNP1a	GTGRTCAACTAGACCTGA[2]	TC/C	TC/C	415
Pc94-SNP1	ACTTTGAGATGGCGATTT	GC/G	GC/G	186
Pc94-SNP2c	CATCTCAAAGTCAAGGGA	GA/G	GA/G	194
Pc94-SNP3	GATGYCGCACTATGTCCT[3]	GC/C	GC/C	240

[1]The underlined SNP bases represent the *Pc94* resistant allele found in resistant oat genotypes; the susceptible genotypes have the non-underlined base.
[2]R was G/A, degenerate SBE primers.
[3]Y was C/T, degenerate SBE primers.

in all ten genotypes (two parents, four resistant and four susceptible lines) selected from the Asb/S42 population. The remaining primer pairs showed either multiple or weak band(s) on some of the genotypes and these were not used further. Direct sequencing of this band amplified by primer pair *Pc94*-577-500 on the 10 genotypes revealed 519 bp amplicons. From BLAST (Basic Local Alignment Search Tool) search, this 519 amplicon was found to have a 78% identity over a 321 base alignment to a nucleotide sequence in *Triticum monococcum* actin (ACT) gene (GenBank accession No AF326781.1) and a 78% identity over a 321 base alignment to a *Triticum aestivum* resistance protein T10rga2-1A gene (GenBank accession No. AY270159.1). Within the 519 bp amplicon, more than 50 putative SNP sites were identified between the resistant and susceptible genotypes. Since base calling errors can affect identifying true SNP sites, it is suggested that SNP bases surrounded by perfectly aligned, consistently high quality sequence would have less base calling errors and suitable for SNP identification. This kind of sequence region has been termed good neighbourhood (Altshuler et al. 2000). Four SNP sites from the good neighbourhood regions were selected for SBE validation (Table 2). Two of these SNP sites (*Pc94*-SNP1a and *Pc94*-SNP3) had additional single base differences within the SBE primer sequence between genotypes, thus degenerate SBE primers were designed for these sites.

The sequence data showed that SNP sites may consist of multiple or alternative SNP bases at the same base position within the amplicon(s) for a single genotype. For example, at the SNP site *Pc94*-SNP1a located at 415 bp within the 519 bp amplicon (Fig. 1), the T and C bases were present at the same base position in all the resistance genotypes while only C bases were found in the susceptible genotypes. These multiple bases at a single site were probably due to the expected heterogeneity of PCR products derived from near-identical homoeologous or paralogous sequences (Chen 2000).

SNP validation by SBE

The four selected SNP sites identified by comparative sequence alignment were corroborated by the SBE assay, an independent SNP detection method for culling SNP base-calling errors

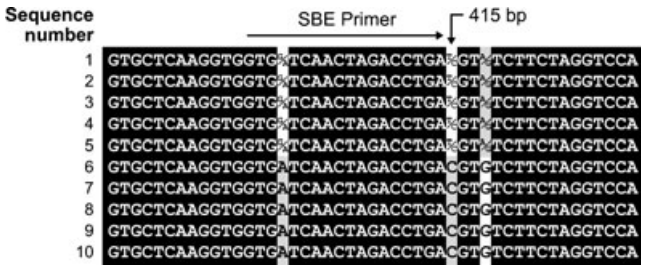

Fig. 1: Alignment of partial sequences for amplicon(s) harbouring single nucleotide polymorphism (SNP) site *Pc94*-SNP1a. Sequences 1–5 are from resistant genotypes and sequences 6–10, from susceptible genotypes. Sequences are in the 5′ to 3′ direction and the single base extension (SBE) primer sequence annealed to the complementary strand of the given sequence

from the putative SNP sites. All four SNP sites were validated for their base variations and the SNP markers showed co-segregation with *Pc*94 gene in the panel of 16 genotypes with and without *Pc94* (Table 2). For example, on SNP site *Pc94*-SNP1a, two SBE bases (T and C) derived from PCR amplicons were observed for the parent S42 and the seven resistance lines, whereas a single SBE base (C) was found in the parent 'Calibre' and the seven susceptible lines (Fig. 2). Similarly, *Pc94*-SNP1, *Pc94*-SNP2c and *Pc94*-SNP3 also had alternative bases for the resistance genotypes and a single base for the susceptible genotypes (Table 2).

Linkage analysis and genotyping

The map distances and recombination rates between the SNP marker *Pc94*-SNP1a and gene *Pc94* were estimated at 2.1 cM and 2.3% for Cal/S42, and 5.4 cM and 6.1% for Asb/S42 populations. The segregating patterns in the two populations with the SCAR marker primer pairs (CR24f/CR477r and CR127f/CR612r) were identical to the patterns with SNP marker *Pc94*-SNP1a (Table 3). Whenever an oat line showed a + allele with the SCAR markers, this line showed TC bases extension with the SBE reaction. Conversely, whenever an oat line showed a 0 allele with the SCAR markers, it showed a C base with SBE reaction. The applicability of using the newly

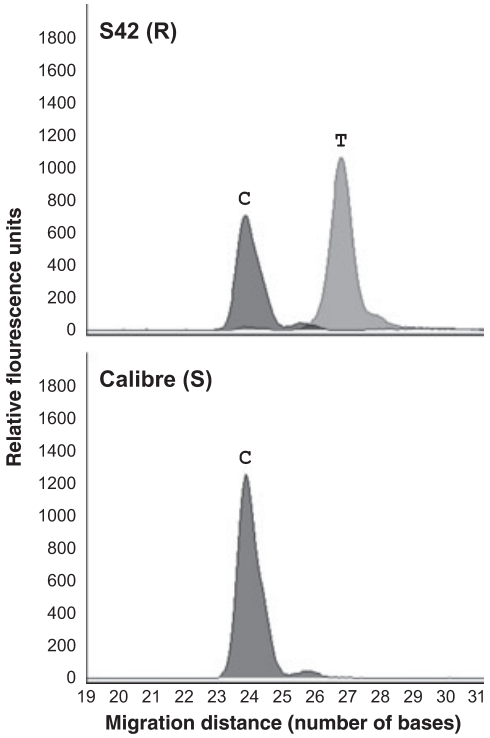

Fig. 2: Example of single nucleotide polymorphism (SNP) site validation by the single base extension (SBE) reaction and heterogeneity of genomic PCR amplicons. SBE product(s) from near-identical PCR amplicons for a single SNP site (*Pc94*-SNP1a) were separated by capillary electrophoresis. The resistance parent (S42) showed both the T and C bases and susceptible parent ('Calibre') had a C base. The *x*-axis was the migration distance in bases and the *y*-axis, the relative fluorescent units (rfu)

identified SNP markers for testing other oat cultivars/lines for presence of *Pc94* was also investigated. Using the *Pc94*-SNP1a site, 20 cultivars lacking the *Pc94* gene showed the expected C allele. The resistant cultivar 'Leggett' had TC alleles and the resistance donor parent *A. strigosa* accession RL1697 had a T allele (Table 4).

Discussion

This study uses AFLP sequence information to discover SNP markers linked to crown rust resistance gene *Pc94* in oat. It converted previously associated AFLP markers to SNP markers by exploiting pre-existing linkage information of important trait(s). Comparisons of amplicon sequences between five resistant and five susceptible genotypes resulted in the identification of putative SNP markers for *Pc94*. With the fairly high-throughput procedure for SNP verification as the SBE assay, the putative SNP markers were validated and shown to be linked to *Pc94* using segregating populations.

Developing SNP markers is technically challenging in polyploidy plants due to the homoeologous and paralogous sequences in the genomes. The genome of hexaploid oat is comprised of three homoeologous genomes (AACCDD) and numerous gene families (paralogs) are present. The PCR generates near-identical gene sequences from the genome resulting in multiple, near-identical PCR amplification products. This difficulty was demonstrated in this study in that, out of the eight primer sets designed from the two linked AFLP bands, only one primer pair amplified a single, clean band from the five resistant and five susceptible genotypes tested. In addition, even this visually identified single agarose band contained at least two different amplicon sequences of identical size. Thus multiple or alternative SNP bases at the same base position within the amplicons are present for a single genotype (Figs 1 and 2). Different genotypes can have unique single/multiple bases at this SNP site and this fingerprints these genotypes. For the tetraploid plant potato, alternative bases at a single SNP amplicon site were also observed (Chen 2000).

The comparative sequence alignment approach for identifying SNPs from PCR amplicons derived from AFLP marker information offers a rapid and efficient method for the conversion, as no laborious cloning procedure is required. Often a single AFLP band was shown to contain multiple fragments (4–30) of similar size co-migrating with the target AFLP band during electrophoresis (Meksem et al. 2001, Xu and Ban 2004). These contaminating fragments may be preferentially cloned thus hampering the isolation of the sequence that gave the original polymorphism. Other cloning AFLP conversion techniques, such as inverse PCR (Bradeen and Simon 1998) and the extension-AFLP method (Xu and Ban 2004) reconstruct the original fragment-end polymorphism. However, the multiple PCR fragments generated from complex, highly repetitive genomes makes this kind of conversion difficult.

Within the 519 bp amplicon, more than 50 putative SNP sites were found. This number is much higher than SNP

Table 3: Segregation of single nucleotide polymorphism (SNP) and SCAR markers in the two $F_{2:3}$ populations

		SBE bases from *Pc94*-SNP1a		SCAR bands from CR24f/CR477r and CR127f/CR612r			
Population	No. of lines	TC	C	+	0	$X^2_{(3:1)}$	$P_{(3:1)}$-value
Calibre/S42							
Resistant	30	30	0	30	0		
Segregating	70	69	1	69	1		
Susceptible	30	2	28	2	28		
Total		101	29	101	29	0.503	0.4784
Assiniboia/S42							
Resistant	30	29	1	29	1		
Segregating	67	63	4	63	4		
Susceptible	32	2	30	2	30		
Total		94	35	94	35	0.313	0.5761

Table 4: Assignment of single nucleotide polymorphism (SNP) alleles for the *Pc94*-SNP1a site of oat cultivars/lines with and without gene *Pc94*

Cultivars/lines	Gene *Pc*94	SBE base (s)	Cultivars/lines	Gene *Pc*94	SBE base (s)
RL1697 (*Avena strigosa)*	*Pc*94	T	Goslin	–	C
Leggett	*Pc*94	TC	Gwen	–	C
AC Aylmer	–	C	HiFi	–	C
AC Francis	–	C	Kaufmann	–	C
AC Morgan	–	C	Makuru	–	C
AC Pinnacle	–	C	Marion	–	C
AC Stewart	–	C	Ogle	–	C
Boudias	–	C	Riel	–	C
Calibre	–	C	Robert	–	C
CDC Boyer	–	C	Ronald	–	C
CDC Dancer	–	C	Steele	–	C
Dumount	–	C	Triple crown	–	C

frequencies reported from other plant species. On average, corn has one SNP per 48 bp in the non-coding regions and one SNP per 131 bp in the coding regions (Rafalski 2002). Barley has one SNP per 189 bp (Kanazin et al. 2002). A plausible explanation is that for the resistant genotypes, one of the PCR sequences was amplified from *A. strigosa* DNA harbouring *Pc94*. For the susceptible genotypes, all PCR sequences were amplified from cultivated oat DNA. Thus the sequence alignments between resistant and susceptible genotypes were actually between fragments from alien (*A. strigosa*) and cultivated oat DNA.

The ABI 3100 Genetic Analyzer used for SBE test was able to detect simultaneously all four possible bases at a single SNP site. This is especially significant for the simultaneous detection of alternative bases at homoeologous and paralogous sequence sites, characteristic of polyploid genomes in plants. The equipment used 16 capillaries simultaneously and approximately 768 samples (single well/capillary) can be interrogated in 24 h after the PCR set-up and SAP clean-up phases. Furthermore, by multiplex loading (up to 10-plex) of SBE fragments of varying lengths in one capillary, an even higher throughput can be achieved. The SBE detection platform using fluorescent technology and capillary electrophoresis allowed for high-throughput and reliable marker scoring system.

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