Identification and Characterization of Random Amplified Polymorphic DNA Markers Linked to a Major Gene (*Cr2*) for Resistance to *Cronartium ribicola* in *Pinus monticola*

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ABSTRACT

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DNA markers tightly linked to resistance (R) genes provide a very powerful tool for both marker-assisted selection in plant breeding and positional cloning of R genes. In the present study, a linkage of random amplified polymorphic DNA (RAPD) markers to the single dominant gene (*Cr2*) for resistance to white pine blister rust fungus (*Cronartium ribicola*) was investigated in western white pine (*Pinus monticola*). A mapping population of 128 individual megagametophytes was generated from seeds of a heterozygous resistant tree (*Cr2*/*cr2*), and the corresponding seedlings of each megagametophyte were subjected to the test of phenotype segregation by inoculation with *C. ribicola*. Bulked segregant analysis and haploid segregation analysis identified eight robust RAPD markers linked to *Cr2*. This constitutes the first *Cr2* genetic linkage map spanning 84.7 cM with four markers only 3.2 cM from *Cr2*. One sequence (U256-1385) of these linked markers was significantly similar to the *Ty3*/*gypsy*-like long terminal direct repeats retrotransposons. Another marker, U570-843, had no significant similarity to any entry in either GenBank or the loblolly genomics data bank. As presumed that the average physical distance per centimorgan is about 10 Mb in *P. monticola*, it is probably unrealistic to use these DNA markers for positional cloning of the *Cr2* gene.

White pine blister rust is a serious disease of western white pine (*Pinus monticola* D. Don.) and other five-needle pine species. It is caused by fungal pathogen *Cronartium ribicola* (J.C. Fisch). Since its introduction to North America in the early 1900s, the spread of *C. ribicola* has devastated western white pine. Western white pine is a fast-growing and highly valuable forest timber species. The dramatic reduction of western white pine has caused a serious impact on both the forest industry and ecosystems of North America. Discovery and deployment of pathogen-resistant western white pine is believed to be a reasonable way to restore this highly valuable species in ecosystems and commercial plantations (8,25). Therefore, breeding and selection for white pine blister rust resistance is one of the important objectives in western white pine research programs.

A typical gene-for-gene system has been identified in the white pine blister rust pathosystem. A dominant gene (*Cr1*) was first discovered in sugar pine (*P. lambertiana* L.) for major gene resistance that triggers a typical hypersensitive response (HR), i.e., rapid localized cell death at the sites of *C. ribicola* infection on needles (18,19). Single dominant resistance (R) genes also have been demonstrated in other five-needle pines, including the *Cr2* gene from western white pine (20) and the *Cr3* gene from southwestern white pine (*P. strobiformis* Engel.) that confer a complete resistance to *C. ribicola* (17). Performance of the *Cr2* gene in seven plantations in British Columbia (BC), Canada over 13 years showed that no virulent pathotype of *C. ribicola* was present and that the *Cr2* gene appeared to be stable in BC (14).

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Resistance conferred by single dominant R genes is specific to a particular pathogen race that expresses the corresponding avirulence gene. Usually pathogen avirulence (*Avr*) genes have coevolved with host R genes. Evidence from the flax rust pathosystem has showed that *Avr* genes have been under strong positive selection, and that adaptive evolution has occurred in both components of an *R-Avir* gene pair (6). Therefore, protection conferred by individual R genes may not be durable (27). Two virulence genes (*Vcr1* and *Vcr2*) are known to overcome the resistance conferred by *Cr1* in sugar pine and *Cr2* in western white pine, respectively, but no virulence gene has yet been found to negate *Cr3* in *P. strobiformis* (17). The pyramiding of several pathogen race-specific resistance genes into one single plant genotype in a seed orchard will theoretically improve white pine blister rust resistance. Marker-assisted selection will make it possible to breed trees with a desired genotype. However, genetic analyses of conifer species are hampered by long generation times and relatively high cost of experimental hybridization. In addition, it is a challenge to explore and develop genomic resources in conifers because of the extremely large sizes of their nuclear genomes. Therefore, a molecular marker tool would save time and cost in forest-tree breeding.

To effectively apply marker-assisted selection in a breeding program, a molecular marker tightly linked to a target gene would be most reliable and cost-effective because of the elimination or reduced chance of recombination between the marker and the desired gene. This is especially important for the application of marker-assisted selection for the breeding programs using open-pollinated populations such as those in conifers. Random amplified polymorphic DNA (RAPD) markers have been widely used in the construction of genetic maps. RAPD markers linked to major R genes have been identified in two conifer species, sugar pine (5,12) and loblolly pine (*P. taeda* L.) (35). An RAPD linkage map of a gene for resistance to pine needle gall midge has also been constructed in Japanese black pine (*P. thunbergii*) (21).

We report here the use of a PCR-based genetic marker technique in one western white pine family that showed segregation (*Cr2/cr2*) for white pine blister rust resistance. The aim of the present study was to identify and characterize molecular markers linked to the *R* gene (*Cr2*) in western white pine as a part of our ongoing program with the long-term target to understand the molecular mechanisms of host genetic resistance in the white pine blister rust pathosystem.

MATERIALS AND METHODS

Plant material. A western white pine tree, 3566, originally from a bulked Dorena seed orchard collection (U.S. Department of Agriculture [USDA], Forest Service), was previously identified as a heterozygous resistant tree (*Cr2/cr2*) because typical Mendelian segregation in the family of this parent tree was observed in our breeding program (R. S. Hunt, *unpublished data*). Open-pollinated seeds of tree 3566 were germinated in June 2002. Haploid megagametophytes were harvested individually during seed germination and stored at –20°C for genomic DNA extraction. Seedlings corresponding to each megagametophyte were labeled and transplanted into styroblocks and grown in a greenhouse at the Pacific Forestry Centre (Victoria, BC, Canada). These styroblocks were moved to the Saanichton *Ribes* garden (Saanichton, BC, Canada) for *C. ribicola* infection in September 2002. Inoculation was performed by placing blocks beneath *C. ribicola*-infected *Ribes* spp. for 2 months during basidiospore release. The styroblocks were transferred back into the greenhouse where seedlings were observed for disease-symptom development on needles. The phenotype of each seedling was assessed for normal infection spots or HR spots in March 2003. The susceptible phenotype was further confirmed by stem-canker development, whereas the HR phenotype lacked cankers throughout the summer of 2003.

DNA isolation and RAPD analysis. Genomic DNA was isolated from megagametophyte tissues with a DNeasy plant mini kit (Qiagen, Mississauga, Ontario, Canada). A conifer RAPD primer kit (100 primers), designed specifically for conifer species, was obtained from the University of British Columbia (Vancouver, BC, Canada). Another set of 160 10-base random primers (Kits D, E, F, G, H, I, J, and K) was obtained from Operon Technologies (Alameda, CA).

Bulked segregant analysis (BSA) (28) and haploid segregation analysis (5) were used to map R gene linkage in western white pine. BSA was carried out to screen random primers able to detect polymorphism. For this purpose, bulked DNA samples were equally mixed from 15 resistant or 15 susceptible individuals. *Taq* master mix kits (Qiagen) were used for RAPD PCR in a total volume of 25 µl with about 5 ng of megagametophyte DNA from either individual megagametophytes or bulked samples. PCR conditions consisted of pre-denature at 94°C for 3 min, 45 cycles of 30 s at 94°C, 30 s at 36°C, and 2 min at 72°C with a final step of 7 min at 72°C. The amplified products of 5 µl were separated electrophoretically on 1.5% agarose gels and visualized by ethidium bromide fluorescence. The remaining PCR products were saved at –20°C for DNA cloning and sequencing to characterize *Cr2*-linked DNA markers.

DNA markers with segregation in the population were scored for presence (1) or absence (0) of the amplified fragments. The genotype of the haploid megagametophyte is the same as that one in the corresponding diploid seedling contributed by the maternal parent. A heterozygous locus segregates in a ratio of 1:1 in haploid megagametophytes of conifers. Each RAPD marker was tested for Mendelian segregation by χ^2 test ($\rho = 0.05$). The chi-squares and significance values (*P*) were analyzed with Yate's adjustment for the segregation of each RAPD marker in this population. Only those DNA markers that fit the Mendelian ratio (1:1) significantly ($\rho = 0.05$) were selected for further genetic mapping analysis. To avoid resistance phenotype from pollen containing the R gene (*Cr2*), only megagametophyte samples corresponding to susceptible individual seedlings were included to determine genetic linkage between any DNA marker and the *Cr2* gene. The whole population, including megagametophytes corresponding to both resistant and susceptible seedlings, was used for cosegregation analysis for any two DNA markers. Recombination fraction (RF) and relative LOD (the log of the odds ratio) scores of each DNA marker to the *Cr2* gene were calculated using "lod table" command in the Mapmaker program (22). To construct the linkage map, data were analyzed using MAPMAKER (22) version 3.0, with parameters of a LOD score larger than 3 and map distance below Haldane 50 cM.

Characterization of *Cr2*-linked DNA markers. Partial original PCR products were separated by agarose gel fractionation. The polymorphic DNA fragments were purified using a QIAquick gel extraction kit (Qiagen) and ligated with pGEM-T easy vector (Promega, Madison, WI), according to the manufacturer's instructions. Ligation-reaction mixtures were transformed into chemical competent cells *Escherichia coli* DH5α (Invitrogen, Burlington, ON, Canada). White clones were selected for plasmid extraction using a QIAprep spin miniprep kit (Qiagen). Recombinant clones with inserts of expected sizes were determined with restriction enzyme analysis. Nucleotide sequences of a few DNA clones from each fragment were determined on both strands at Macrogen, Inc. (Seoul, South Korea).

DNA sequence data were compiled and analyzed using DNASTAR programs (DNASTAR, Inc., Madison, WI) and ExPASy Proteomics tools (available online from the Swiss Institute of Bioinformatics). Protein sequences were aligned with the Clustal W network service at the European Bioinformatics Institute (EBI, Cambridge, UK). DNA sequences were searched for homology to entries in GenBank at the National Center for Biotechnology Information (NCBI) (available online from NCBI) and those in the loblolly genomics data bank at the Center for Computational Genomics and Bioinformatics (CCGB) (available online from CCGB, University of Minnesota) using programs of BLAST (1). Nucleotide sequences of characterized DNA markers were deposited as GenBank accession nos. DQ144972 and DQ144973.

RESULTS

Disease symptom assessment. Periodically all needles of each seedling were assessed for disease-symptom development. By spring, there were 128 infected seedlings; of these, 67 were identified as resistant because HR spots indicative of the *Cr2* gene were observed on their needles and no stem cankers subsequently developed during the following summer and fall. The remaining 61 seedlings were classified as susceptible because typical yellow disease spots developed on their needles, and stem cankers appeared during the following summer. The segregation of resistant versus susceptible progeny fit the 1:1 ratio expected for a single major resistance gene ($\chi^2 = 0.062$, $\rho = 0.80$); it also suggests that the contamination by open pollen with the *Cr2* gene was not significant.

Identification of RAPD markers linked to the *Cr2* gene. The genomic DNA samples were extracted from megagametophyte tissues of individual seeds that constituted the whole population of 128 seedlings with identified phenotypes for genetic mapping. To identify DNA markers linked to the *Cr2* locus, BSA was first performed to detect polymorphism between resistant and susceptible bulks that were equally pooled from 15 samples. Initial screening with the 100 conifer-characterized primers, as well as the 160 randomly selected primers, revealed that 27 of them produced DNA polymorphic fragments between resistant and susceptible bulks. When these 27 primers were used further to uncover DNA markers in individual megagametophytes that constituted the bulks, 53 DNA fragments were identified as polymorphic in

this small population of 30 samples. Figure 1 shows examples of RAPD profiles generated by 10-mer primers U181, U256, U550, and OA11 that produced RAPD markers U181-420, U256-1385, U550-2200r, and OA11-2500, respectively. Seventeen primers were selected to characterize DNA markers in the whole population of 128 individuals. RAPD profile analysis of the whole population led to the identification of eight DNA markers derived from seven random primers that showed recombination with *Cr2* from 0.033 to 0.311 (Table 1). These eight markers segregated at an expected ratio of 1:1. No distorted segregation was detected for these markers by the χ^2 test (Table 1).

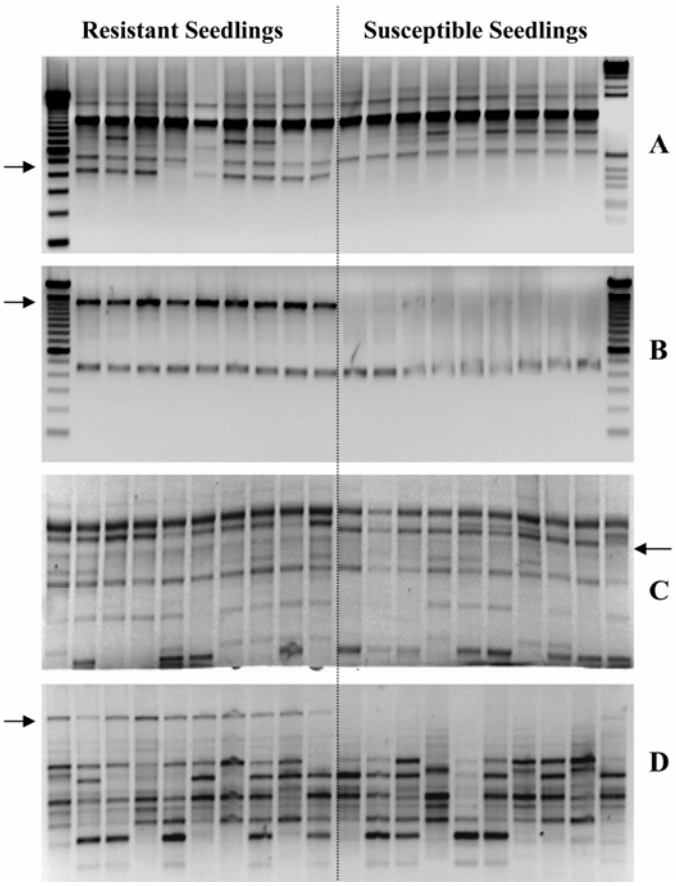

Fig. 1. Random primer amplification of DNA isolated from individual seed megagametophytes from resistant tree 3566. **A,** Polymerase chain reaction (PCR) profiles of primer U181, a DNA marker of 420 bp (U181-420), is linked with *Cr2* in coupling. **B,** PCR profiles of primer U256, a DNA marker of 1,385 bp (U256-1385), is linked with *Cr2* in coupling. **C,** PCR profiles of primer U550, a DNA marker of 2,200 bp (U550-2200r), is linked with *Cr2* in repulsion. **D,** PCR profiles of primer OA11, a DNA marker of 2,500 bp (OA11-2500), cosegregates with *Cr2* in coupling. The arrows indicate these random amplified polymorphic DNA (RAPD) markers.

A genetic map of the *Cr2* linkage was constructed with these eight DNA markers that spanned 84.7 cM (Fig. 2). Six of them (U181-420, U256-1385, U550-2500, U570-843, OA11-2500, and OI10-1250) were linked to *Cr2* in coupling, and the other two (U550-2200r and U561-690r) in repulsion. Seven markers were located on one side of the *Cr2* locus, and only one on the other side, far from the location of *Cr2* at 40.0 cM. Four RAPD markers, U256-1385, U550-2200r, U570-843, and OA11-2500, inherited as one block, were located 3.2 cM from *Cr2*. Another RAPD marker, U181-420, showed no recombination with the above four RAPD markers in 61 susceptible seedlings (Table 1), but showed 4.4% recombination in 67 resistant seedlings. Therefore, the U181-420 marker was mapped 6.5 cM from the *Cr2* gene based on its recombination with other DNA markers (Fig. 2).

RAPD marker sequences. To develop single copy DNA markers for both potential marker-assisted selection in breeding

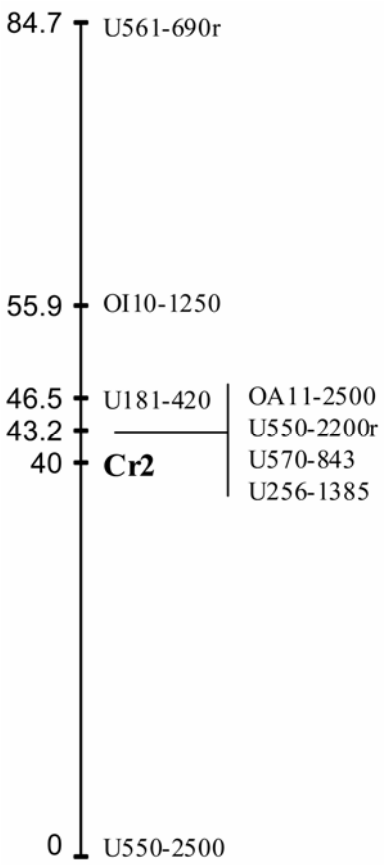

Fig. 2. Linkage map of random amplified polymorphic DNA (RAPD) markers around the *Cr2* gene that confers major gene resistance to *Cronartium ribicola* in western white pine (*Pinus monticola*). Eight RAPD markers were mapped to five distinct loci. Genetic map distances are in Haldane centimorgans (cM).

TABLE 1. Segregation ratios of eight random amplified polymorphic DNA (RAPD) markers and their linkage association with a western white pine major gene (*Cr2*) for resistance to *Cronartium ribicola*

Marker	Segregation frequencies[a]		Marker ratio (+/–)	χ^2 (1:1)	*P*	Recombination fraction	LOD score
	Susceptible	Resistant					
OA11-2500	2(+)/59(–)	60(+)/7(–)	0.939	0.015	0.90	0.033	14.54
U256-1385	2(+)/59(–)	59(+)/7(–)	0.924	0.035	0.85	0.033	14.54
U550-2200r	59(+)/2(–)	7(+)/60(–)	1.064	0.015	0.90	0.033	14.54
U570-843	2(+)/59(–)	60(+)/7(–)	0.939	0.015	0.90	0.033	14.54
U181-420	2(+)/59(–)	56(+)/11(–)	0.828	0.391	0.53	0.033	14.54
OI10-1250	8(+)/53(–)	51(+)/16(–)	0.855	0.250	0.61	0.131	8.07
U561-690r	43(+)/18(–)	30(+)/37(–)	1.327	1.004	0.31	0.295	2.29
U550-2500	19(+)/42(–)	53(+)/14(–)	1.285	0.768	0.38	0.311	1.93

[a] Marker presence (+) or absence (–) of RAPD markers in total numbers of individual megagametophytes (61 susceptible + 67 resistant).

programs and positional characterization of the *Cr2* gene in the future, the DNA bands of two RAPD markers, U256-1385 and U570-843, were cloned and sequenced. Multiple clones showed identical sequences for each fragment. A homology search using BLAST (1) revealed that U570-843 (843 bp in length) showed no significant similarity to any entry in either GenBank or the loblolly genomics data bank. However, the putative amino acid sequence of U256-1385 (1,385 bp in length) had significant similarities (~6e-09) to the putative gag/pol polyproteins of *Ty3/gypsy*-like long terminal direct repeats (LTR) retrotransposon from *Pisum sativum* L. (29) and other angiosperm species.

DISCUSSION

This is the first report of genetic mapping of the *Cr2* gene. We used both haploid segregation analysis and BSA to identify RAPD markers tightly linked to *Cr2*. Because of their high variability, primary neutrality and inheritance in a dominant Mendelian manner, RAPD markers are widely applied to the genetic characterization of populations from a variety of plant species (9). Since maternal haploid tissue is available from seed megagametophytes, the RAPD technique has especially provided a powerful tool to investigate genetic markers linked to major resistance genes in conifer species (5,12,21,35). RAPD markers also have been identified for the mapping of disease resistance genes in woody angiosperms, including Chinese elm (*Ulmus parvifolia*) (3), *Populus deltoides* (32), *Corylus avellana* (4,26), *Eucalyptus* (16), and apple (15). Short primer length (10- or 12-mer) and low annealing temperatures in PCR amplification confer low reproducibility on some RAPD markers, which limits the effective application of this technique in marker-assisted selection for breeding programs. Therefore, we selected just those RAPD markers that are strictly repeatable and easy to score for our current work. Of the 260 random primers used for BSA, only 17 primers were further used to reveal polymorphisms in the whole population of 128 megagametophytes. Ultimately, we identified eight robust RAPD markers that were linked to the *Cr2* gene.

In the population of 128 individuals, four RAPD markers (OA11-2500, U550-2200r, U570-843, and U256-1385) were inherited as one block and located on one side of the *Cr2* locus. These four DNA markers showed only 3.3% recombination with white pine blister rust resistance (Table 1), and they were mapped 3.2 Haldane cM from *Cr2*. Short genetic distance of these RAPD markers to *Cr2* makes them potentially useful for marker-assisted selection within the defined pedigree arising from tree 3566. However, the average genome size estimated from feulgen microdensitometry and flow cytometry for *P. monticola* is 28.25 pg/1C (34), here 1C value means DNA content of one nonreplicated holoploid genome with the chromosome number *n*. Since 1.0 pg of DNA is equivalent to 965 million bases (Mb) (2), the predicted length of *P. monticola* genome is about 2.7×10^4 Mb per 1C genome. If the recombinational genome size of western white pine were the same as 2,600 Haldane cM of eastern white pine (7), the average physical distance per Haldane centimorgan would be roughly 10 Mb in *P. monticola* genome, suggesting that the closest markers we identified may be about 32 Mb from *Cr2*. In *Pinus* species, the recombination rate is very low related to their genome sizes, as indicated by the larger million bases number per centimorgan. It is necessary to obtain a genetic map with resolution about 0.01 cM for conifer loci of interest before any attempt at physical mapping is made; therefore, a map-based cloning strategy for *Cr2* characterization is very challenging. Although it seems unrealistic to use the DNA markers identified here for positional cloning of the *Cr2* gene, the *Cr2* genetic linkage map constructed in the current work constitutes a comprehensive basis for further fine mapping of the *Cr2* locus at both sides, using a population with at least thousands of individuals. Our previous work found that there is a complex gene family of resistance gene analogs (RGAs) in the western white pine genome (23). A class IV chitinase isoform has been identified to be associated with slow-canker-growth (SCG) resistance to *C. ribicola* in western white pine (24). The identified RAPD markers that were linked or unlinked to *Cr2* will provide anchored loci for the genetic mapping of RGAs and other functional genes related to disease resistance (such as chitinase) in western white pine.

One of the sequenced RAPD markers, U256-1385, has significant homology with *Ty3/gypsy*-like retrotransposons that belong to one of two retrotransposon groups with LTR. This finding is not surprising, as both *gypsy*-like and *copi*-like LTR retrotransposons are present as highly heterogeneous and diverse genetic elements that are distributed broadly in higher plants, including gymnosperms (10,11,31,33). Retrotransposon-based molecular marker systems have been developed for high-throughput marker analysis in higher plants, and have revealed more information than other marker systems (30). Although the presence of retroelement-like sequences in some markers essentially renders them useless as probes for positional cloning, the use of genome-walking strategies may identify single-copy sequences adjacent to sequences containing retroelement-like markers. For the RAPD marker U570-843, no homologous sequence was found in GenBank or in the data bank of conifer genomics, suggesting that it may be unique. Genomic blots will be probed to assess the copy number of the DNA markers of interest before their application for further physical mapping. Cloning and sequencing of these DNA markers will make feasible the development of sequence-characterized amplified region (SCAR) markers to allow targeted selection of the resistance allele by PCR. The sequence information of these western white pine DNA markers will facilitate further investigations of *Cr2* locus identification, as well as marker-assisted selection in white pine.

Different genetic mechanisms confer resistance to white pine blister rust, including the *Cr2* major dominant gene, partial resistance by SCG, and others (13,25). Observations over several years, of plantations located across large geographic areas, confirm that the *Cr2* gene provides complete protection against diverse isolates of *C. ribicola* from BC (14). One way to improve the durability of white pine blister rust resistance is to pyramid resistance genes to provide multigenic resistance against a wide spectrum of *C. ribicola* isolates (25). For this purpose, the *Cr2* gene is being crossed into SCG to establish *Cr2* × SCG pollen sources for SCG seed orchards in BC. To test outcomes of these crosses by traditional methods is laborious and time-consuming because it takes at least 7 years for cankers to develop after infection. The DNA markers identified in this study, or SCAR markers derived from them, will be tested for possible application in screening out susceptible seedlings without *Cr2*. Based on these DNA markers, we will develop additional DNA markers specific for SCG resistance for the selection of seedlings with both *Cr2* and SCG resistances.

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