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Characterisation of Gene H in Red Raspberry: Explaining its Role in Cane Morphology, Disease Resistance and Timing of Fruit Ripening

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Abstract

The Gene H region on raspberry linkage group 2 is an interesting region associated with a number of diverse traits. Cane pubescence is determined by Gene H and cultivars and selections with fine hairs (pubescent canes) are more resistant to cane botrytis and spur blight than non-hairy ones. Gene H is the most significant marker in a QTL on Linkage Group (LG) 2 that control cane pubescence and disease resistances and has also been demonstrated to delay ripening. Gene H phenotype for disease resistance is now used as a visual marker in resistance breeding. This study set out to explain the role of Gene H by characterising gene content in this region through identifying and sequencing BAC clones spanning the region to explain the phenotypic effects on cane hairiness, disease resistance and timing of ripening. This has allowed us to propose that PDF2/GLABROUS2 is Gene H and has also provided an insight into the effects the region has on ripening and disease resistance.

Keywords: Gene H; Raspberry; PDF2; DIVIA; Molecular breeding

Introduction

The *Gene H* region on raspberry linkage group 2 is interesting as it is associated with a number of diverse traits [1]. It has been known for some time that pubescence in raspberry is determined by Gene *H* (genotype *HH* or *Hh*), the recessive allele of which gives glabrous canes (genotype *hh*). Raspberry cultivars and selections with fine hairs (pubescent canes) were previously reported to be more resistant to cane botrytis (*Botrytis cinerea*), and spur blight (*Didymella applanata* (Niessl) Sacc.) than non-hairy ones [2,3] but more susceptible to cane spot (*Elsinoe veneta*), powdery mildew (*Sphaerotheca macularis*) and yellow rust (*Phragmidium rubi-idaei*) [4-8]. *Gene H* was shown to be the most significant marker in a QTL on Linkage Group (LG) 2 that control cane pubescence (hairs) and disease resistances [1] and also delayed ripening [9].

In terms of disease resistance, how Gene *H* results in the large increase or decrease has not been determined. It has been suggested that it is due to linkage with major resistance genes or minor gene complexes that independently contribute to the resistance or susceptibilities of the diseases affected. An alternative explanation is that the gene itself is responsible through pleiotrophic effects on each of the resistances [10]. The gene is known to have other effects besides its main effect on cane pubescence: it is associated with a small increase in spine frequency and decrease in spine size [1,4,11]. Hairs and spines are both outgrowths of epidermal cells and their early development is inter-related [12]. It would therefore seem likely that *Gene H* acts early in development and affects several cell characteristics. Resistance to *B. cinerea* and *D. applanata* is highest in immature tissues and Williamson and Jennings [10] have postulated that the gene increases resistance by delaying cell maturity. An unexpected effect on the timing of fruit ripening by *Gene H* was subsequently demonstrated, which supports this hypothesis [9]. Alternatively cane hairiness itself could affect the ability of fungi to adhere and infect tissues though experiments have failed to clearly demonstrate this [4]. Jennings also reported that the genes for fertility Cr (fertility) cr (semi-sterility) which affect fruit development resulting in a crumbly fruit phenotype were linked to G*ene H* (pubescent canes) and also *gene T* (colour) though no association was identified through QTL analysis of the crumbly phenotype [7].

This work set out to identify gene content in this region and thus attempt to explain the phenotypic effects on cane hairiness, disease resistance and timing of ripening.

Materials and Methods

Raspberry crosses

The mapping population, as described previously [1,9,13,14] used to identify the *Gene H* region, consists of a full sib family generated from a cross between the European red raspberry cv. Glen Moy (genotype *Hh*) and the North American red raspberry cv. Latham (genotype *hh*).

Sequencing across the Gene H region

Markers in the map region on LG 2 closely associated with Gene *H* were used to screen clones from a large insert library (BAC library) constructed from Glen Moy [15] spanning the *Gene H* region using probes prepared using standard procedures as briefly described as follows: Markers with maximum linkages to *Gene H* were identified in JoinMap 4 [16] and used to screen the BAC library. Probes were prepared using the primers shown in Table 1. The products were amplified in Glen Moy using 25 ng DNA, 1.0 uM primer, 0.2 mM dNTPs and 0.1 units Taq polymerase (Roche) on a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystems, Foster City, CA, USA). Amplification was performed as follows: 5 min at 95°C; 60 s at 94°C, 60 s at 57°C, 60 s at 72°C for 35 cycles, followed by 8 min at

72°C. The PCR product was then purified using a Qiagen MinElute PCR purification kit. The purified product was then used to probe the raspberry BAC library [15] using standard protocols. Positive clones were identified and BAC DNA was prepared using the Sigma PhasePrep kit according to the manufacturer's instructions.

BAC	Primers used to screen BAC library	Further information
20B21	Rub6a Left: TGCATGTGACTTTGCATCTCT Right: GCACTGAAAATCATGCATCTG	Rubus SSR library Within QTL for Ripening/ hairs
20D22	CAF1 (ERubLR_SQ12.1_B12) Left: TGGGAGTTCAACTTCAACGA Right: CGGCCCATCATTTACAAAATA	Rubus EST library Within QTL for Ripening/ hairs
24N24	Hairy01 Left: CAGCATTTCAGCTTGCTTTG Right: GGGAGCGAGAGAGACAACTG	AFLP E40M12-256 (Hairy-01 256SNP) Within QTL for Ripening/ hairs
11G23	FG215 Left: TTGGGCCCTGATAGTGTCTC Right: ACCTTACGAACCCTCGTCAC	FG215 originates from prunus LG6 (GenBank accession no. BH023827) Within QTL for Ripening/ hairs/colour

Table 1: Primer sequences used to screen BAC library and relevant information regarding the source of the sequence.

Confirmation of BAC sequence locations on linkage groups

To confirm BAC locations, primers were designed to putative open reading frames from BAC sequences using Primer 3 (Table 2) and the corresponding regions amplified in Glen Moy, Latham and progeny DNA as described previously [9] to determine polymorphisms. Where size polymorphisms were observed, primers were end-labelled with FAM or HEX and PCRs performed on the mapping population and analysed on the ABI3730 DNA Analyser (Applied Biosystems) as described in Graham et al. [13] using Genemapper v 3 (Applied Biosystems). Where SNPs were identified, primers were designed for Pyrosequencing assays which were performed as described previously using the PSQ Assay Design Software 1.0 (Biotage, Uppsala, Sweden) [14].

Marker	Left Primer Sequence	Right Primer Sequence
20B21bacF	CTGGTTCCCTGAAGACGTTC	CATAGTCGGTGCCCAGTCTT
20B21_cont25_SSR	CATTCCGTCAATCTGCCATA	GTGGTGGTGCCTTATGGTG
20b21c4ssr	TCACTACTCACCAACACCAATCC	ATCGGAAAAATCGGGAAGAA
20b21csbp2	AATGCTTCCAGTCATTTTGGA	AAGCCCGATGCCCTTTTTAAT
20B21_P3.1_SSR	CTCCGAGTAAAGCATCACGA	GCTGTCTGAGTGTTGCTAGTGC
20B21cont19SSR	AAAGCCGGATTTGGATAACA	TGCTACTGGTGCAACTCGAT
20B21_Medea	GGTCCTAATTCCTCCGAAGC	CCCCAATTCATCTACGCTCA
bes_Ri20B21R	ATGGAGCTGCCCATACAATC	GGAGAACCTCGGTCAATGAG
20D22c01634_103	AGGATGAGAACTTTGGACCAG	AGCTTGAACCCTGGATCACA
ERubLR_SQ12.1_12CAF (20D22)	TGGGAGTTCAACTTCAACGA	CGGCCCATCATTTACAAAATA
11G23c0064_snp149	TGTTTGGCAAAGCATTCCTAAA	AGTTGGGTGTGTGAAGTACTT
11g23c0064_snp126	TGTTTGGCAAAGCATTCCTAAA	AGTTGGGTGTGTGAAGTACTT
11g23c0064_snp93	TGTTTGGCAAAGCATTCCTAAA	AGTTGGGTGTGTGAAGTACTT
11G23c0003p2a2	ATTGCAGGTCGGAGTCTTCA	CATCATCAA GGT TGG CAC GT
ERubLR_SQ5.3_E0360S (11G23)	TCGTCTCTTGGGAGAAGGAA	CGGTCATTAAAATACAAATGCAA
FG215 (11G23)	TTGGGCCCTGATAGTGTCTC	ACCTTACGAACCCTCGTCAC
24N24c0001SNP_snp80	CATCATGCATGTCTTCTTTGCT	TCAAAGGGGAGGCTAGGAGA
24n24c0006_1snp141	CTCCCTTGTGCTTGCAGTAC	ATCGGATCATGGCTCGTCC
24N24c0006_1snp2002	CTCCCTTGTGCTTGCAGTAC	ATCGGATCATGGCTCGTCC
24n24c0006_snp3742	CTCTTTGAGCATCGCACCTC	TCTTCAAACCTCTCGCCTGT
24n24c0006_snp374	CTCTTTGAGCATCGCACCTC	TCTTCAAACCTCTCGCCTGT
24N24c0006_snp241	CTCTTTGAGCATCGCACCTC	TCTTCAAACCTCTCGCCTGT
24N24c0002ethover	CCA TGG AGC TTA TGG TTT CC	TGC AGA GTG CAC AAG GTA AA
AFLP_E40M12_256 (24N24)	CAGCATTTCAGCTTGCTTTG	GGGAGCGAGAGAGACAACTG

Table 2: Markers generated from BAC sequences and primers for mapping.

Sequencing of BAC DNA

BAC DNA (5 µg) was subject to 454 sequencing at the University of Liverpool (UK). An assembly was generated using Newbler (v. 1.1.03.24). Visual inspection of the contigs produced was performed using the assembly viewer 'Tablet' [17]. Further sequencing was carried out using Sanger sequencing and added to the assembly using 'phrap' (www.phrap.org/phredphrapconsed.html) which yielded large contigs representing the four BACs. Finally the sequence was subjected to a BLAST based vector screening using the NCBI univec database as the target (www.ncbi.nlm.nih.gov/VecScreen/UniVec.html) to identify vector contamination. The vector sequences were manually removed using BioEdit software (www.mbio.ncsu.edu/BioEdit/bioedit.html) to produce the final, cleaned version of the BAC sequences'.

Gene prediction

Open reading frames from the four BAC sequences were identified using the Softberry gene structure prediction program FGENESH (http://linux1.softberry.com/berry.phtml) using *Vitis vinifera* as the reference and subject to gene prediction using blastX (http://blast.ncbi.nlm.nih.gov).

Results

The allele frequencies at markers across the *Gene H* region were examined and there was no evidence of segregation distortion in the markers from the Glen Moy x Latham cross (other than SSR107b) (Table 3).

Locus	Distance from Gene H	Glen Moy x Latham (signif.)
Rub107a	83.6	3.84 (*)
Ri20B21_cont25SSR	32.4	2.74 (-)
Ri20B21_P3.1_SSR	4.66	3.40 (-)
Ri20B21cont19SSR	4.1	2.09 (-)
Ri20B21_medea	3.4	0.39 (-)
GeneH	0.0	2.89 (*)
RhiM001	1.7	0.58 (-)
Rub4a	17.9	0.15 (-)
bes_Ri29G13	19.5	1.08 (-)

Table 3: Segregation distortion in the Gene H region.

BAC identification and sequencing

Hairy01-256SNP, Rub6a, ERubLR_SQ12.1_B12 and FG215Probes Hairy01-256SNP, ERubLR_SQ12.1_B12, Rub6a and FG215 significantly linked to the *Gene H* region identified four BAC clones 24N24, 20B21, 20D22 and 11G23 (Table 1) for further analysis.

These were mapped in the Latham x Glen Moy population to confirm locations using primers in Table 2, shown in Figure 1. Gene predictions were carried out (Appendix 1-4) and alignments determined (Figure 2).

The four BAC clones were each estimated to be between 120-180 kb.

BAC clones, 20B21 and 20D22 overlap, as do 24N24 and 11G23. Interestingly the overlap between 20B21 and 20D22 contained a gene with significant homology to PDF2/GLABROUS 2 (GL2) and we suggest that GLABROUS2 is in fact *Gene H*. The remainder of BAC 20B21 contained genes shown in Supplementary Table 1. Genes from 20D22 are shown in Supplementary Table 2, 11G23 in Supplementary Table 3 and 24N24 in Supplementary Table 4.

The ordering of the BAC markers on the linkage group was close to the physical ordering from BAC sequencing Figure 2.

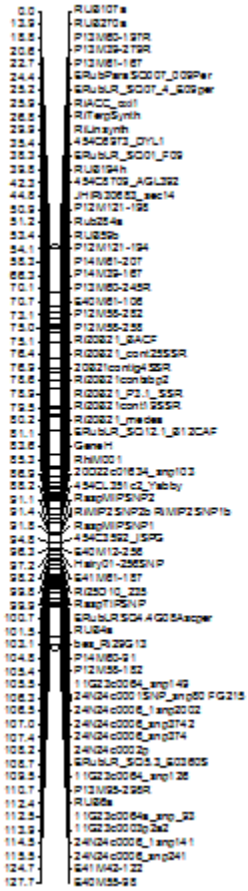

Figure 1: Linkage map of LG 2 with the Gene H region.

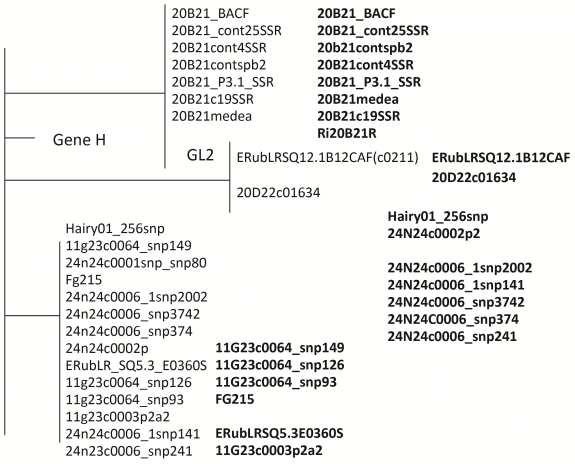

Figure 2: Linkage map order of individual BACs across Gene H region in black with physical map order in bold black if known.

Discussion

A number of interesting genes were identified which may go some way to explain the phenotypes associated with the *Gene H* region and may have utility in future breeding.

A full list of predicted genes is shown in the Supplementary Tables 1-4 and genes with potential relevance to the phenotypes of interest are discussed below.

Gene H and cane pubescence

This work has allowed us to suggest that PDF2/GLABROUS2 is Gene H.

The *Gene H* region in raspberry is named after the cane phenotype it controls and determines whether the plant has pubescence (genotype *HH* or *Hh*), or glabrous canes (genotype *hh*). In higher plants, the outermost cell layer (L1) of the shoot apex gives rise to the epidermis of shoot organs. PROTODERMAL FACTOR2 (PDF2), a member of the HD-GL2 class of homeobox genes, is expressed exclusively in the L1 of shoot meristems and plays a critical role in maintaining the identity of L1 cells, possibly by interacting with their L1 box and those of downstream target-gene promoters. PDF2/GLABRA 2 (GL2) has been found on LG 2 in a region where Bac 20B21 and 20D22 overlap and clones from each of these BAC span the *Gene H* region [18]. Recent plant development studies have identified regulatory pathways for epidermal cell differentiation in Arabidopsis thaliana [19]. Such pathways contain transcriptional networks with a common structure in which the homeobox gene GLABLA2 (GL2) is downstream of the transactivation complex consisting of MYB, bHLH, and WD40 proteins [20]. Despite the consistent position of GL2 within the network, its role in epidermal tissues varies; in the root epidermis, GL2 promotes non-hair cell differentiation after cell pattern formation, whereas in the leaf epidermis, it is likely to be involved in both pattern formation and differentiation of trichomes. It was previously shown to be required for trichome formation and seed mucilage production. A DIVIA like Myb transcription factor was identified on BAC 11G23 with similarity to WEREWOLF, a regulator of root hair pattern. WER forms a transcriptional complex with other factors which positively regulate GL2 inhibiting the generation of hairs. Another gene with similarity to MyB39 like sequence was also on the overlap between BACs 20D22 and 20B21.

Gene H and Ripening

The *Hh* genotype of *Gene H* is also associated with a slowing down of ripening across all stages from open flowers to the green/red stage compared to the *hh* genotype [9]. It is interesting to speculate that the DIVIA like Myb transcription factor on BAC 11G23 could be controlling both trichomes and flowering time, as WEREWOLF, a regulator of root hair pattern has also been shown to be a posttranscriptional regulator of FT, a key floral regulator. WER forms a transcriptional complex with other factors which positively regulate GL2 inhibiting the generation of hairs. A late flowering WER mutant phenotype has been reported with hairy roots. This occurred in long but not short days so can be classified as a photoperiod pathway mutant. DIV encodes a protein belonging to the MYB family of transcription factors [21]. By later stages of corolla development, effects of DIV on cell types become manifest. The most obvious effect is the formation of stripes of trichomes on the inner surface of the corolla to either side of the boundary between ventral and lateral petals.

Gene H and disease resistance

In terms of potential disease resistance a number of candidates have been identified that may suggest, genetic resistance in the region is a possibility, rather than a delay to maturity. 20D22 has a CCR4 association factor. CCR4-associated factor 1 (CAF1) is a sub-unit of CCR4-NOT, an enzyme complex mRNA deadenylase [22]. Expression of CAF1 has been shown to affect response to pathogen infection [23]. T-DNA insertion mutants disrupting the expression of AtCAF1a and/or AtCAF1b are defective in deadenylation of stress-related mRNAs, indicating that the two AtCAF1 proteins are involved in regulated mRNA deadenylation in vivo. The single and double mutants of AtCAF1a and AtCAF1b showed reduced expression of pathogenesis-related (PR) genes PR1 and PR2 and were more susceptible to *Pseudomonas syringae* pv infection, whereas transgenic plants over-expressing AtCAF1a shoed elevated expression of PR1 and PR2 and increased resistance to the same pathogen. This suggests roles of the AtCAF1 proteins in regulated mRNA deadenylation and defence responses to pathogen infections.

Another gene with homology to a disease resistance protein was identified on BAC 11G23.

Other genes in BAC regions: *Signalling pathways.*

A number of other genes were identified across the four BAC clones including two ubiquitin conjugating enzymes on 20D22 and a ubiquitin activating enzyme on 24N24. Selective modification of proteins by ubiquitination is directed by diverse families of ubiquitin-protein ligases. These have a diverse set of cellular functions and are important for the development of signalling pathways. A Josephin domain which functions in deubiquitination activity was also identified on 11G23 [24].

A RAB 3 GTPase was identified on 24N24. These small GTP binding proteins function as molecular switches performing diverse cellular functions with the Rab group thought to be involved in membrane trafficking.

PTR1 on BAC 11G23 is a small peptide of two to three amino acids which are posited as an important alternative to amino acids. A PTR1 transporter mediating the uptake of di- and tripeptides from Arabidopsis thaliana was shown to be expressed in the vascular tissue throughout the plant, indicative of a role in long-distance transport of di- and tripeptides [25].

Two leucine-rich repeat receptor kinases (LRR-RKs), the largest subfamily of transmembrane receptor-like kinases in plants, were identified on 24N24. LRR-RKs regulate a wide variety of developmental and defence-related processes including cell proliferation, stem cell maintenance, hormone perception, host-specific as well as non-host-specific defence response, wounding response, and symbiosis.

Ethylene overproduction protein identified on BAC 11G23 is involved in the regulation of ethylene production. Studies have shown that overexpression of ETO1 inhibited induction of ethylene production by the plant growth regulator cytokinin, and promoted ACS5 degradation by a proteasome-dependent pathway. ETO1 was also shown to interact with CUL3, a constituent of ubiquitin ligase complexes. ETO1 thus has a dual mechanism, inhibiting ACS enzyme activity and targeting it for protein degradation. This permits rapid modulation of the concentration of ethylene [26].

This work further expands the information and utility of the Gene H region with applications for breeding specific characteristics other than disease resistance. The phenotype is now used as a visual screen as part of the breeding process for material more resistant to cane diseases. This may now be considered in terms of timing of ripening for varieties better adapted to particular growing seasons.

The raspberry cultivar Glen Moy has recently been sequenced at the James Hutton Institute and is currently being aligned and the emerging genome sequence will be valuable for future research and can be used to fully align the BACs from Glen Moy across the Gene H region as well as align the Latham x Glen Moy linkage map to the genome sequence. This has considerable consequences for raspberry breeding, growth and management as all phenotypic associations on linkage groups can now be directly linked to the genome sequence and thus the regulatory genes themselves.

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