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ORIGINAL RESEARCH ARTICLE

Fine mapping identifies significantly associating markers for resistance to the honey bee brood fungal disease, Chalkbrood

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Summary

Chalkbrood infection of honey bee (*Apis mellifera*) brood by the fungus *Ascosphaera apis* results in fatal encapsulation of susceptible larvae with a mycelial coat. Recent QTL analysis indicates that some level of physiological resistance exists in individual larvae. We performed a fine mapping analysis to define the genetic interval that confers resistance in the larvae and identify the strongest association molecular markers that could by useful for marker assisted selection of the trait. Evaluation of the interval suggests that only two possible genes (*single Ig IL-related receptor-like*, XM_003251514.1 and juvenile hormone-binding protein, XM_391872.4) are likely to be responsible for the resistance. Both genes are strong physiological candidates and potentially function as modulators of the antifungal-specific innate immunity pathway in insects.

El mapeo detallado identifica de manera significativa marcadores asociados a la resistencia a la enfermedad fúngica de la cría de la abeja de la miel, cría yesificada

Resumen

La infección de la cría yesificada de la abeja de la miel (*Apis mellifera*) causada por el hongo *Ascosphaera apis* resulta en la encapsulación fatal de larvas susceptibles con una capa de micelio. Recientes análisis de QTL indican que existe un cierto nivel de resistencia fisiológica en larvas al nivel individual. Se realizó un análisis de mapeo detallado para definir el intervalo genético que confiere resistencia en las larvas e identificar los marcadores moleculares que muestren una asociación más fuerte y que puedan ser útiles para la selección asistida por marcadores del caracter. La evaluación del intervalo sugiere que sólo dos genes posibles (el similar al receptor relacionado con Ig Il, XM_003251514.1 y la proteína de unión de la hormona juvenil, XM_391872.4) son probablemente los responsables de la resistencia. Ambos genes son fuertes candidatos fisiológicos y potencialmente funcionan como moduladores de la vía de la inmunidad innata antifúngica-específica en los insectos.

Keywords: honey bee, chalkbrood, MAS, marker-assisted selection, fine mapping, Toll pathway, immunity, *Apis mellifera*, *Ascosphaera apis*

Introduction

Infection of honey bee (*Apis mellifera*) brood by the fungus *Ascosphaera apis* kills susceptible larvae and pupae by the mycelial encapsulation and desiccation of infected individuals, resulting in chalkbrood disease. The disease is particularly prevalent in colonies that are already under stress by a variety disease challenges, or less-than-ideal environmental conditions (reviewed in Heath, 1982). However, endemic infections had been rare due to the hygienic behaviour of worker bees that remove diseased larvae/pupae prior to the sporulation stage of the fungus (Invernizzi *et al.*, 2011), as well as the probable genetic component of resistance conferred by the larvae themselves (Holloway *et al.*, 2012). Beekeeper-mediated requeening of susceptible colonies also mitigates much of the infection rates and frequencies by altering the genetic basis of susceptibility in managed colonies.

Despite the basal-level tolerance to chalkbrood, overall colony health is continually being challenged by economic, agronomic, and environmental demands. The increased stresses on bees are beginning to result in higher prevalence of the disease and likely higher economic costs. Migratory beekeeping, monoculture crop production, agrochemical exposure, pest infestations, pathogen infections, and other environmental stresses continue to challenge the physiological response mechanisms in bees, including their innate immunity defences. Identifying mechanisms to control the disease would positively impact colony health, pollination capabilities, and honey production.

Considering that a strong genetic basis for chalkbrood resistance has been suggested through quantitative trail loci (QTL) analysis in a mapping population (Holloway *et al.*, 2012), the potential exists to breed resistant bees using marker assisted selection (MAS) to increase the prevalence of a trait of importance to honey bee health. MAS is a highly utilized genetic tool with proven value in a variety of agricultural fields from milk production, and fat and protein content in cattle (Boichard *et al.*, 2002) to disease resistance in cereal crops (reviewed in Miedaner and Korzun, 2012). Generating pathogen resistant honey bee stocks may also prove to be economically valuable by increasing colony longevity, colony health, and economic productivity.

In this paper, we report the results of fine mapping of the genetic basis of larva-mediated chalkbrood resistance (LMCR) by the use of associated SNPs utilized as CAPs (cleaved amplified polymorphism) markers. Resolution of the causative genetic interval strongly implicates two coded proteins, single Ig IL-related receptor like, and a predicted juvenile hormone-binding protein, that may be responsible for the trait. The known anti-fungal functionalities of both proteins suggest that an innate immunity to chalkbrood could function within challenged larvae.

Materials and methods

Evaluation of resistance and SNP analysis

Population development, phenotyping, and QTL identification in regards to chalkbrood resistance was previously described (Holloway *et al.*, 2012). Briefly, brood from a backcrossed population generated by single-drone insemination with Russian and various commercial bees was inoculated with chalkbrood spores and analysed for the development of the disease. DNA from individually phenotyped individuals (92 susceptible, 92 resistant) was purified and subjected to SNP genotyping and QTL analysis. The trait was considered to be binary where resistant individuals survived the challenge whereas susceptible individuals did not (effectively functioning as a qualitative trait). DNA samples remaining following QTL analysis (91 susceptible, 88 resistant) were subjected to fine mapping and further analyses.

Development of CAP molecular markers and fine mapping of the locus

Available genomic sequences (Amel 4.0 and Amel 4.5, NCBI honey bee genome) underlying the defined QTL were BLASTed for known SNPs within the interval. Polymorphisms and the surrounding sequence information for thousands of SNPs were collected and screened for a creation/destruction of restriction sites by the SNPs themselves, thereby being classified as potential CAPs (cleaved amplified polymorphisms) markers. Restriction sites were prioritized such that fewer enzymes were able to screen a large number of SNPs. Primers were designed to amplify over the SNPs and to generate amplicons 300-500 bp in length and for the digestion products of the CAPs markers to be easily resolved on 1-3% agarose gels. Amplification and restriction enzyme digestion of each CAP marker was tested on a subset of individuals to determine if the polymorphism existed within the chalkbrood mapping population (Table 1). Successful CAPs were used to genotype the entire mapping population. CAPs genotype information as compared to the phenotype of the mapping population was analysed for the decrease in the number of recombinant individuals as the interval narrowed from the original QTL. By this methodology, the interval was narrowed according to standard qualitative trait mapping practices.

Evaluation of candidate genes

RNA was purified from tissue samples from a subset of the original mapping population and samples of aged brood from unrelated Russian honey bee colonies using the Maxwell 16 nucleotide purification system (Promega Corporation, Madison, WI). Extracted RNA was then quantified (ng/µl) and quality (260/280 ratio) was assessed on a NanoDrop (NanoDrop Technologies Inc., Wilmington, DE). cDNA syntheses were performed using a QuantiTect Reverse Transcription Kit (Qiagen Inc., Valencia, CA). cDNA from the mapping population was subjected to non-quantitative real-time PCR to determine presence/absence of gene expression without regard to controlling for developmental stage of the individuals or quantity/quality of cDNA. cDNA from the unrelated Russian aged brood series was subjected to quantitative real-time PCR (qPCR) and statistical analysis according to the following methods. An Alien Reference RNA qRT-PCR Detection Kit (Agilent Technologies Inc., Santa Clara, CA) was used for an exogenous control according to the manufacturer's protocols. Genes remaining within the fine mapping-defined interval were screened for expression using primers designed to specifically amplify from cDNA and legitimize candidacy for chalkbrood resistance by real-time PCR analysis (Table 2).

The qPCR reactions were performed in a Bio-Rad CFX96 real time PCR detection system (Bio-Rad Laboratories, Hercules, CA) using the fast protocol with iTaq™ Fast SYBR® Green Supermix (Bio-Rad

Table 1. CAPs markers designed from available SNPs within the QTL interval.

SNP name	Forward primer 5'-3'	Reverse primer 5'-3'	amplicon length, bp	CAP enzyme	predicted digestion products, bp	polymorphic and informative in mapping population
AMB-00902430	TGATATACCTGAGCACTAAGCTTGTAA	GGCCTCAAACGAATCTACAGAAGT	300	ApaLI	93/207	-
AMB-01122531	TCTCACACTCGATCTCATTCGCGT	ACTCACCGGATACTCGTCGTACTT	314	ApaLI	142/172	-
AMB-00625115	CGCGAGGAAGGGTAACGGTATAAA	TCCTCGAGTGATTCTCGTCTCGAA	356	BamHI	140/216	-
AMB-00858666	GGTCTCCAACATTTGTTCAGATTCGAC	GCAACTCGAGATGATTTCGCGCAT	307	BamHI	132/175	-
AMB-00686049	ACGATGAAATGGCCACGGAGGAAA	TTCCTTCGAGTCAGCCTCGTCTTT	461	BamHI	219/242	-
AMB-01010082	ACGCCGTTGTGTCAGGATCAAGAA	TCTCGAATAGATAGATTCGGAACGC	339	BbsI	142/197	-
AMB-00631272	ACTTTCTCCCACGGTGCTACGAAT	TCCAACCCTTCGTGCGCTGTTAAT	428	BbsI	214/214	-
AMB-00612238	TGGGAGGAGGTGACGAATTTCAGA	TACGACCACCTCCAAGTCCTCAAT	313	BbsI	118/195	-
AMB-00858616	TCGTTGCATTCTTCTTCACACACG	ATGGAACGGCATCCGCGTTTATTG	350	BbsI	112/238	-
AMB-01019072	ATGTGTGCGACACGATGGGTAACT	CGGCATGCTAAATATATGTGTTCATTCT	414	BstBI	193/221	-
AMB-01018524	AGGAGTACCATCTCCGCTAAGAGA	ATGTCAACACCTGGCGATCCTATG	337	BstBI	90/247	-
AMB-00902509	TGAACAGAGACGTGTACGCGAGAA	CGTTCGCATCAACGGTTTCATCGT	387	BstBI	143/244	-
AMB-00858574	TCATTGTTCCCACCGATCGAGCAT	CGCTGTTTGGCATTCGACACTTTC	309	BstBI	115/194	Yes
AMB-00858654	GGTCTCCAACATTTGTTCAGATTCGAC	GCAACTCGAGATGATTTCGCGCAT	307	BstBI	115/192	-
AMB-00902548	TCCATTATTGCGACTGCGCAGGAT	ACAAGTTGTGTGTACCGGTGGTTG	307	ClaI	91/216	Yes
AMB-01118908	CAGATTGCACTGCCAAGCCTTCAA	TGTCCTTCGGATTTATCGGGCGAA	353	ClaI	175/178	Yes
AMB-00769758	TCCGTCGTTGAAAGACCTGCCAAA	TCGGCCAAGGAAGAAGTAGAGAAC	464	ClaI	215/249	-
AMB-00902552	TGCGCCAGAATGGTAAACGGATTG	GTTGGTAACGCAAGCTAAATCTACG	372	DraI	174/198	Yes
AMB-01128891	AGATGGAGATTGGTCTCCAGATG	CCTCTTGAATCCGTAACACAACCAGC	440	DraI	202/238	-
AMB-01136379	CGCGTTGAAACAATTGCAAGAATGCTG	AGTGGAAAGTGTATCGACGAAAGT	322	EcoRI	91/231	-
AMB-00631190	TGTTCGGTGGTGAAACGAGTGGTA	AACCAGCGTTAAGGGAGGAACAGT	374	EcoRI	170/204	Yes
AMB-00612262	TGGCTACAAACTGTTGCTCCATCT	GAGAGCGCGAAATCACCGATGAAA	300	EcoRV	87/213	Yes
AMB-00686140	CGATAACGTCCGAAATCGCAAATCC	TCGGTTAATTTACGAACGAAATACATGG	317	HinDIII	150/167	Yes
AMB-00858553	AGCAAGAGAGGTTCGTTCTGGCAA	CTGCGAACATCTCGTCCCTTTCTT	300	HinDIII	79/221	-
AMB-00703872	AATATAGCAACAGCCTAGCCCGCA	TCGGCGATCAAGTAGAACGGGTTT	413	HinDIII	172/241	-
AMB-01122520	TTGCAAAGCTGTTGTTCGATCCCG	GAGGATCGTTGGATATTCGAGTAGG	307	KpnI	109/198	-
AMB-00682233	CGCGGATCGACGAAATTTCTGTAACG	CAACTTCTAGGCTTCTAGACGATCA	372	NruI	147/225	-
AMB-00625093	GAAACGTTGAAAGGCATGGCCGTA	TTTGCACGCGTACGTTCGCTAATC	302	NruI	116/186	-
AMB-01018606	ACGTGAGTATCGAGCAACCGCATA	TGATTAATTCGCGTTCGCTCACGG	320	NruI	129/191	-
AMB-00680431	GCTCACGAACTCGGATCTAACTGT	TTATCGGACGAGAGACAACGCCAT	316	NruI	147/169	-
AMB-00972134	GCATAATGAGCATTGTAGCTAATAGTCCC	GGAATTGCATTTGCGATTAATGGTG	364	NruI	133/231	-
AMB-01151447	ATGATTCGCCTTGAACTTGCGACC	TGAACCTCAAAGACTACCACGCCA	368	NruI	124/244	Yes
AMB-00902518	ACGATGACGGTAACAACGCAGGAA	TTTCTTCTACGCGTGTGCGGTCTA	344	NruI	145/199	-
AMB-01122512	AGAGATAGGCCATCAACGCTGGAA	ACTCGTTCTCGATTGCTCGTTCGT	480	NruI	233/247	-
AMB-01075289	TCCATCGTGCGCTCAATTAGAGGT	TCGAAAGCTCTTGAAGCGACGAGT	339	NruI	113/226	-
AMB-00602804	ACGACGGTTCCTTCGTTCGATTCT	TCCCGTGCATATCCCGTATTCAGT	365	SnaBI	182/183	-
AMB-00640650	GTTGAGGAAGATGGAGATGTTGAGG	CCGCGGATTCTTGATAGAGATACCGAA	494	SnaBI	246/248	Yes
AMB-00674355	ATTGAATCGCGCGCGGAAAGAGAT	AAGAAAGATCGACGCACGATTGGC	303	SnaBI	141/162	Yes
AMB-00919983	TCGCCAGAAATTACCTCGAGCAGT	GGAAGAACAATTTAAGAACCGGGACAG	344	SnaBI	155/189	-
AMB-00631311	ACGGAAATCCTCGTCTTCCACGAA	ACGGACACGCGTTACCATTGTCTA	342	SnaBI	106/236	-
AMB-00858568	TTTCTCCATCCTCCACGCGACATA	ATGCTCGATCGGTGGGAACAATGA	316	SnaBI	156/160	-
AMB-00858821	TTTGGCTACTCGAGGGAAGA	CTTTGCGAGAGAGTCGAGTTTCAA	306	SnaBI	64/242	-
AMB-00858470	TTGCCTCTCGAGGGAAGAAT	TCGAAATCGATCGAGAAACGCTCG	330	SnaBI	109/221	-
AMB-01128890	TGCTGTTATTGGAAGACAAGCATT	AAGCAGAAGCTCTTCTAGCACGTC	318	XbaI	129/189	-
AMB-00858514	TCTTTGGAACGTAAACGGCGCTTC	TCGCATCGCTATCGATCGGACAAT	308	XbaI	97/211	-

Table 2. Gene specific primers for evaluation of expression.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	amplicon length, bp
SIGIRR	TTCAAAGCCCTTCATTGCTGCCTG	TCACCTTCTGCGCGTAAATCGTCT	cDNA = 145
JHBP-like	AATGTAACCGGCACACTCGTCGAT	TTGGACACCTCCTCGTCGTTGTAT	cDNA = 206, gDNA = 683
Actin	TGCCAACACTGTCCTTTCTG	AGAATTGACCCACCAATCCA	cDNA, gDNA = 155

Laboratories). All samples had three biological replicates per treatment and three technical replicates were run for each biological sample. A dissociation curve was generated and used to validate that a single amplicon was present for each qPCR reaction.

Reference gene (β-*actin*) and the Alien exogenous control gene were used to calculate the relative expression of the target genes *SIGIRR* and *JHBP-like* according to the ABI Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR (Applied Biosystems, Foster City, CA) with the following modification: the geometric mean of the reference gene and Alien exogenous control gene was used to calculate the ΔC_t values for the individual target genes (Vandesompele *et al.*, 2002). The ΔC_t values were normalized to the first instar larvae treatment to calculate the $\Delta\Delta C_t$. All qPCR primer sets were verified to have similar amplification efficiencies using the template-standard dilution approach. The values were then graphed using the SigmaPlot 11.0 software (Systat Software; Inc., San Jose, CA). Statistical differences were calculated by analysis of variance (ANOVA) and Tukey's test using JMP® 8.0 (SAS Institute Inc., Cary, NC, USA). Statistical differences were not calculated using the $\Delta\Delta C_t$ values because normalization using the $\Delta\Delta C_t$ leads to incorporation of values from one of the treatments and violates the ANOVA rules.

Results

Fine mapping of the larval-mediated chalkbrood resistance interval

The larval-mediated chalkbrood resistance (LMCR) QTL (Holloway *et al.*, 2012) was determined reside on chromosome 11. The published QTL interval was further refined utilizing the currently available genome (Amel4.5 publically available on NCBI) and re-alignment of the SNP panel markers. The newly refined interval was found to overlay the single Amel4.5 whole genome shotgun scaffold NW_003378155 spanning nearly 1Mb and containing 107 known, predicted or hypothetical genes. Fine mapping of the region was initiated using 45 CAPs markers designed from the available honey bee genome SNP information (Table 1). Ten of the CAPs markers were polymorphic within the population and useful to define the interval responsible for chalkbrood resistance (Fig. 1A) when considered a binary trait. Markers from the original SNP panel that defined the interval were used to anchor the CAPs markers to the genome, and served as the baseline outer flanking markers. Chi-square statistical analysis of only the non-recombinant individuals (n = 171) between the two innermost markers showed that the smallest defined interval significantly associated to the phenotype (χ = 20.13; P = 7.21 x 10^{-6}). When fine mapped in this manner, the interval spans approximately 36Kb and implicates only two genes as potential candidates: *single Ig IL-related receptor-like* (*SIGIRR*; XM_003251514.1), and a hypothetical gene with a juvenile-hormone-binding-protein conserved domain ("*JHBP-like*"; XM_391872.4). The two innermost markers reside close to or within these genes, further defining the interval for the phenotype-determining nucleotide change(s), or quantitative trait nucleotides (QTN), to predictions of either the

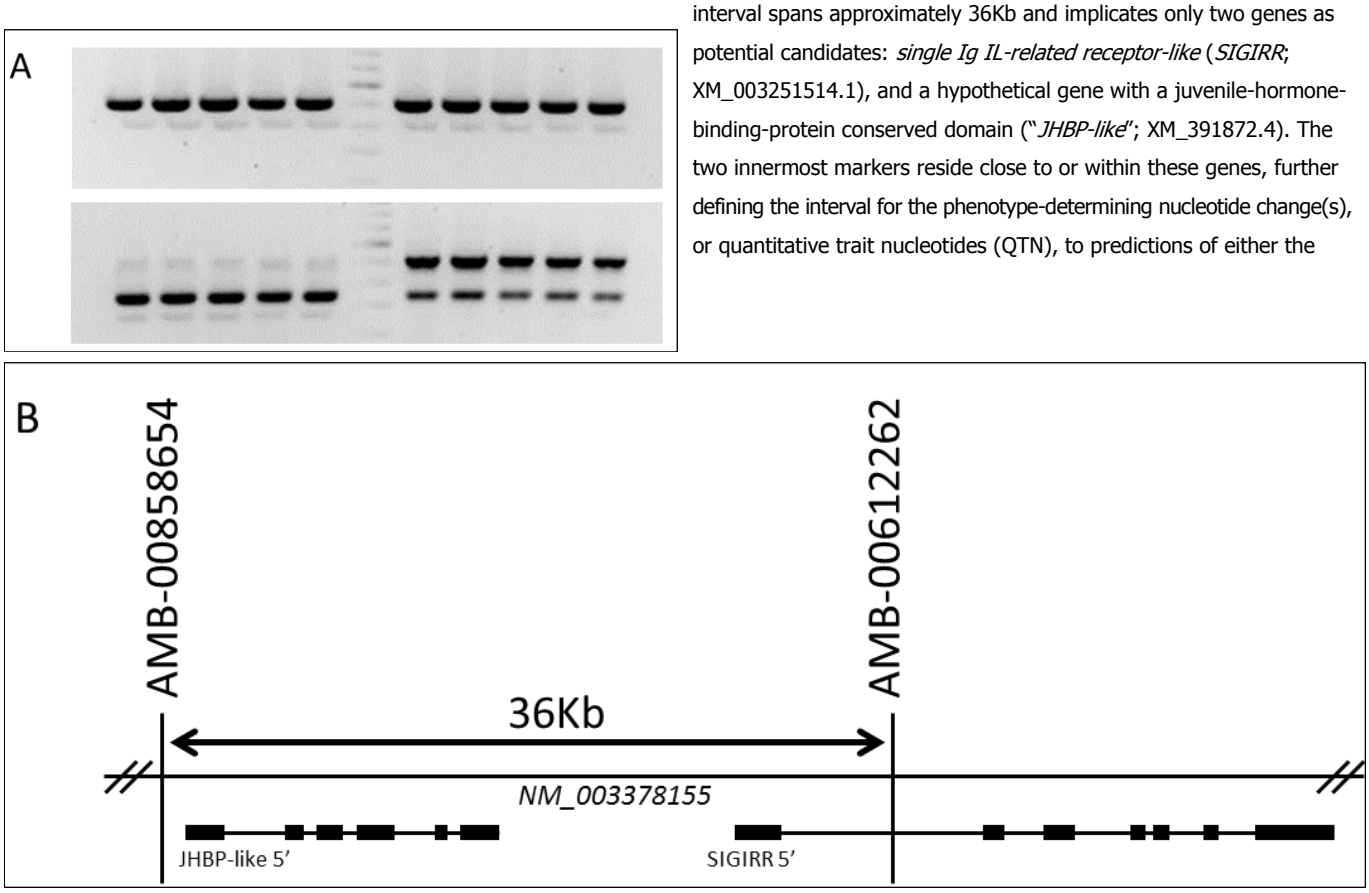

Fig. 1. Fine mapping of chalkbrood resistance interval using CAPs markers. **A** Example of CAPs genotyping of AMB-00902548. Lanes left to right: 5 homozygous susceptible individuals, 50bp ladder (dense band=350bp), 5 heterozygous resistant individuals. Top panel: undigested PCR product of 307 bp; bottom panel: ClaI digestion products that can be scored and segregates with the phenotypes. **B** Representation of the fine mapping interval defined by CAP markers AMB-00858654 and AMB-00612262. The markers delineate a 36Kb critical interval on whole genome shotgun scaffold NM_003378155 and contains two genes: JHBP-like and SIGIRR (not drawn to scale).

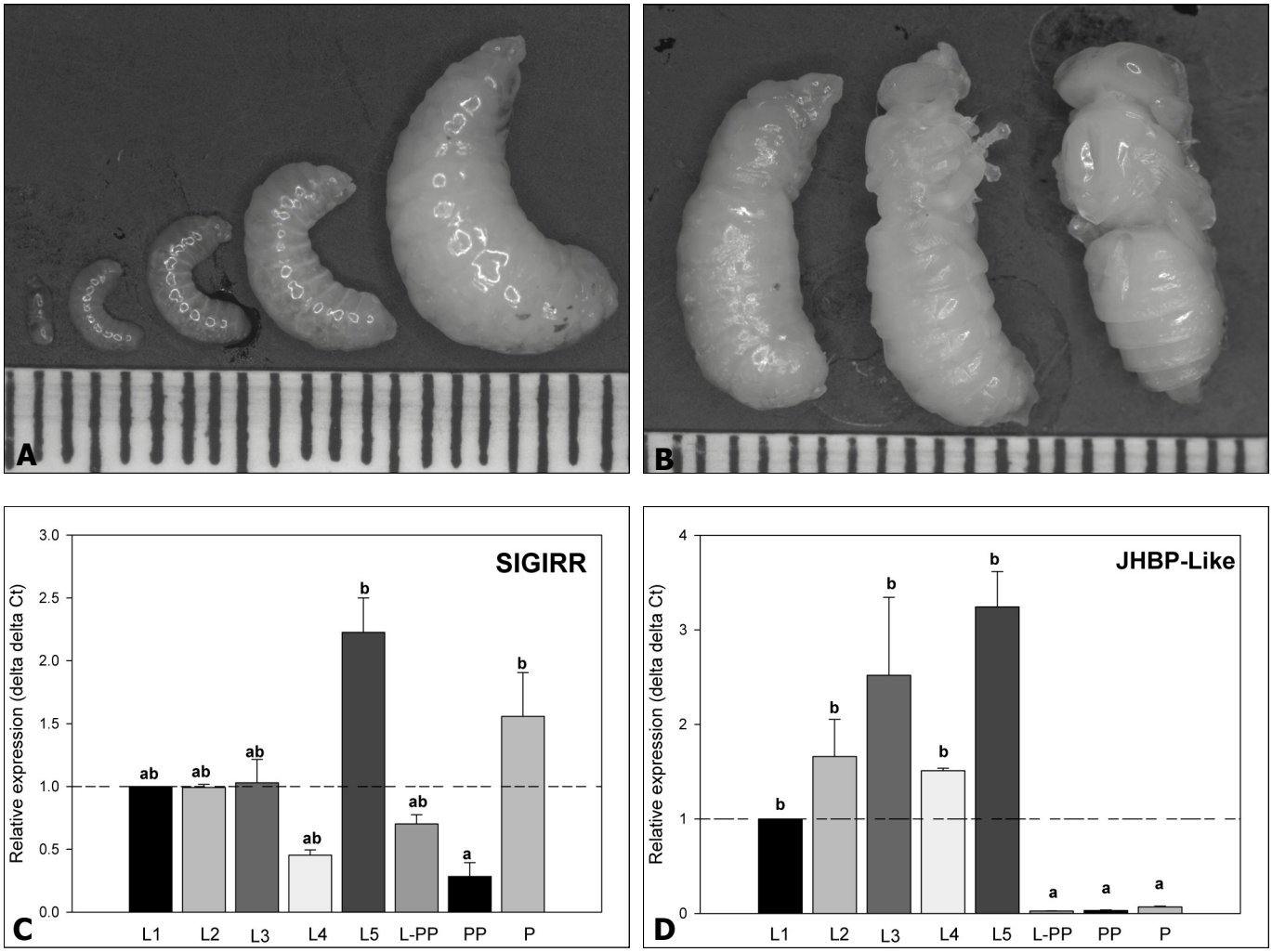

Fig. 2. Candidate gene expression in a honey bee brood developmental series. **A** Representative image of average age of ten individuals pooled to make a cDNA temporal expression library. First instar larvae, 2nd instar larvae, 3rd instar larvae, 4th instar larvae, and 5th instar pools (left to right in image) were tested. Actual age of individuals in each pool ranges ± 0.5 days from mid-point of instar time period. Ruler lines denote 1mm. **B** Representative image of average age of five individuals pooled to make a cDNA temporal expression library. Late stage 5th instar larvae/early pre-pupae, pre-pupae, and white-eyed pupae pools (left to right in image) were tested. Actual age of individuals in each pool ranges ± 0.5 days from mid-point of life-stage time period. Ruler lines denote 1mm. **C,D** Relative expression of candidate genes *SIGIRR* (**C**) and *JHBP-like* (**D**) in the developmental time series: L1 = 1st instar larvae; L2 = 2nd instar larvae; L3 = 3rd instar larvae; L4 = 4th instar larvae; L5 = 5th instar larvae; L - PP = Late stage 5th instar larvae/early pre-pupae; PP = pre-pupae; and P = white-eyed pupae. Relative expression levels (DDCt) are calculated relative to β-*actin* and Alien exogenous control as reference genes then normalized to L1 instar. Error bars indicate the standard error of the mean from the three biological replicates from each life stage. Statistical differences between samples were normalized to the expression of the target genes compared to control genes (DCt). SIGIRR-ANOVA whole model d.f.= 9,23; $F = 3.5442$; $p = 0.0170$; treatment d.f.=2,7; $F = 3.7824$; $p = 0.0164$. JHBP-ANOVA whole model d.f.= 9,23; $F = 18.4241$; $p < 0.0001$; treatment d.f.=2,7; $F = 22.6743$; $p < 0.0001$.

entire *JHBP-like* gene or the promoter, 5'UTR or first exon of *SIGIRR* (Fig. 1B). Analysis of all individuals (n = 178) and the individual CAPs markers showed that phenotype associates with both the marker present in *SIGIRR* (AMB-00612262) ($\chi = 16.22$; $P = 5.64 \times 10^{-5}$) and the marker present flanking the *JHBP-like* (AMB-00858654) ($\chi = 17.48$; $P = 2.95 \times 10^{-5}$). This suggests that the QTN may reside closer to, or within, the *JHBP-like* gene.

Analysis of candidate genes

Legitimacy of the candidate genes is dependent on the appropriate spatial and temporal expression of the gene relevant to the larval need for the physiological initiation of resistance. Larvae are susceptible to chalkbrood infection when 3-4 days old (Flores *et al.*, 1996) and any innate resistance likely functions then, or soon after. Tissue samples remaining from the mapping population represent larvae at least

8 days old and pupae up to 14 days old (Holloway *et al.*, 2012) and so remaining samples were not age- appropriate for gene expression analyses relevant to initial resistance mechanisms. However, the non-quantitative analysis of expression showed that the two candidate genes are both expressed in the mapping population albeit temporally inappropriate (data not shown). Instead, a developmental series of unrelated Russian honey bee brood was screened for the expression of the candidate genes to help determine legitimacy and temporal expression patterns. Because the SNP/LMCR association has not yet been validated in a secondary population, the resistance status of current Russian honey bee populations remains unknown. Therefore, pools of ungenotyped (hence, unbiased) larvae best represent the current population regardless of potential chalkbrood response. Three biological replicates of cDNA pools from ten individuals of each of the following stages were evaluated: 1st instar larvae, 2nd instar larvae, 3rd instar larvae, 4th instar larvae, 5th instar larvae (Fig. 2A); as well as five individuals each of the following stages: late-stage 5th instar larvae/pre-pupae, pre-pupae, and white eyed pupae (Fig. 2B). Real-time PCR of candidate genes during the tested developmental time course shows that both the *SIGIRR* (Figure 2C) and *JHBP-like* (Fig. 2D) genes are stably expressed in Russian honey bees (the source of the resistance) at the expected time of the immune response (i.e., 4-day-old, 1st instar larvae). The instabilities in candidate gene expression during the later stages of development likely play no role in the initiation of the immune response, therefore, both *SIGIRR* and *JHBP-like* are viable candidates for LMCR.

Discussion

Here we report the identification of molecular markers strongly associated with chalkbrood resistance in a mapping population. The nature of the honey bee genetics and the inability to generate recombinant inbred lines hinders the simplicity of traditional mapping, yet the analysis of a major quantitative trait locus by this method of fine mapping in a *qualitative-trait* manner identified significant molecular markers for subsequent marker-assisted selection.

The critical interval determined by the fine mapping of LMCR contained regions of two genes, *SIGIRR* and *JHBP-like*. The current version of the genome (Amel4.5) is validated by, and superimposed with, gene expression evidence and known or predicted gene annotations homologous to genes in other organisms. Additionally, unconfirmed algorithmic gene predictions, EST reads, and prospective nucleotide sequences based on reverse-translated conserved protein domains are aligned to the genomic scaffolds. Available gene annotations and predictions at the time of fine mapping only showed confidence in the expression of *SIGIRR;* however, very little EST information exists for honey bees. Meanwhile, the *JHBP-like* gene was considered a hypothetical protein, yet a large data set of EST reads suggested that *JHBP-like* was more likely a true gene expressed from within the interval. Despite the conflicting evidence available, both genes were evaluated for candidacy during the mapping process and both were expressed during the expected larval stage when a resistance response would be initiated.

Any expected function of SIGIRR in honey bee LMCR is based on the known functions of mammalian SIGIRR in the innate immunity Toll-like receptor (TLR) signalling pathway and a potential homologous role in the *Drosophila* Toll pathway. SIGIRR has been shown to function in the mouse gut epithelial tissue (Garlanda *et al.*, 2004) and antagonizes the activation of the TLR pathway following an inflammatory stimulus, thus tempering, or preventing, an appropriate immunity response (Wald *et al.*, 2003). The *Drosophila* Toll pathway is activated by bacterial and fungal infection and initiates the production of peptides (in particular, the anti-fungal peptide drosomycin) that inhibit fungal spore germination and hyphae growth, or can cause lysing of the fungal cells (reviewed in Zhang and Zhu, 2009). In honey bee LMCR, any altered gene expression or amino acid sequence of SIGIRR might, for instance, lead to an attenuation of the antagonist function on the Toll pathway, thus increasing the innate immune response against fungal infections. Further characterization of the protein sequence or functional domains of SIGIRR expressed in susceptible versus resistant individuals may enhance the understanding of the mechanisms of chalkbrood resistance.

Hormones modulate innate immunity in insects in other ways. In *Drosophila*, the steroid hormone 20-hydroxy-ecdysone (20E) acts to increase both the phagocytosis of fungal cells and the expression of the Toll pathway-mediated drosomycin anti-fungal peptide (Dimarcq *et al.*, 1997). The 20E-induced immunity response can be suppressed by interactions with juvenile hormone by downregulating the expression of *drosomycin* (Flatt *et al.*, 2008). The LMCR mapping interval contains a juvenile hormone-binding protein domain containing gene. While little information exists on the actual function of the *JHBP-like* gene, any modification of its interactions with JH would likely result in a change in the immune response of challenged larvae.

Considering that the two LMCR candidate genes are both strong components of the innate immunity in insects, it is possible that pathogens in addition to *A. apis* could be targeted for the anti-microbial Toll-induced immune response. Specifically, the microsporidian fungi *Nosema ceranae* and *N. apis* are widespread and presumably deadly challenge to honey bees. While the economic threshold of *Nosema spp*. is yet unknown, it is believed that infections are contributing to the ongoing loss of honey bee colonies throughout the world and are fuelling research to understand the infection process, tolerance levels, and any genetic basis of resistance. An increase in prevalence of the LMCR responsible alleles has the potential to lessen the impact of *Nosema* spp. on overall colony health and productivity.

While the exact gene or QTN responsible for the LMCR remain unknown, the critical interval has a strong predictive quality on the larval phenotype following a chalkbrood challenge. Prescreening breeding stocks for the resistance-associated alleles of the interval, particularly SNP AMB-00858654, could ultimately serve for marker-

assisted selection of highly resistant colonies. While the fine mapping was successful in this reported population, the interval will need to be validated in additional chalkbrood-challenged mapping populations to determine if LMCR is controlled by this same locus. Future populations will be developed that also enable the evaluation of chalkbrood response in homozygous resistant bees which were genetically unavailable in this study. In addition to the validation of the locus, the allelic associations must be evaluated. Considering the high recombination rate in the honey bee, one cannot assume that the same CAPs marker genotypes will associate with the phenotypes in all populations. Recombination around the LMCR interval may cause other populations to have the alternative SNP allele associate with the resistance. For instance, in this tested population, the homozygous susceptible individuals exhibited a BstBI restriction digest site at AMB-00858654, while the heterozygous resistant individuals contained both the digestible and non-digestible alleles. In other bee stocks and populations, an inherited and stable recombination event could result in the homozygous susceptible individuals having the non-digestible SNP at that marker. Further study of the locus in varied honey bee stocks, followed by selective breeding, may result in improved resistance of honey bees to chalkbrood and other fungal infections.

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