

CSP and Takeout Genes Modulate the Switch between Attraction and Repulsion during Behavioral Phase Change in the Migratory Locust

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Abstract

Behavioral plasticity is the most striking trait in locust phase transition. However, the genetic basis for behavioral plasticity in locusts is largely unknown. To unravel the molecular mechanisms underlying the behavioral phase change in the migratory locust *Locusta migratoria*, the gene expression patterns over the time courses of solitarization and gregarization were compared by oligonucleotide microarray analysis. Data analysis revealed that several gene categories relevant to peripheral olfactory perception are strongly regulated in a total of 1,444 differentially expressed genes during both time courses. Among these candidate genes, several CSP (chemosensory protein) genes and one takeout gene, *LmigTO1*, showed higher expression in gregarious and solitarious locusts, respectively, and displayed opposite expression trends during solitarization and gregarization. qRT-PCR experiments revealed that most CSP members and *LmigTO1* exhibited antenna-rich expressions. RNA interference combined with olfactory behavioral experiments confirmed that the CSP gene family and one takeout gene, *LmigTO1*, are involved in the shift from repulsion to attraction between individuals during gregarization and in the reverse transition during solitarization. These findings suggest that the response to locust-emitted olfactory cues regulated by CSP and takeout genes is involved in the behavioral phase change in the migratory locust and provide a previously undescribed molecular mechanism linked to the formation of locust aggregations.

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Introduction

Aggregation of animals is a common phenomenon that can be found in herds, shoals, flocks, schools and swarms of various species [1]. During aggregating, many species exhibit behavioral plasticity as an adaptive response to environmental changes [2]. Recently, several genes associated with behavioral plasticity have been identified, such as *Cyp6a20* gene in fruit flies [3], *foraging* gene in honey bees [4], and *Zenk* gene in zebra finches [5], but the temporal dynamics of the interactions between genetic and environmental factors on behavioral plasticity are still largely unknown.

The migratory locust, *Locusta migratoria*, is a worldwide agricultural pest, which exhibits severe plagues during which they migrate in marching juvenile bands and adult migratory swarms [6]. In response to the population density changes, they exhibit extreme phenotypic plasticity between the quiet, cryptically colored “solitarious” phase and the swarm-forming, conspicuously colored “gregarious” phase. Of all phase-related characteristics, behavioral changes are the most significant traits involved in the swarm formation in all locust species, particularly the changes in activity levels and in responses to other individuals [7]. Although the phase transition theory was originally established in the

migratory locust by Uvarov [8], the best characterized model is the desert locust, *Schistocerca gregaria*. It is revealed that the behavioral gregarization in the desert locust can be triggered by a combination of sensory inputs from head (visual and olfactory) or by the mechanosensory stimulation of hind legs [9,10]. These cues, which are stimulated by social environmental factors, are integrated by the nervous system to initiate behavioral phase change [11].

Recent genomic approaches have significantly improved our understanding in the phenotypic plasticity of the migratory locust. The application of large-scale expressed sequence tags (ESTs) revealed that 532 genes show different expression levels between the two phases of the migratory locust [12,13]. Several *hsp* genes display higher expression levels in gregarious locusts and are regulated during phase change [14]. The two extreme phases exhibit different transcriptomic profiles of small RNAs, among which longer small RNAs, possibly piRNAs, are more abundant in the solitarious phase of the migratory locust [15]. As important sources of small RNA, transposable elements also show different expression patterns between the two phases, especially in central nervous and peripheral olfactory tissues [16]. However, to further understand the molecular mechanism of phase change, it is required to address the relationship between gene expression and

Author Summary

The migratory locust, *Locusta migratoria*, is a worldwide agricultural pest whose outbreaks can result in plagues during which locusts mass migrate in marching bands and flying swarms. They exhibit striking phenotypic plasticity depending on the population density. They can transform between the barely-visible solitary phase and the swarm-forming gregarious phase. A key step in the formation of large aggregation is the initial shift from the strong mutual aversion in solitary locusts to the attraction to each other in gregarious locusts. Previous genomics studies have revealed 532 differentially expressed genes and many regulating small RNAs between the two phases of the migratory locust. Here, we developed a large-scale oligonucleotide microarray to quantify the expression of 9,154 genes during the time courses of isolating gregarious locusts and crowding solitary locusts. We found that several olfaction-related genes, several CSPs and one *takeout*, are strongly regulated during both processes and display opposite expression trends in response to population density change. We then identified that these genes mediate the rapid switch of attraction/repulsion behaviors in the migratory locust. Our findings are particularly significant for understanding the process of locust aggregation and may provide new targets to manipulate locust behavior as part of novel management strategies.

behavioral changes, especially the time window when sensory cues and crowding trigger the behavioral transition.

In this study, we developed a large-scale oligonucleotide microarray in the migratory locust and investigated the genome-wide expression profiles in heads of fourth-instar nymphs during the time courses of behavioral solitarization and gregarization. A binary logistic regression model was used to quantify the state of behavioral phase and the time course of behavioral phase change [17]. Furthermore, we used RNAi approach to explore the roles of candidate genes in phase-related behavior. The data from genome-wide expression profiling, the knockdown of olfactory-related genes and behavioral assays revealed the importance of olfactory-related genes, several CSPs and one *takeout*, in the fundamental phase-related behaviors of the migratory locust.

Results

The time course of behavioral phase change

Based on the data collected from 86 gregarious and 69 solitary fourth-instar migratory locust nymphs, we built a binary logistic model that can accurately categorize 87.2% of

gregarious nymphs and 87.0% of solitary nymphs, to summarize the behavioral phase state as a single probabilistic metric of gregariousness— P_{greg} . The model retained three variables: attraction index, total distance moved and total duration of movement (Table 1). Using this model, we investigated the behavioral phase changes of fourth-instar nymphs during the time courses of solitarization (Isolation of gregarious locusts, IG) and gregarization (Crowding of solitary locusts, CS). Gregarious nymphs showed significant behavioral solitarization after IG for only 1 h, which was in strong contrast to gregarious controls (Mann-Whitney $U = 240$, $p = 0.001$, Figure 1A). Full behavioral solitarization was acquired after IG for 16 h, exhibiting no significant difference with solitary controls (Mann-Whitney $U = 366.5$, $p = 0.110$). However, solitary nymphs did not display significant behavioral changes in gregarization until CS for 32 h as compared with solitary controls (Mann-Whitney $U = 261$, $p = 0.002$, Figure 1B). Full behavioral gregarization was not acquired even after CS for 64 h as compared with gregarious controls (Mann-Whitney $U = 115$, $p = 0.000$).

In addition, we also examined the time course of three behavioral variables that were significant predictors of phase-related behavior as revealed by logistic regression analysis. During the IG process, attraction index declined rapidly in 1 h to a stable level, but not to the level of solitary controls even after IG for 64 h (Mann-Whitney $U = 336$, $p = 0.037$, Figure S1A). However, total distance moved and total duration of movement decreased to the level of solitary controls in 4 h (Mann-Whitney $U = 446$, 375 , $p = 0.632$, 0.092 , Figure S1B and S1C). During the CS process, these three behavioral variables did not increase until 32 h (Mann-Whitney $U = 318$, 249 , 292 , $p = 0.021$, 0.001 , 0.004 , Figure S1D, S1E, S1F), but were far below the level of gregarious controls (Mann-Whitney $U = 319$, 220 , 239 , $p = 0.035$, 0.0004 , 0.001 , respectively). After CS for 64 h, only attraction index increased to the level of gregarious controls (Mann-Whitney $U = 387.5$, $p = 0.359$, Figure S1D).

Gene expression profiling during the time course of phase change

To investigate the global gene expression patterns related to behavioral phase change, we performed comparative gene expression profiling in heads of gregarious and solitary nymphs that underwent population density changes. During the time courses of IG and CS, 794 and 1103 genes, respectively, were differentially regulated (Table S1, Dataset S1), and they fell into a diversity of ontological categories (Figure S2) and pathways (Table S2). The number of differentially expressed genes exhibited a wave-like pattern during the IG process, with the highest level at 16 h. The number of differentially expressed genes increased along with the time course of CS, with the highest level at 32 h

Table 1. Variables retained in binary logistic model.

Model		Model if term removed								
Variables in equation	β	Standard error	Wald	df	Sig.	Exp(β)	Model Log Likelihood	Change in -2 Log Likelihood	Sig. of the Change	
X_1	attraction index	0.005	0.001	12.755	1	0.000	1.005	-56.557	15.875	0.000
X_2	total distance moved	0.012	0.005	5.972	1	0.015	1.012	-52.005	6.770	0.009
X_3	total duration of movement	0.015	0.007	4.694	1	0.030	1.015	-51.362	5.484	0.019
	Constant	-2.110	0.425	24.615	1	0.000	0.121			

$P_{\text{greg}} = e^{\eta} / (1 + e^{\eta})$, $\eta = -2.110 + 0.005 \times \text{attraction index} + 0.012 \times \text{total distance moved} + 0.015 \times \text{total duration of movement}$.
doi:10.1371/journal.pgen.1001291.t001

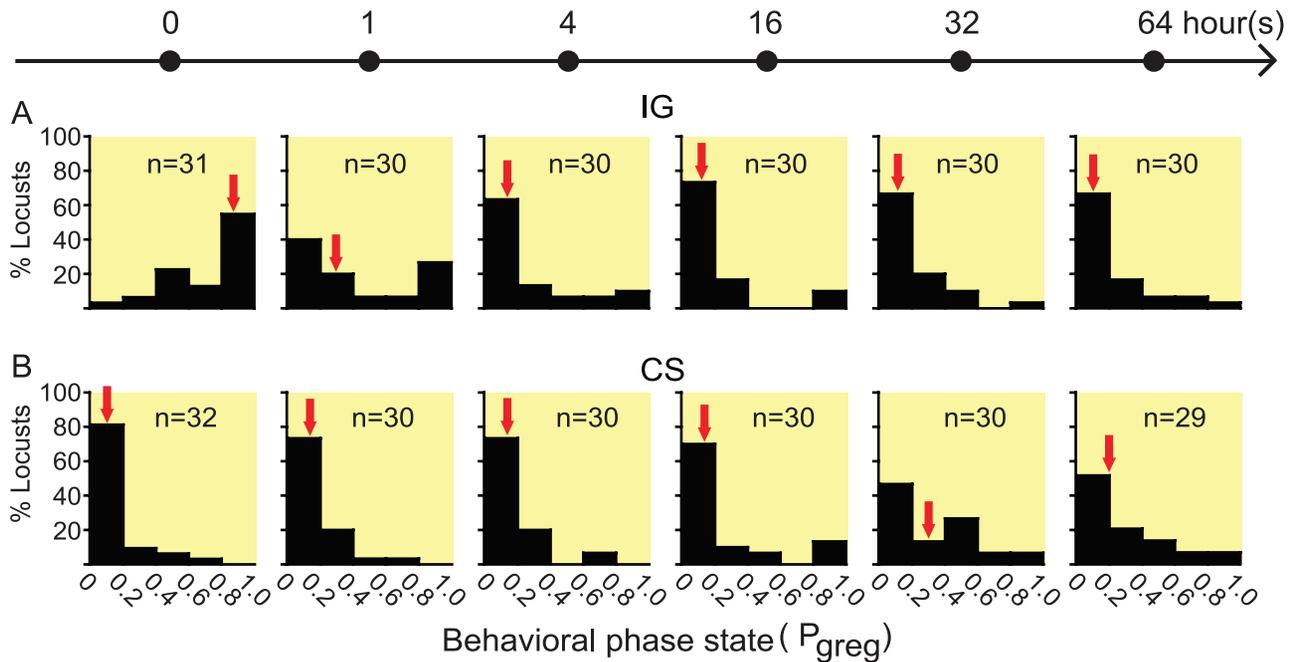


Figure 1. The time course of behavioral phase change in fourth-instar nymphs of *Locusta migratoria*. (A) Isolation of gregarious locusts (IG). (B) Crowding of solitary locusts (CS). P_{greg} , probabilistic metric of gregariousness. Arrows indicate median P_{greg} values.
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(Figure 2A). Further hierarchical cluster analysis for all differentially expressed genes revealed fluctuating patterns for most genes during the IG process, but a relatively stable trend during the CS process (Figure S3).

Enrichment analysis of differentially expressed genes showed that insect cuticle proteins, insect pheromone-binding family A10/OS-D, odorant binding proteins and arthropod hemocyanin/insect LSPs were the top four enriched InterPro categories in both time courses (Table 2). The BLAST and phylogenetic analysis revealed that insect pheromone-binding proteins and odorant binding proteins represented two gene families, chemosensory proteins (CSPs) and takeout proteins, respectively (Figures S4, S5, S6, Table S3). We further clustered 453 mutually differentially expressed genes during IG and CS processes into eight categories from A to H (Figure 2B, Dataset S1). Of these categories, A and H clusters showed consistent gene expression patterns during the time course of treatments while negative correlation of expression trends (Pearson correlation, $r = -0.896$, $p < 0.0001$). In these two categories, *CSP* and *takeout* genes represented the largest numbers—25% of the 40 annotated genes. Microarray results of 12 selected genes, including *CSP* and *takeout*, were validated by qRT-PCR (Table S4). Therefore, we hypothesized that *CSP* and *takeout* genes play important roles in behavioral phase changes.

Tissue-specific and time course expression of *CSP* and *takeout* genes

To reveal the potential involvement of *CSP* and *takeout* genes in the locust behaviors, in both gregarious and solitary nymphs, we investigated the tissue-specific expression of representative *CSP* and *takeout* genes (Figure 3A, Figure S7) that showed consistent expression patterns in qRT-PCR and microarray analysis, including *LmigCSP1*, *LmigCSP2*, *LmigCSP3*, *LmigCSP4* and *LmigTO1* (Table S4). As shown by Figure 3A, *CSP* and *takeout* genes were highly expressed in peripheral tissues (antenna, labial palp, wing

and hind leg) with abundant sensilla, but not in internal tissues (brain and fat body). Among all these tissues, the highest RNA expression levels of *CSP* and *takeout* genes were detected in antennal tissues, except for *LmigCSP1* that exhibited the highest expression in wing and hind leg tissues. Only *LmigCSP3* and *LmigTO1* showed significantly different expression levels in antennal tissues between gregarious and solitary nymphs. Relative expression level of *LmigCSP3* RNA was higher in gregarious nymphs (Student's t-test, $t = 8.893$, $p = 0.001$, Figure 3A Left), while *LmigTO1* exhibited higher expression levels in solitary nymphs (Student's t-test, $t = 3.670$, $p = 0.021$, Figure 3A Right). Between gregarious and solitary nymphs, *LmigCSP3* and *LmigTO1* were also differentially expressed in hind leg (Student's t-test, $t = 5.479$, $p = 0.003$) and labial palp (Student's t-test, $t = 5.812$, $p = 0.004$), respectively; however, the RNA relative expression levels in these tissues were relatively low. Significantly different expression levels of three other *CSP* family members were mainly detected in wing and hind leg (Figure S7).

We further investigated the expression patterns of these five genes in antennal tissues during the time courses of IG and CS (Figure 3B, Figure S8). As shown by Figure 3B, the overall expression of *LmigCSP3* and *LmigTO1* exhibited an opposite pattern in the two time courses. During the IG process, RNA relative expression levels of *LmigCSP3* increased slightly at 4 h, and then rapidly decreased until 64 h (ANOVA, $F_{5,18} = 15.266$, $p < 0.0001$). RNA relative expression levels of *LmigTO1* increased significantly from 1 h to 4 h, and then stayed at a stable level (ANOVA, $F_{5,18} = 11.154$, $p < 0.0001$). During the CS process, RNA relative expression levels of *LmigCSP3* increased significantly at 4 h (ANOVA, $F_{5,18} = 4.151$, $p = 0.020$). In contrast, RNA relative expression levels of *LmigTO1* decreased until 64 h (ANOVA, $F_{5,18} = 22.091$, $p < 0.0001$). Three other *CSP* gene family members showed similar expression patterns as *LmigCSP3* (Figure S8).

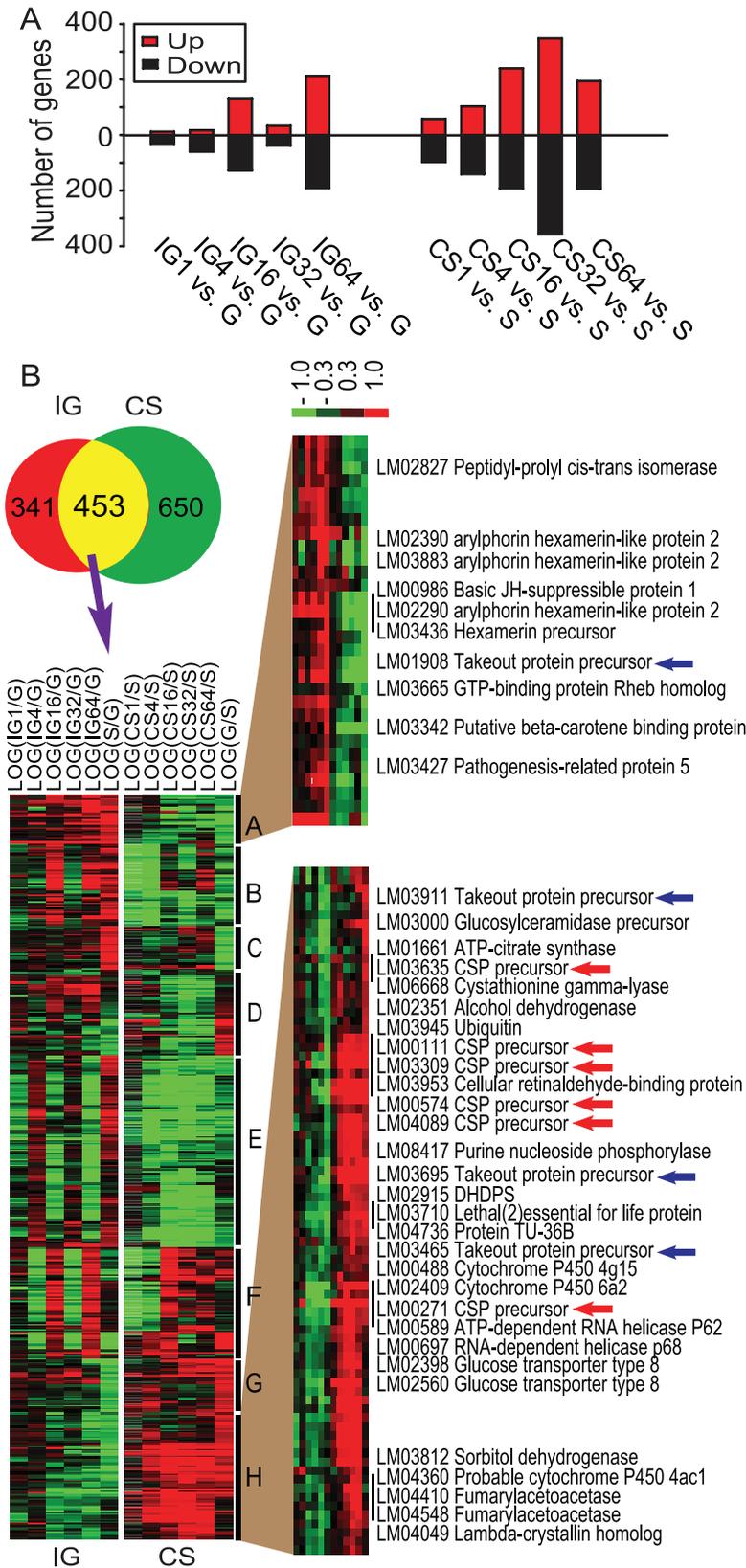


Figure 2. Candidate genes involved in locust phase change. (A) The numbers of up- and down-regulated genes during the time course of IG or CS. Numbers following IG or CS indicate hours of treatment. (B) Venn diagram indicating 453 mutually differentially expressed genes during the time courses of IG and CS. These 453 genes were categorized into 8 clusters by hierarchical clustering using a complete linkage algorithm. Cluster A and cluster H exhibit consistent changes over the time course of treatments and display a negative correlation of expression trends (Pearson

correlation, $r = -0.896$, $p < 0.0001$). *CSP* and *takeout* genes are highlighted in these two categories (red arrows for *CSP* and blue arrows for *takeout*). Horizontal stripes represent genes, and columns represent experimental treatments. Logarithmic fold change of treatment vs. control (gregarious during IG or solitary during CS) is shown in the heat map using red and green colors to indicate up- and down-regulation, respectively. doi:10.1371/journal.pgen.1001291.g002

Functional investigation of *LmigCSP3* and *LmigTO1*

To explore the physiological significance of *CSP* and *takeout* genes, RNAi and behavioral analysis were performed to identify their *in vivo* functions. RNAi experiments focused on *LmigCSP3* and *LmigTO1*, which exhibited antenna-rich expression and substantial expression difference between gregarious and solitary nymphs. In comparison with non-injected controls (non-inj), RNA relative expression levels of *LmigCSP3* and *LmigTO1* decreased significantly after injection of double-stranded *LmigCSP3* (ds*LmigCSP3*), *LmigTO1* (ds*LmigTO1*) and *LmigCSP3*&*LmigTO1* (simultaneous injection of ds*LmigCSP3* and ds*LmigTO1*), but no change was detected in double-stranded GFP-injected controls (Figure 4A and 4B). This silencing effect of ds*LmigCSP3* and ds*LmigTO1* was also observed in several other tissues, such as antenna, labial palp and hind leg (Figure S9). The expression of five other *CSP* genes, *LmigCSP1*, *LmigCSP2*, *LmigCSP4*, *LmigCSP5* and *LmigCSP6*, were significantly suppressed by ds*LmigCSP3* injection (Figure 4C) due to the high sequence similarity among *CSP* family members (Figure S10). However, the RNA relative expression levels of another two *takeout* family members, *LmigTO2* and *LmigTO3*, were barely affected by ds*LmigTO1* injection (Figure S11). Interactions of silencing effects between *LmigCSP3* and *LmigTO1* were not detected (Figure S12).

Behavioral assay revealed that attraction/repulsion behaviors of fourth-instar nymphs were influenced by ds*LmigCSP3* or ds*LmigTO1* injection (Figure 5). In comparison to the non-injected controls, the attraction index significantly decreased when

gregarious nymphs were injected with ds*LmigCSP3* (Mann-Whitney $U = 545$, $p < 0.0001$) and ds*LmigCSP3*&*LmigTO1* (Mann-Whitney $U = 387.5$, $p < 0.0001$), but no behavioral changes were detected upon dsGFP or ds*LmigTO1* injection (Mann-Whitney $U = 1442.5$, 1177.5 , $p = 0.926$, 0.915 , respectively. Figure 5A). The change extent of attraction index induced by the ds*LmigCSP3*&*LmigTO1* injection was less than that induced by the injection of ds*LmigCSP3* in gregarious locusts (Mann-Whitney $U = 587$, $p = 0.013$). Solitary nymphs injected with ds*LmigTO1* and ds*LmigCSP3*&*LmigTO1* exhibited a significant increase in the attraction index as compared with non-injected controls (Mann-Whitney $U = 985.5$, 567.5 , $p = 0.001$, 0.019 , respectively), but no behavioral changes were observed upon dsGFP or ds*LmigCSP3* injection (Mann-Whitney $U = 1519.5$, 770.5 , $p = 0.900$, 0.895 , respectively. Figure 5D). The ds*LmigTO1*-injected solitary nymphs spent significantly less time in the opposite end of test arena (away from the stimulus group) and tended to stay in the middle area of arena compared to non-injected and dsGFP-injected nymphs. However, we did not detect the influence of ds*LmigCSP3*, ds*LmigTO1* or ds*LmigCSP3*&*LmigTO1* injection on the total distance moved (Figure 5B and 5E) and total duration of movement (Figure 5C and 5F) in nymphs of both phases.

To investigate whether the behavioral effects of *LmigCSP3* and *LmigTO1* RNAi were correlated with olfactory perception, a Y-tube olfactometer was used to examine the behavioral response to either volatiles of gregarious nymphs or a clean air control (Figure S14B). More gregarious nymphs chose the volatile arm (Chi

Table 2. Top ten gene categories enriched in InterPro annotations during the time course of IG or CS.

Time course	InterPro ID	Interpro Name	P value	No. of differentially expressed genes	No. of annotated genes
IG	IPR000618	Insect cuticle protein	2.41E-27	28	49
	IPR005055	Insect pheromone-binding family A10/OS-D	4.63E-16	13	19
	IPR004272	Odorant binding protein	3.40E-09	11	22
	IPR000896	Arthropod hemocyanin/insect LSP	3.51E-07	8	10
	IPR005203	Hemocyanin, C-terminal	4.56E-04	4	5
	IPR005204	Hemocyanin, N-terminal	4.56E-04	4	5
	IPR000886	Endoplasmic reticulum targeting sequence	5.70E-04	6	15
	IPR001548	Peptidase M2, peptidyl-dipeptidase A	9.97E-04	3	3
	IPR008938	ARM repeat fold	1.26E-03	4	6
	IPR008037	Pacifastin inhibitor	2.71E-03	4	7
CS	IPR000618	Insect cuticle protein	9.97E-19	30	49
	IPR005055	Insect pheromone-binding family A10/OS-D	1.09E-06	11	19
	IPR004272	Odorant binding protein	1.20E-06	12	22
	IPR000896	Arthropod hemocyanin/insect LSP	9.26E-06	8	10
	IPR002068	Heat shock protein Hsp20	5.31E-04	4	4
	IPR005203	Hemocyanin, C-terminal	2.33E-03	4	5
	IPR005204	Hemocyanin, N-terminal	2.33E-03	4	5
	IPR005804	Fatty acid desaturase	2.33E-03	4	5
	IPR001436	Alpha crystallin	3.51E-03	3	3
	IPR001548	Peptidase M2, peptidyl-dipeptidase A	3.51E-03	3	3

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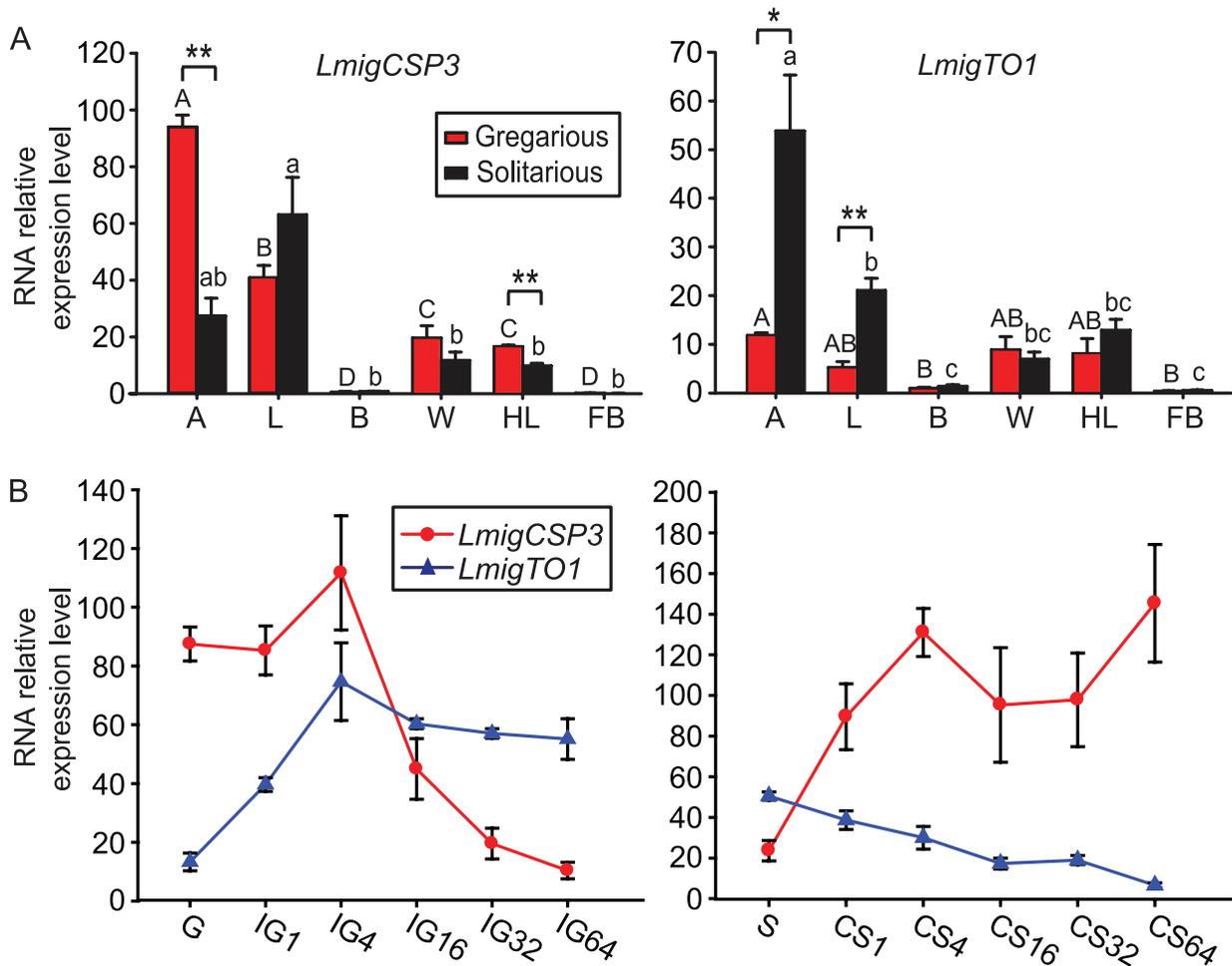


Figure 3. The tissue-specific and time course expression of *LmigCSP3* and *LmigTO1* genes in fourth-instar nymphs. (A) RNA relative expression levels of *LmigCSP3* (Left) and *LmigTO1* (Right) in six selected tissues. A, antenna; L, labial palp; B, brain; W, wing; HL, hind leg; FB, fat body. Means labeled with the same letter within each treatment are not significantly different and error bars represent SEM. *, $p < 0.05$; **, $p < 0.01$. (B) RNA relative expression levels of *LmigCSP3* and *LmigTO1* in antennal tissues during the time course of IG (Left) or CS (Right). doi:10.1371/journal.pgen.1001291.g003

square = 10.373, $p = 0.001$. Figure 6A) and more solitary nymphs chose the air control arm (Chi square = 12.500, $p < 0.0001$. Figure 6B). After *dsLmigCSP3* injection, gregarious nymphs tended to choose the air control arm (Chi square = 6.480, $p = 0.011$) and significantly decreased their preference to volatiles as compared with non-injected controls (Mann-Whitney $U = 758$, $p = 0.000$), but no behavioral changes were detected upon *dsGFP* or *dsLmigTO1* injection (Mann-Whitney $U = 1282$, 1175.5, $p = 0.712$, 0.831, respectively. Figure 6A). After *dsLmigTO1* injection, solitary nymphs randomly chose the volatile or air control arm (Chi square = 0.032, $p = 0.857$), and significantly decreased their behavioral aversion to volatiles as compared with non-injected controls (Mann-Whitney $U = 333$, $p = 0.007$); no behavioral changes were detected upon *dsGFP* or *dsLmigCSP3* injection (Mann-Whitney $U = 502$, 445, $p = 0.458$, 0.342, respectively. Figure 6B).

Discussion

We have demonstrated here that the migratory locust, *Locusta migratoria*, can alter the phase-related behaviors in response to population density changes, particularly the activity levels and the

attraction/repulsion responses to other individuals. Dramatically different gene expression was also detected during the time courses of solitarization and gregarization. Bioinformatics analysis of microarray data and behavioral assays indicated that several *CSP* genes and one *takeout* gene, affect the attraction/repulsion response to other individuals by regulating peripheral olfactory sensitivity. This is the first evidence to establish the relationship between olfactory-related genes and phase-related behaviors in the migratory locust.

Although a logistic regression model has been built for the migratory locust in previous study [18], an integrated time course analysis of behavioral phase change was not conducted. Our results showed that the behavioral gregarization of fourth-instar solitary nymphs occurred slowly, but the solitarization of gregarious nymphs was much faster (Figure 1). However, not all of the three key behavioral variables (attraction index, total distance moved and total duration of movement) comprising the linear predictor of phase state— P_{greg} , displayed parallel patterns with P_{greg} during the time courses (Figure S1). For example, although the P_{greg} values from the logistic regression model only modestly shifted, the attraction index of solitary locusts increased significantly during 64 h crowding. The total distance moved

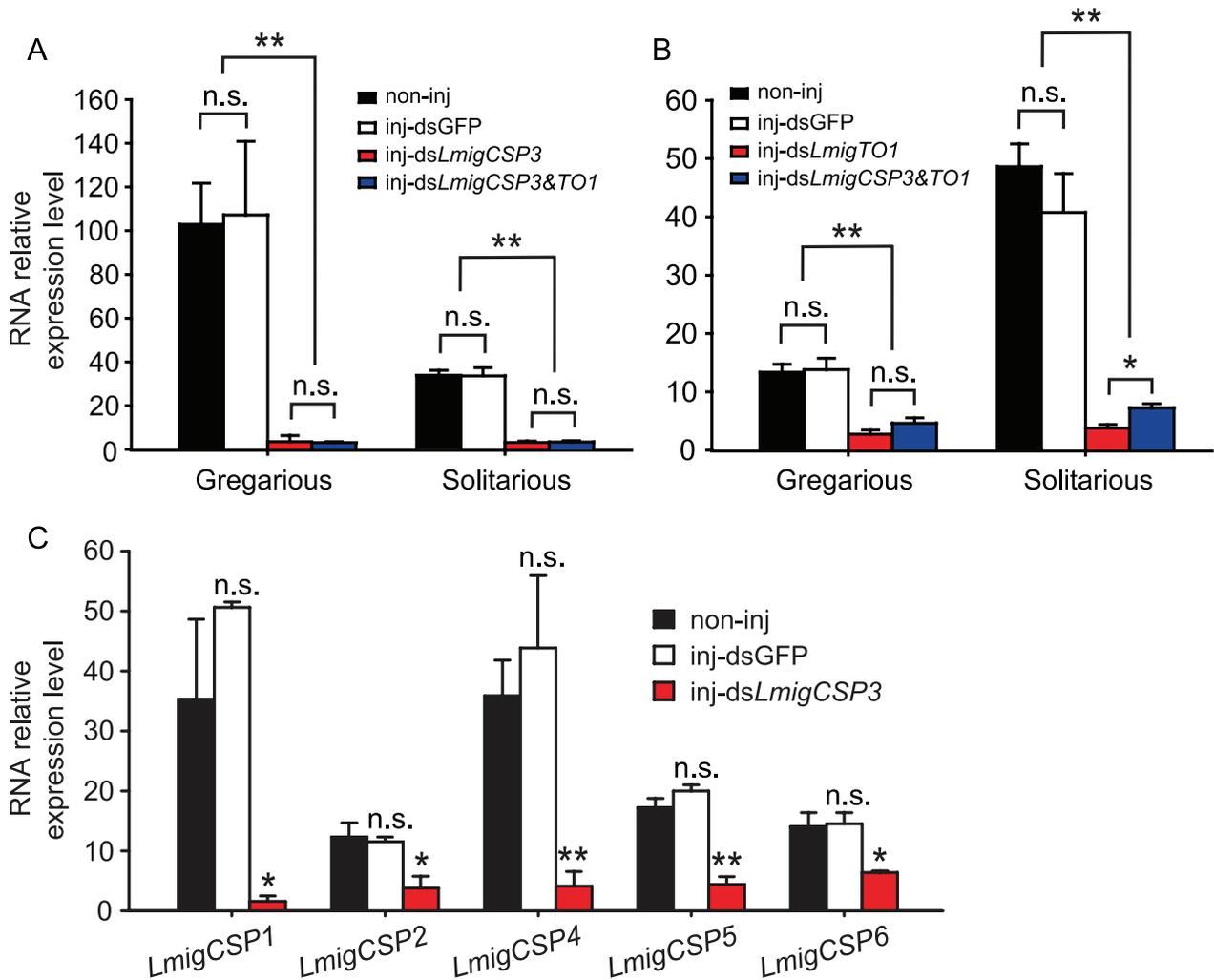


Figure 4. The effects of RNAi knockdown of *LmigCSP3* and *LmigTO1* genes on their expression levels. (A) The expression levels of *LmigCSP3* in gregarious (Student's t-test, $t = 5.308, 5.229, p = 0.006, 0.006$) or solitarious (Student's t-test, $t = 10.880, 12.731, p < 0.0001, 0.0001$) fourth-instar nymphs after ds*LmigCSP3* or ds*LmigCSP3&TO1* injection, respectively (B) The expression levels of *LmigTO1* in gregarious (Student's t-test, $t = 4.825, 6.852, p = 0.005, 0.002$) or solitarious (Student's t-test, $t = 10.401, 13.366, p < 0.0001, 0.0001$) fourth-instar nymphs after ds*LmigTO1* or ds*LmigCSP3&TO1* injection, respectively (C) Effects of ds*LmigCSP3* injection on RNA relative expression levels of five other CSP genes (Student's t-test, $t = 4.353, 3.496, 4.347, 6.315, 3.271, p = 0.012, 0.025, 0.007, 0.001, 0.017$, respectively). In all groups, treatment (inj-dsGFP, inj-ds*LmigCSP3*, inj-ds*LmigTO1* or inj-ds*LmigCSP3&TO1*) compared with the corresponding non-injected controls. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. doi:10.1371/journal.pgen.1001291.g004

and total duration of movement of solitarious locusts showed remarkable change only at 32 h time point. This indicates that the full behavioral change (as defined by the behavior of the gregarious control group used to build the logistic regression model) did not occur by 64 h, but the various behaviors reached the final gregarious condition at different rates, with attraction index shifting the most rapid one.

Fourth-instar nymphs of the migratory locust exhibited rapid and complicated transcriptomic changes (15.8% of total genes) in response to altered population density. Previous reports in several other insect species, such as fruit fly [3] and honeybee [19], suggest that social stimuli, like other environmental factors, can induce a high level of transcriptional regulation. The overall gene expression pattern showed that the transcriptional response of gregarious nymphs to solitarization is weak and dynamic while that of solitarious nymphs to gregarization is relatively intensive and stable (Figure 2A). Clearly, the trends of overall gene expression are not completely consistent with the rate of

behavioral change. One possible explanation is that the acquisition or loss of phase-related behavior in the migratory locust may involve different genes and pathways. Alternatively, some differentially expressed genes do not regulate behavioral change, but they could be involved in other phase-related traits, such as colour, immunity and nutrition.

The present results highlighted several gene categories whose expressions were highly and robustly correlated with time course processes in response to population density change, for example, cuticle proteins, pheromone-binding proteins and hexamerins. In these categories, odorant/pheromone binding protein genes, which are suggested to be involved in olfactory perception [20–22], were differentially regulated and more abundant (Figure 2, Table 2). Similar results were observed by our previous studies on the comparison of EST data between two extreme phases of the migratory locust [13]. These findings imply that the change of olfactory pathways may occur rapidly when the locusts experience population density changes. A role of olfactory pathways in

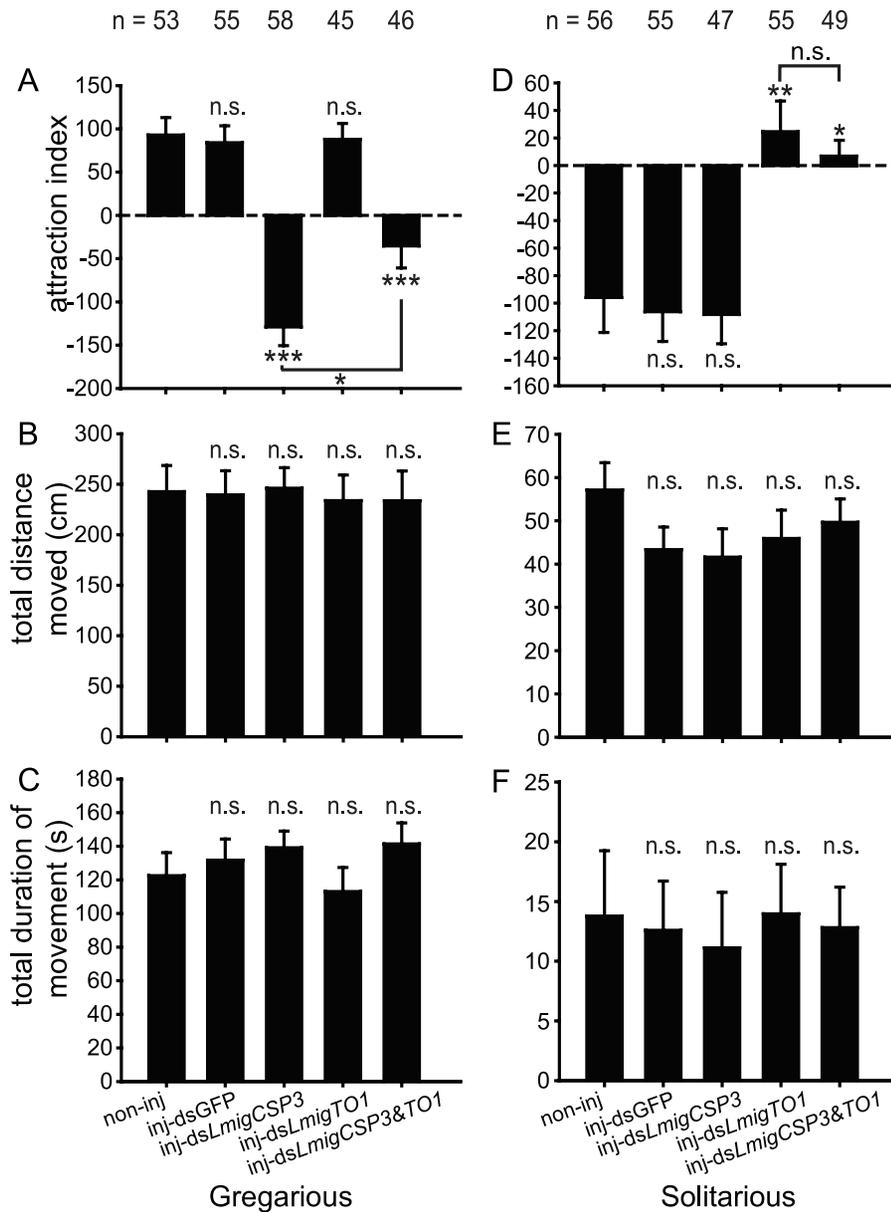


Figure 5. Behavioral phenotype of locust nymphs in arena after RNAi knockdown of *LmigCSP3/LmigTO1*. (A) Changes of attraction behavior induced by *LmigCSP3*, *LmigTO1* or *LmigCSP3&TO1* knockdown in gregarious nymphs. (B) Effects of *LmigCSP3*, *LmigTO1* or *LmigCSP3&TO1* knockdown on total distance moved in gregarious nymphs. (C) Effects of *LmigCSP3*, *LmigTO1* or *LmigCSP3&TO1* knockdown on total duration of movement in gregarious nymphs. (D) Change of repulsion behavior induced by *LmigCSP3*, *LmigTO1* or *LmigCSP3&TO1* knockdown in solitarious nymphs. (E) Effects of *LmigCSP3*, *LmigTO1* or *LmigCSP3&TO1* knockdown on total distance moved in solitarious nymphs. (F) Effects of *LmigCSP3*, *LmigTO1* or *LmigCSP3&TO1* knockdown on total duration of movement in solitarious nymphs. In all groups, treatment (inj-dsGFP, inj-ds*LmigCSP3*, inj-ds*LmigTO1* or inj-*LmigCSP3&TO1*) compared with non-injected controls. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant. doi:10.1371/journal.pgen.1001291.g005

triggering behavioral changes and mediating social interactions has been proposed in the desert locust [10,23,24]. Further functional analysis is required to elucidate whether these genes are involved in one or both of the two processes, i.e., triggering behavioral changes and mediating social interactions, in the migratory locust.

Analyses of two highly regulated gene categories, *CSPs* and one *takeout*, indicated an opposite expression pattern to modulate the shift of attraction/repulsion behavior in response to conspecifics. Most *CSP* genes and one *takeout* gene exhibit antenna-rich expression in fourth-instar nymphs and markedly different

expression levels between gregarious and solitarious locusts (Figure 3A). In addition, these genes displayed altered expression levels during the time course of IG or CS (Figure 3B). More importantly, knockdown of *LmigCSP3* in gregarious locusts led to a shift towards solitarious-phase-like behavior (i.e., being repelled by conspecific volatiles) (Figure 5A, Figure 6A). Similarly, knockdown of *LmigTO1* in solitarious locusts suppressed the solitarious behavior (Figure 5D, Figure 6B). However, in both cases, we failed to detect any effect on other phase-related variables (total distance moved and total duration of movement—both associated with activity levels rather than position relative to the stimulus

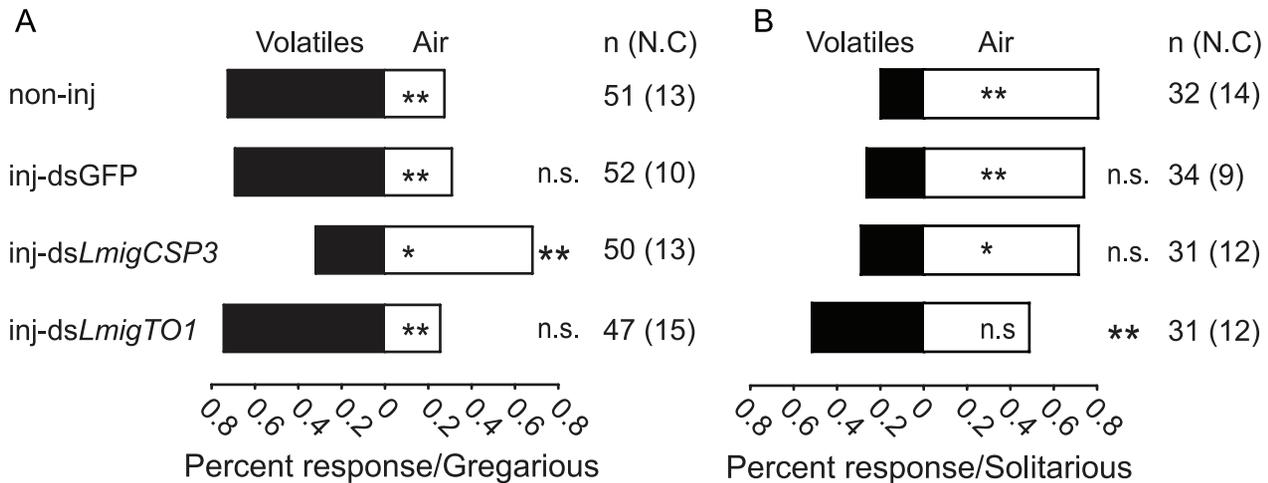


Figure 6. Dual-choice of locust nymphs in Y-tube olfactometer after RNAi knockdown of *LmigCSP3* or *LmigTO1*. (A) Choice behavior of gregarious locusts injected by *dsLmigCSP3* or *dsLmigTO1*. (B) Choice behavior of solitary locusts injected by *dsLmigCSP3* or *dsLmigTO1*. Volatiles represent volatiles from 30 gregarious fourth-instar nymphs. Marks (*, ** and n.s.) inside white stripe indicate the significance of Chi square test for comparing individual numbers in each arm. Marks (** and n.s.) outside the stripe indicate the significance of treatment (inj-dsGFP, inj-dsLmigCSP3 or inj-dsLmigTO1) compared with non-injected controls. n, individual numbers used in significance test. N.C., no choice, excluded in significance test. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. doi:10.1371/journal.pgen.1001291.g006

group), suggesting that various phase-related behavioral traits may be regulated by different genes.

It is evident that the overall tendency of *CSPs* expression during solitarization is similar to behavioral changes, but with a several hour delay. This time delay might be due to the changes of the ratio of the expression levels of *CSP* and *takeout* genes. Our results from the single gene knockdown or the double gene knockdown experiments have suggested that the levels of attraction/repulsion may be dependent on the relative expression of *CSP* and *takeout* genes (Figure 4, Figure 5). Thus, although expression levels of *CSP* genes are not significantly decline during 4 h solitarization, the immediate increase in *takeout* expression could be sufficient for the behavioral change towards repulsion.

Previous studies suggested that both *CSP* and *takeout* proteins are large families of soluble binding proteins secreted into the lymph fluid in various insect species [20,25]. They were proposed to have important roles in olfactory reception [26], development [27], feeding [28], courtship [29] and aging [30]. For instance, in the fire ant *Solenopsis invicta*, allele variation of an OBP Gp-9 gene can cause differences in workers' abilities to recognize queens and to regulate their numbers [31]. It is proposed that *CSP* genes also have a role in chemoreception [32–34] and locust phase change [35]. The first member of *takeout* family was discovered in *Drosophila melanogaster* as a clock-regulated gene that modulates feeding and mating behaviors [28,36]. Following that, more *takeout* genes were found to be specifically expressed in chemosensory organs, such as antenna and labellum, in many insect species, suggesting their functions in olfactory perception [21,37]. Here, we provided direct evidence for the first time that *CSP* and *takeout* genes are involved in locust social interactions.

Our results suggested that the differential expressions of *CSP* and *takeout* genes led to opposite behavioral responses of gregarious and solitary nymphs to the odour from gregarious locusts by regulating the peripheral olfactory sensitivity to pheromones. Consistent with this hypothesis, studies in adult desert locusts showed that the regulation of aggregation behavior involves pheromonal communication and that a key compound produced by mature gregarious males, phenylacetoneitrile, appears to have a

concentration threshold beyond which the pheromone arrest becomes repulsion [38,39]. Previous studies also suggested that gregarious and solitary nymphs displayed different neural sensitivity in antennal sensilla basiconica to aggregation pheromone in the desert locust [40]. It is found that *CSPs* are present at the tips of sensilla in the migratory locust [41] and *takeout* is selectively localized around the auxiliary cell membranes at the base of sensilla in the blowfly [21]. The changes in *CSP* and *takeout* expression may together influence the pheromone sensitivity threshold beyond which attraction becomes repulsion. Alternatively, *CSP* and *takeout* genes may determine the sensitivity of different olfactory receptor cells (ORCs) that specifically drive attraction or repulsion, or that produces different firing patterns across olfactory neurons to regulate these two opposing behaviors [40]. Clearly, many neurotransmitters and hormones are involved in the regulation of olfactory sensitivity [42,43]. Serotonin has recently been revealed to be a key neurochemical mediator of behavioral phase change in the desert locust [11] and has been suggested to be not only involved in the establishment of short-term effects, but also activate second messenger cascades and synthesis of proteins that result in long-term effects [7]. Thus, the differential olfactory sensitivity between gregarious and solitary locusts regulated by *CSP* and *takeout* genes may be modulated by the serotonin and other neurotransmitters. Further investigation of the link between *CSPs/takeout* expression and some neurotransmitters may lead to a more comprehensive understanding of behavioral phase change.

Behavioral change is the first phase trait in response to crowding and it is crucial for swarm formation and migration in locusts [7]. Our study suggested that the migratory locust displays a different time course of behavioral phase change as compared with the desert locust, *Schistocerca gregaria* [44], and the Australian plague locust, *Chortoicetes terminifera* [45]. We identified several candidate gene categories involved in the fundamental phase-defining behavioral differences in locusts, and characterized the functions of *CSP* and *takeout* genes, which mediate the rapid switch of attraction/repulsion behaviors. This molecular mechanism is particularly significant for understanding locust aggregation and

potentially provides new targets to manipulate locust behavior as novel management strategies.

Materials and Methods

Insects

Gregarious and solitary locusts used in experiments were from the same stock at the Institute of Zoology, CAS, China. The gregarious cultures were reared in large, well ventilated, cages (25 cm×25 cm×25 cm) at densities of 200–300 insects per cage. The solitary cultures were reared in isolation for more than 10 generations which remain the phase-traits for reversible phase transition (Figure S13) in physical, visual and olfactory isolation, achieved by ventilating each cage (10 cm×10 cm×25 cm) with charcoal-filtered compressed air. Both colonies were reared under a 14:10 light/dark photo regime at 30±2°C and on a diet of fresh greenhouse-grown wheat seedlings and wheat bran.

Experimental samples

Isolation of gregarious locusts. The fourth-instar gregarious nymphs were separately reared in the solitary rearing cages supplied with food. After 0, 1, 4, 16, 32 or 64 h of treatments, their behavioral state was measured, or their heads (including antennae) or antennae were dissected and immediately put into liquid nitrogen for microarray and qRT-PCR experiments. All the insects were sampled at the same time point (9:00 am) for 4–5 biological replicates (4 replicates in CS and 5 replicates in IG), and equal numbers of male and female insects (six insects in total) were sampled in each biological replicate (the same applies for the tissue sampling below). Each biological replicate was individually hybridized to the array; separate replicates were used for qRT-PCR validation.

Crowding of solitary locusts. Two solitary fourth-instar locusts were introduced into the solitary rearing cage, which contained 20 gregarious nymphs of the same developmental stage supplied with food. After staying with the stimulus group for 0, 1, 4, 16, 32 or 64 h, locusts were taken for behavioral assays, or their heads (including antennae) or antennae were dissected and immediately put into liquid nitrogen for microarray and qRT-PCR experiments.

Tissue sampling. Six tissues, including antenna, labial palp, brain, wing, hind leg and fat body, from gregarious and solitary fourth-instar nymphs were dissected and put into liquid nitrogen.

Behavioral assay

The arena assay experiment was performed in a rectangular perspex arena (40 cm length×30 cm width×10 cm height) with opaque walls and a clear top (Figure S14A) according to previous study [17]. One of the separated chambers (7.5 cm length×30 cm width×10 cm height) contained 15 fourth-instar gregarious locusts as a stimulus group, and the other chamber was left empty. Before measurement, the locusts were restricted in a Perspex cylinder for 2 min. Then, locusts were released into the arena and monitored for 300 s. EthoVision video tracking system (Netherlands, Noldus Information Technology) was used to automatically record individual behavior. Eleven behavioral variables were acquired: entry frequency in stimulus area (stimulus area was defined as 25% of the arena closest to the stimulus group, Figure S14A in red), latency of first occurrence in stimulus area, total duration in area close to the wall, entry frequency in area close to the wall, entry frequency in the region opposite to stimulus area (opposite of stimulus area was defined as the 25% of the arena at the opposite end to the stimulus group, Figure S14A in blue), latency of first occurrence in the opposite of stimulus area, mean distance to the

stimulus group, attraction index (total duration in stimulus area, total duration in the opposite of stimulus area and total duration in middle area were weighted by 1, -1 and 0, respectively). Attraction index = 1×total duration in stimulus area+(-1)×total duration in the opposite of stimulus area+0×total duration in middle area), total distance moved, total duration of movement, and frequency of movement. Based on the data from 86 gregarious and 69 solitary fourth-instar nymphs, a forward stepwise approach was performed to construct the binary logistic regression model: $P_{\text{greg}} = e^{\eta} / (1 + e^{\eta})$, where $\eta = \beta_0 + \beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \dots + \beta_k \cdot X_k$. P_{greg} indicates the probability of a locust being considered as the gregarious. $P_{\text{greg}} = 1$ means fully gregarious behavior and $P_{\text{greg}} = 0$ means fully solitary behavior. This model shared similar features with previous logistic model [11,17] as the retained variables indicated: total distance moved and total duration of movement represent the activity levels, and attraction index represent the attraction or repulsion to the stimulus group.

A Y-tube olfactometer was used to analyze the behavioral responses of individual locust to volatiles (including volatiles from body and feces) from 30 fourth-instar gregarious locusts in the absence of any visual cues (Figure S14B). Individual locust was recorded as “first choice” for volatile or air (whenever the locust moved more than 5 cm into either arm) or “no choice” (N.C) in 5 min.

Oligonucleotide microarray design and construction

All sequences for probe design were derived from 9,154 locust unigenes [13]. The 60-mer oligonucleotide probes were designed and synthesized by Beijing Genomics Institute, CAS. The probes were dissolved in 3×SSC solution at 40 μM and finally spotted on poly-L-lysine coated slides using a SpotArray Enterprise microarrayer (PerkinElmer Life Sciences Inc.). Each slide contained 48 blocks, with 20 columns and 20 rows in each block. Every probe had duplicate spots. The probes of three house-keeping genes (beta-actin, GAPDH and 18S RNA) and two rice genes were spotted on each block as positive and negative controls, respectively.

RNA preparation, cDNA labeling, and microarray hybridization

Total RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s protocol. cDNA integrated with aminoallyl-dUTP (Sigma) was prepared from total RNA by reverse transcription (Invitrogen). The purified probes were labeled covalently with Cy3 and Cy5 (Amersham Pharmacia Biotech). Subsequently, mixed probes were hybridized to the microarray slide for 16–18 hours at 42°C under darkness. A combined loop hybridization design was adopted in the microarray hybridization experiments (Figure S15). For dye bias control, dye-swap strategy was used for microarray hybridization.

Microarray data processing

Microarray slides were scanned by a GenePix 4000B scanner and raw data was acquired with GenePix 5.0 software (Axon Instrument). After background correction and data normalization, significance analysis was performed using a fix model in MAANOVA (1.4.1) software package [46] in R 2.6.0 platform. All data were transformed by Bayesian methods [47] to compare with references (gregarious locusts in IG and solitary locusts in CS). Genes with significance levels of $p < 0.005$ and fold change > 1.5 were considered as differentially expressed. Unsupervised hierarchical clustering [48] was performed with cluster 3.0 software using uncentered Pearson correlation and complete

linkage, and presented by Java Treeview software [49]. Significant pathways were analyzed by KEGG Orthology-Based Annotation System (KOBAS, <http://kobas.cbi.pku.edu.cn>). InterPro categories were enriched for supplied gene list based on the algorithm presented by GOstat [50]. For each InterPro term, the difference between tested and reference gene groups was represented by a p value, which is estimated by chi square test. Fisher exact test was used when any expected value of count is <5. The p values of multitest were corrected by FDR [51]. Categories were considered as significantly enriched at $p < 0.05$.

Quantitative RT-PCR (qRT-PCR)

cDNA was reverse-transcribed from 2 μ g total RNA using MMLV reverse transcriptase (Promega). The standard curve method [52] was used to measure the mRNA relative expression levels, which were normalized by beta-actin [16,42]. We adjusted all values of relative expression levels of these genes for all qRT-PCR experiments based on the starting concentration of plasmids initially used to dilute a series of standard curves in tissue-specific experiments. PCR amplification was conducted using Mx3000P spectrofluorometric thermal cycler (Stratagene) and RealMaster-Mix (SYBR Green) kit (Tiangen), initiated with a 2-min incubation at 95°C, followed by 40 cycles of 95°C, 20 s; 58°C, 20 s; 68°C, 20 s. Melting curve analysis was performed to confirm the specificity of amplification.

RNA interference (RNAi)

Double-stranded RNA (dsRNA) of green fluorescent protein (GFP), *LmigCSP3* or *LmigTO1* were prepared using T7 RiboMAX Express RNAi system (Promega) following the manufacturer's instructions. For both gregarious and solitary locusts, fourth-instar locusts remained uninjected or were injected with 18 μ g (6 μ g/ μ l) of dsGFP, ds*LmigCSP3*, ds*LmigTO1* or mixed ds*LmigCSP3* and ds*LmigTO1* (ds*LmigCSP3*&*TO1*) in the second ventral segment of the abdomen. Then, the injected gregarious locusts were marked and put back into gregarious-rearing cages, and the injected solitary locusts were put back into solitary-rearing cages. Three days later, the effects of RNAi on mRNA relative expression levels were investigated by qRT-PCR and the behavior was examined as described above.

Statistical analysis

Differences between treatments were compared either by Student's t-test or by one-way analysis of variance (ANOVA) followed by a Tukey's test for multiple comparisons. Behavior-related data were analyzed by Mann-Whitney U test because of its non-normal distribution characteristics. Chi square test was used for significance test between numbers of locusts in each arm in Y-tube assay. Differences were considered significant at $p < 0.05$. Values were reported as mean \pm SEM. Data were analyzed using SPSS 15.0 software (SPSS Inc., Chicago, IL).

Supporting Information

Dataset S1 Differentially expressed genes during time course of IG and CS.

Found at: doi:10.1371/journal.pgen.1001291.s001 (0.47 MB XLS)

Figure S1 Change trends of three retained variables during the time course of IG or CS. Changes of attraction index (A), total distance moved (B) and total duration of movement (C) during the IG process. Changes of attraction index (D), total distance moved (E) and total duration of movement (F) during the CS process.

Treatments are compared with gregarious control during the IG process or solitary control during the CS process. *, $p < 0.05$; **, $p < 0.01$.

Found at: doi:10.1371/journal.pgen.1001291.s002 (1.00 MB EPS)

Figure S2 Gene ontological categories of differentially expressed genes during the time course of IG or CS. Only differentially regulated gene categories ($p < 0.05$) are shown here.

Found at: doi:10.1371/journal.pgen.1001291.s003 (2.97 MB EPS)

Figure S3 Hierarchical clustering of differentially expressed genes during the time course of IG or CS. A complete linkage algorithm was used to cluster the 794 (IG) or 1103 (CS) differentially expressed genes. Horizontal stripes represent genes and columns show experimental treatments. Logarithmic fold change of treatment vs. reference (gregarious during IG or solitary during CS) are shown in the heat map using red and green color codes for up- and down-regulation, respectively.

Found at: doi:10.1371/journal.pgen.1001291.s004 (3.11 MB EPS)

Figure S4 Phylogenetic tree of representative CSP (A) and takeout (B) protein sequences. Consensus unrooted trees were generated with 1000 bootstrap trials using the neighbor-joining method and presented with a cutoff value of 50.

Found at: doi:10.1371/journal.pgen.1001291.s005 (0.98 MB EPS)

Figure S5 Alignment of deduced protein sequences of CSPs from locust EST database. Putative signal peptides (shaded in yellow) were predicted using SignalP V3.0 web server, and conserved cysteine residues and sequence regions are indicated by red letters.

Found at: doi:10.1371/journal.pgen.1001291.s006 (6.31 MB EPS)

Figure S6 Alignment of deduced protein sequences of takeout from locust EST database. Putative signal peptides (shaded in yellow) were predicted using SignalP V3.0 web server, and conserved cysteine residues are indicated by red letters.

Found at: doi:10.1371/journal.pgen.1001291.s007 (7.91 MB EPS)

Figure S7 Tissue-specific expression of *LmigCSP1*, *LmigCSP2*, and *LmigCSP4* in gregarious and solitary fourth-instar nymphs. A, antenna; L, labial palp; B, brain; W, wing; HL, hind leg; FB, fat body. Means labeled with the same letter within each treatment are not significantly different and error bars represent SEM. *, $p < 0.05$; **, $p < 0.01$.

Found at: doi:10.1371/journal.pgen.1001291.s008 (0.87 MB EPS)

Figure S8 Gene expression of three other CSP genes in antennal tissue during the time course of IG or CS. (A) RNA relative expression levels of *LmigCSP1*, *LmigCSP2* and *LmigCSP4* during the time course of IG (ANOVA, $F_{5,18} = 2.810, 18.346, 9.439$, $p = 0.066, 0.000, 0.001$, respectively). (B) RNA relative expression levels of *LmigCSP1*, *LmigCSP2* and *LmigCSP4* during the time course of CS (ANOVA, $F_{5,18} = 1.474, 4.827, 8.020$, $p = 0.269, 0.012, 0.002$, respectively).

Found at: doi:10.1371/journal.pgen.1001291.s009 (0.82 MB EPS)

Figure S9 Systemic effects of RNAi. (A) RNA relative expression levels of *LmigCSP3* (Upper) and *LmigTO1* (Lower) genes after injection of ds*LmigCSP3* and ds*LmigTO1*, respectively, in the four selected tissues in gregarious nymphs. (B) RNA relative expression levels of *LmigCSP3* (Upper) and *LmigTO1* (Lower) genes after injection of ds*LmigCSP3* and ds*LmigTO1*, respectively, in the four selected tissues in solitary nymphs. A, antenna; L, labial palp; B, brain; HL, hind leg. In all groups, treatment (inj-dsGFP, inj-ds*LmigCSP3* or inj-ds*LmigTO1*) compared with respective non-injected controls. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant.

Found at: doi:10.1371/journal.pgen.1001291.s010 (0.99 MB EPS)

Figure S10 Six *CSP* genes have a high sequence identity of 78.18%. GenBank accession number: *LmigCSP5*, CO835786; *LmigCSP6*, CO852124.

Found at: doi:10.1371/journal.pgen.1001291.s011 (2.12 MB EPS)

Figure S11 Effect of ds*LmigTO1* injection on RNA relative expression levels of *LmigTO2* and *LmigTO3*. Student's t-test, $t = 0.372$, 0.770 , $p = 0.723$, 0.471 , respectively. In all groups, treatment (inj-dsGFP or inj-ds*LmigTO1*) compared with respective non-injected controls. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant.

Found at: doi:10.1371/journal.pgen.1001291.s012 (0.66 MB EPS)

Figure S12 Interaction of *LmigCSP3* and *LmigTO1*. (A) RNA relative expression level of *LmigCSP3* after ds*LmigTO1* injection in gregarious or solitary nymphs. (B) RNA relative expression level of *LmigTO1* after ds*LmigCSP3* injection in gregarious or solitary nymphs. In all groups, treatments (inj-dsGFP, inj-ds*LmigCSP3* or inj-ds*LmigTO1*) are compared with respective non-injected controls. n.s., not significant.

Found at: doi:10.1371/journal.pgen.1001291.s013 (0.81 MB EPS)

Figure S13 The behavioral phase state of gregarious nymphs (A) and solitary nymphs crowding for 4 instars (B). P_{greg} , probabilistic metric of gregariousness. Arrows indicate median P_{greg} values.

Found at: doi:10.1371/journal.pgen.1001291.s014 (0.63 MB EPS)

Figure S14 Schematic diagram of behavioral assay system. (A) Behavioral assay arena. (B) Y-tube olfactometer assay set. Individual nymph was released from Y-tube adapter to observe their choice.

Found at: doi:10.1371/journal.pgen.1001291.s015 (8.20 MB EPS)

Figure S15 Schematic diagram of experimental design of behavioral assay and microarray hybridization. IG (left of the dashed), isolation of gregarious locusts; CS (right of the dashed), crowding of solitary locusts. Numbers following IG or CS

represent hours of treatment. Arrows indicate microarray hybridization of neighboring treatments.

Found at: doi:10.1371/journal.pgen.1001291.s016 (1.01 MB EPS)

Table S1 Number of differentially expressed genes during the time course of IG or CS.

Found at: doi:10.1371/journal.pgen.1001291.s017 (0.05 MB DOC)

Table S2 Top ten regulated pathways during the time course of IG or CS.

Found at: doi:10.1371/journal.pgen.1001291.s018 (0.07 MB DOC)

Table S3 Genes used in phylogenetic tree reconstruction.

Found at: doi:10.1371/journal.pgen.1001291.s019 (0.07 MB DOC)

Table S4 qRT-PCR validation of microarray data and primer sequences.

Found at: doi:10.1371/journal.pgen.1001291.s020 (0.17 MB DOC)

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Author Contributions

Conceived and designed the experiments: WG XW LK. Performed the experiments: WG XW ZM LX JH DY. Analyzed the data: WG XW ZM. Wrote the paper: WG XW LK.

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