FACULTY OPINIONS

LANDMARKS: PRELIMINARY MANUSCRIPT

Peter W.H. Holland - University of Oxford, UK (* Lead author, drafted commentary)

Jane A. Langdale – University of Oxford, UK

Nipam H. Patel – Marine Biological Laboratory, Woods Hole; & University of Chicago

Graham N. Stone – University of Edinburgh

An evaluation of: A novel family of secreted insect proteins linked to plant gall development

Korgaonkar A, Han C, Lemire AL, Siwanowicz I, Bennouna D, Kopec RE, Andolfatto P, Shigenobu S, Stern DL. Curr Biol 2021;31:1836-1849.e12

Background:

When we think about the genetic control of growth and development in plants or animals, we naturally turn to the genome of the organism that is developing. But the development of one species can sometimes be controlled by the genes of a quite different species. Galls present an interesting example. Galls are unusual plant tissues whose development is induced by an interacting organism. Well-known galls include the root nodules of legumes induced by *Rhizobium* bacteria. The most complex galls are induced by animals, including certain nematodes, flies, wasps, aphids and mites, for which the gall structure provides protection and food for the larva (and sometimes adults) of the gall-inducing species. Importantly, galls are not amorphous growths but are intricately-patterned and reproducible structures with a shape, color and form specific to the animal, not specific to the host plant (one plant species can develop distinct gall structures, induced by different animals). The assumption is that galls represent what Richard Dawkins has termed the 'extended phenotypes' of gall-inducer genes acting in host plant tissues (1-2).

It has long been clear that insects and mites must use molecules to manipulate plant cell biology to drive the development of galls but until now the molecules responsible, and the genes encoding them, have not been identified. Many candidate genes have been proposed, based on transcriptomic and proteomic screens in gall-inducing animals, but linking particular animal genes to specific plant phenotypes has proved elusive (3-11). The genomes of a growing number of insects, including gall inducers, have been shown to encode genes for plant hormones including auxins and cytokinins, but how the products of these genes interact with plant tissues remains little understood (12). The report from Korgaonkar and colleagues (13) makes a leap forward in our understanding by identifying an insect gene that controls the color of galls formed on the leaves of the witch hazel shrub. Notably, the gene is a member of a novel gene family that may encode a suite of secreted proteins that are transferred from the insect into the leaf to manipulate plant development.

MAIN CONTRIBUTIONS AND IMPORTANCE

A powerful approach to finding genes responsible for any phenotype is to exploit naturally occurring polymorphisms within a species. Starting with this logic, the researchers from the laboratory of David Stern observed that 'cone galls' made by the aphid *Hormaphis cornu* on leaves of witch hazel *Hamamelis virginiana* are either red or green. These galls are formed by proliferation of plant cells at the site where female aphids founding a gall insert their piercing mouthparts, suggesting that the red or green galls are induced by chemicals in aphid saliva. The investigators sequenced and assembled a reference genome of the aphid *H. cornu*, then undertook lower coverage genome sequencing of 43 aphids that founded green galls and 47 aphids that founded red galls. A genome wide association study (GWAS) revealed several polymorphisms around a novel gene that correlated strongly with the color difference; this association was strengthened by genetic analysis of hundreds more samples (13). The clear implication is that this aphid gene controls the color of the developing plant structure.

The researchers named the novel gene *determinant of gall color* (*dgc*). Green galls are far more common than red galls. Consistent with this, closer analysis of polymorphisms indicated the 'green' allele is ancestral in this aphid species; furthermore, the 'red' allele is dominant to green. Working out how the aphid *dgc* gene controls plant gall color is not straightforward. The first clue comes from gene expression. The common 'green' allele of the *dgc* gene was found to be expressed in salivary glands, specifically in the generation and life cycle stage that induces galls. (The derived 'red' allele showed low expression and repressed the 'green' allele). The second clue comes from gene expression changes in the plant when a gall is induced. A striking finding is that high levels of *dgc* gene expression in the aphid represses transcription of plant genes encoding enzymes in the biosynthesis pathway for anthocyanins – compounds that include the red pigments found in many insect-induced galls (14,15); conversely low *dgc* expression permits abundant anthocyanin production and a red pigment is formed. These findings suggest that *dgc* encodes a secreted protein that is injected into the plant to suppress the anthocyanin biosynthesis pathway.

There is more to a gall than its color. The aphid *dgc* gene seems to influence color, so how could all the other features of galls be induced, such as the altered rates and planes of cell division that must be necessary to form a cone gall? This study does not address these questions directly, but it provides some tantalizing clues. Transcriptomic analysis of salivary glands, comparing gall-forming and non-gall-forming phases of the life cycle, revealed a large number of novel aphid genes putatively encoding secreted proteins specific to gall-inducing stages. Many of these genes share similar sequence characteristics, indicative of expansion of a gene family by gene duplication. Indeed, over 400 genes encode predicted proteins with a common structure: a diagnostic pair of cysteine-tyrosine-cysteine (CYC) motifs that prompted the authors to give these the appealing name of *bicycle* (*bi-CYC-like*) genes. The color determinant gene *dgc* is one of these genes. Furthermore, the *bicycle* genes show hallmarks of having been subject to positive Darwinian selection, compatible with adaptation or even an evolutionary arms race with plants. The suggestion is that *bicycle*

genes encode a vast suite of proteins that are injected into host plants to orchestrate gall formation.

OPEN QUESTIONS

The strategy used for tracking down the *dgc* gene was essentially a GWAS approach. GWAS has its pitfalls and in some research areas it is not always clear whether a genetic polymorphism correlating with a phenotypic trait is a causative variant or a spurious association, perhaps due to underlying genetic structure in the samples compared (16,17). In our view, this is unlikely to be a problem in the current study. The GWAS analyses in this study are carefully controlled and the subsequent, very extensive, follow-on work summarized above is internally consistent with the association proposed. The link between *dgc* and gall color seems sound.

It could also be argued that rigorous proof that *dgc* controls gall color requires a direct manipulative experiment. For example, since the 'green' allele has far higher expression than the 'red' allele, it might be possible to use CRISPR/Cas9 or RNAi approaches to disrupt *dgc* gene expression in aphids homozygous for the 'green' allele, and then test whether the gene-targeted aphids now induce red galls. A more straightforward alternative would be to compare the effect of the 'red' or 'green' allele in transient expression assays, for example in *Nicotiana benthamiana* leaves (18).

What is not addressed in the study is whether there is any adaptive significance of differences between green and red gall phenotypes for the gall inducer (14,15). Another question that remains puzzling is how the secreted dgc protein controls expression of plant genes. Does *dgc* encode a transcription factor or a co-factor that interacts with plant transcription factors? Does it influence transcriptional pathways more indirectly? Given that expression of eight genes in the anthocyanin pathway are suppressed, it is likely that the dgc protein targets a regulator of the pathway, possibly the MYB-bHLH complex (19). Although impact on the transcription of genes encoding this complex would be easy to assess, biochemical work would be necessary to identify perturbations to protein function.

But perhaps the most tantalizing questions concern the other *bicycle* genes, of which there are hundreds. Are all of these involved in aspects of gall formation? This seems unlikely, since subsequent work has identified some *bicycle* genes in non-gall-forming aphids (20). Another possibility is that *bicycle* genes, and other secreted salivary molecules, have long been used by insects to subtly interact with plant biochemistry during feeding, and then some of these genes were later co-opted for evolutionarily new roles in gall formation. These will be fascinating questions to address.

Conclusion

The *dgc* gene identified by the authors is part of a large multigene family of putatively secreted proteins in the salivary glands of aphids, and is a clear example of an insect gene that controls an aspect of plant development. A hypothesis emerges that there are a huge

number of similar signaling molecules that aphids inject into plants that then change all kinds of plant cellular behaviors. This study could be a major step forward in the field.

References

1. Dawkins, R (1982) The Extended Phenotype

2. Mayr, E. (1963) Animal species and evolution

3. Chen, M.S.; Zhao, H.X.; Zhu, Y.C.; Scheffler, B.; Liu, X.; Liu, X.; Hulbert, S.; Stuart, J.J. Analysis of transcripts and proteins expressed in the salivary glands of Hessian fly (Mayetiola destructor) larvae. J. Insect Physiol. 2008, 54, 1–16. [CrossRef]

4. Zhao, C.; Escalante, L.N.; Chen, H.; Benatti, T.R.; Qu, J.; Chellapilla, S.; Waterhouse, R.M.; Wheeler, D.; Andersson, M.N.; Bao, R.; et al. A massive expansion of effector genes underlies gall-formation in the wheat pest Mayetiola destructor. Curr. Biol. 2015, 25, 613–620. [CrossRef]

5. Yang, Z., Ma, L., Francis, F., Yang, Y., Chen, H., Wu, H., Chen, X., Proteins Identified from Saliva and Salivary Glands of the Chinese Gall Aphid Schlechtendalia chinensis Proteomics 2018, 18, 1700378. https://doi.org/10.1002/pmic.201700378

6. Zhuhong Wang, Jun-Qing Ge, Hang Chen, Xiaoyan Cheng, Yiqun Yang, Jun Li, R. Jeff Whitworth, Ming-Shun Chen, An insect nucleoside diphosphate kinase (NDK) functions as an effector protein in wheat - Hessian fly interactions, Insect Biochemistry and Molecular Biology, Volume 100, 2018, Pages 30-38, ISSN 0965-1748, https://doi.org/10.1016/j.ibmb.2018.06.003.

7. Eitle, M.W.; Carolan, J.C.; Griesser, M.; Forneck, A. The salivary gland proteome of root-galling grape phylloxera (Daktulosphaira vitifoliae Fitch) feeding on Vitis spp. PLoS ONE 2019, 14, e0225881. [CrossRef

8. Cambier, S.; Ginis, O.; Moreau, S.J.M.; Gayral, P.; Hearn, J.; Stone, G.N.; Giron, D.; Huguet, E.; Drezen, J.M. Gall wasp transcriptomes unravel potential effectors involved in molecular dialogues with oak and rose. Front. Physiol. 2019, 10, 926. [CrossRef] [PubMed]

9. Takeda, S.; Hirano, T.; Ohshima, I.; Sato, M.H. Recent Progress Regarding the Molecular Aspects of Insect Gall Formation. Int. J. Mol. Sci. 2021, 22, 9424. https://doi.org/10.3390/ijms22179424

10 Chromosome-level genome assembly for the horned-gall aphid provides insights into interactions between gall-making insect and its host plant. 2022. https://onlinelibrary.wiley.com/doi/pdf/10.1002/ece3.8815

11. Schultz, J.C., Edger, P.P., Body, M.J.A & Appel, H. M. (2018). A galling insect activates plant reproductive programs during gall development. Scientific Reports 9:1833. https://doi.org/10.1038/s41598-018-38475-6

12. Korgaonkar A, Han C, Lemire AL, Siwanowicz I, Bennouna D, Kopec RE, Andolfatto P, Shigenobu S, Stern DL. A novel family of secreted insect proteins linked to plant gall development. Curr Biol. 2021 May 10;31(9):1836-1849.e12. doi: 10.1016/j.cub.2021.01.104. Epub 2021 Mar 2. Erratum in: Curr Biol. 2021 May 10;31(9):2038. PMID: 33657407; PMCID: PMC8119383.

13. Tokuda, M., Suzuki, Y., Fujita, S., Matsuda, H., Adachi-Fukunaga., S. & Elsayed, A. K. (2022). Terrestrial arthropods broadly possess endogenous phytohormones auxin and cytokinins. Scientific Reports 12:4750. https://doi.org/10.1038/s41598-022-08558-6

14. Miyazaki,S., Kurisu, H., Nakata, M. & Saikawa, Y. (2020). Identification of a cyanidin-3-O-β-galactoside in gall tissue induced by midges on Japanese beech (Fagus crenata Blume). Bioscience, Biotechnology, and Biochemistry 84 (4), 797–799. https://doi.org/10.1080/09168451.2019.1698941

15. Bomfim PMS, Cardoso JCF, Rezende UC, et al. Red galls: the different stories of two gall types on the same host. Plant Biol. 2019;21:284–291.https://doi:10.1111/plb.12915

16. https://www.nature.com/articles/s41576-019-0127-1

17. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3750305/

18. doi: 10.3389/fpls.2014.00519

19. https://doi.org/10.1016/S1360-1385(98)01242-4

20. Stern DL, Han C. Gene structure-based homology search identifies highly divergent putative effector gene family. Genome Biol Evol. 2022 May 9:evac069. doi: 10.1093/gbe/evac069. Epub ahead of print. PMID: 35533072.

Red and green galls induced by *Hormaphis cornus* aphids on leaves of witch hazel *Hamamelis virginiana* at Janelia Research Campus, Ashburn, Virginia. Image copyright David L. Stern under a Creative Commons CC BY license