araGWAB: Network-based boosting of genome-wide association studies in Arabidopsis thaliana

Tak Lee & Insuk Lee

Genome-wide association studies (GWAS) have been applied for the genetic dissection of complex phenotypes in Arabidopsis thaliana. However, the significantly associated single-nucleotide polymorphisms (SNPs) could not explain all the phenotypic variations. A major reason for missing true phenotype-associated loci is the strict P-value threshold after adjustment for multiple hypothesis tests to reduce false positives. This statistical limitation can be partly overcome by increasing the sample size, but at a much higher cost. Alternatively, weak phenotype-association signals can be boosted by integrating other types of data. Here, we present a web application for network-based Arabidopsis genome-wide association boosting—araGWAB—which augments the likelihood of association with the given phenotype by integrating GWAS summary statistics (SNP P-values) and co-functional gene network information. The integration utilized the inherent values of SNPs with subthreshold significance, thus substantially increasing the information usage of GWAS data. We found that araGWAB could more effectively retrieve genes known to be associated with various phenotypes relevant to defense against bacterial pathogens, flowering time regulation, and organ development in A. thaliana. We also found that many of the network-boosted candidate genes for the phenotypes were supported by previous publications. The araGWAB is freely available at http://www.inetbio.org/aragwab/.

Genome-wide association studies (GWAS) have greatly altered the approach to studying complex phenotype genetics. GWAS have been utilized to map >50,000 unique single-nucleotide polymorphism (SNP)-phenotype associations in humans to date. GWAS have also been applied to study complex phenotypes in several animals and plants. As a reference plant, Arabidopsis thaliana is an ideal organism for GWAS because its inbreeding nature allows the preservation of the genotypic information of samples. Therefore, the genotypic information can be reused for association mapping for different phenotypes, enabling cost-effective GWAS. For example, the genotyping of 199 natural accessions using a custom Affymetrix 250 k SNP chip was applied to identify candidate genomic loci and genes associated with 107 distinct phenotypes. With sequencing-based genotyping, GWAS have been applied to various crop species as well.

Despite having an enormous impact on genetics in humans and plants, GWAS are still influenced by “missing heritability,” in which the identified phenotype-associated SNPs cannot explain all of the phenotypic variations. One major reason for missing true phenotype-associated genes is the very strict significance thresholds applied in GWAS to reduce false positives when testing the associations of numerous SNPs simultaneously. The strict P-value thresholds after adjustment for multiple hypothesis tests such as the Bonferroni correction generally allow only a handful of SNPs to be significant (Fig. 1A). Genetic variants associated with complex phenotypes are distributed across many genes in the phenotype-involved pathways, which results in genetic heterogeneity, where phenotype-associated variants occur in only a subset of a population for the phenotype, consequently reducing the statistical power of the association between a single variant and the phenotype. Presumably, this statistical limitation may be overcome somewhat by increasing the population size; but at a much higher cost. The pathway nature of complex phenotypes also provides an opportunity for augmenting GWAS by integrating phenotype-association data via the co-functional gene network, which maps the functional couplings between genes. To rescue highly probable candidates with subthreshold significance by GWAS alone, a method involving network-based boosting of GWAS signals has been proposed, with a companion web application developed for humans.

Department of Biotechnology, College of Life Sciences and Biotechnology, Yonsei University, Seoul, 03722, Korea. Correspondence and requests for materials should be addressed to I.L. (email: insuklee@yonsei.ac.kr)
Because the numbers of GWAS in *A. thaliana* and crop species continue to grow, GWAS augmentation would facilitate the study of complex phenotypes in plants. Here, we present a web application for the network-based *Arabidopsis* genome-wide association boosting, araGWAB (http://www.inetbio.org/aragwab/), which augments the likelihood of association with the given phenotype by integrating GWAS summary statistics (SNP P-values) and co-functional gene network information. We found that araGWAB could more effectively retrieve genes known to be associated with defense against bacterial pathogens, flowering time regulation, and organ development in *A. thaliana*. Many of the network-boosted candidate genes were also supported in the literature.

**Methods**

**Overview of the network-based boosting of GWAS data.** Many previous studies have shown that the genes for the same complex phenotypes tend to be connected in co-functional gene networks. Therefore, we hypothesized that genes that have subthreshold GWAS significances but that functionally connect to significant GWAS candidate genes are also likely to be associated with the phenotype. To use the gene-centric significance information, araGWAB first allocates SNP P-values to genes based on chromosomal proximity (Fig. 1B), by assigning the best P-value within the user-defined distance from the beginning or end of the gene.

The araGWAB boosts the original GWAS signals using “soft” guilt-by-association (GBA) (Fig. 1C–D) with a co-functional gene network of genes in *A. thaliana*. We implemented a soft GBA using 

$$
S_i = \sum_j (2p_j - 1) l_{ij}
$$

where, $l_{ij}$ is the weight of the link that connects genes $i$ and $j$. The soft GBA only sums those $j$ when $2p_j - 1 > 0$. Thus, only genes that are very strongly associated with the phenotype provided full weights during the GBA. Assuming that the network and GWAS data are conditionally independent, they can be integrated by a naïve Bayesian framework. Then, the posterior log odds (the final araGWAB score) that gene $i$ was associated with the phenotype for the given network data ($D_{net}$) and GWAS data ($D_{GWAS}$) was calculated as follows:
P identify the optimal P repeats the whole network boosting process for 100 randomized networks with the same parameter settings. To
to genes, integrating GW AS data and network data, and assessing the boosting efficiency for the given
P provides a summary graph that presents the AUC (5% FPR) scores calculated by AraNet and randomized networks
across the range of log10(P) thresholds and other input parameters used for the given GW AS boosting. AUC (<5% FPR) scores that surpass the deviations of randomized networks indicate a significant GWAS boosting. Users can then download reprioritized genes with the final araGWAB scores for the optimal P-value threshold.

**GWAS data, co-functional gene network, and reference phenotype-associated genes.** We
developed the araGWAB by analyzing the GWAS data from a study of 107 phenotypes3. Because the P-values for all SNPs were not available for the 107 GWAS datasets, we calculated them using Efficient Mixed-Model Association software11. Mixed models are known to over-represent the phenotype associations for SNPs with a
for all SNPs were not available for the 107 GWAS datasets, we calculated them using Efficient Mixed-Model
minor allele frequency ≤0.1. To avoid this over-representation, we used only the 178,623 SNPs that had a minor allele frequency >0.1 to calculate the P-values. The effectiveness of network boosting is significantly influenced by the quality of the co-functional gene network. For network boosting, we applied the latest version of AraNet (version 2)12, which is known as the most accurate and comprehensive co-functional gene network of genes for A. thaliana.

To assess the performance of network boosting, we utilized reference phenotype-associated gene sets for each of the phenotypes. We generated reference phenotype-associated gene sets by compiling Gene Ontology biological process (GOBP)13 terms that were relevant to each phenotype. Because we also utilized GOBP information to train the co-functional gene network for boosting (AraNet), there could be a circularity in the assessment of the boosting effect. To evaluate the boosting effect in a highly conservative manner, we excluded the GOBP genes that were previously utilized for the training of AraNet. We also removed GOBP genes annotated by evidence of low reliability such as ND (No biological data available) and NAS (Non-traceable Author Statement). Finally, we could generate reference phenotype-associated gene sets for only 64 of the 107 phenotypes.

**Web server implementation.** The araGWAB server has a front-end system that provides a user interface and a back-end system that performs data preprocessing and network boosting. To conduct network boosting for a GWAS, users need to submit GWAS summary statistics (P-values) for all tested loci and a set of reference phenotype-associated genes. In addition to the input data, several parameters need to be chosen by users. Genotyping of A. thaliana natural accessions have been conducted based on various genome builds (TAIR714, TAIR814, and TAIR1015). Thus, users need to choose the correct version of the genome build for the given GWAS data. Users can also choose a range for the chromosomal distance between SNPs and genes (10 kb by default) for assigning P-values to the genes. Using the given input data, araGWAB sequentially performs assigning P-values to genes, integrating GWAS data and network data, and assessing the boosting efficiency for the given P-value threshold.

Boosting efficiency is assessed for the user-input reference phenotype-associated genes by the AUC (<5% FPR) score. To measure the significance of the observed boosting efficiency for the given network, araGWAB repeats the whole network boosting process for 100 randomized networks with the same parameter settings. To identify the optimal P-value threshold for boosting a given GWAS, araGWAB repeats the analysis over various P-value thresholds within a given range (−6 < log10(P) < −2 by default) with a set interval (0.3 by default). The P-value that maximizes the AUC (<5% FPR) score is selected as the optimal threshold. Finally, araGWAB provides a summary graph that presents the AUC (<5% FPR) scores calculated by AraNet and randomized networks across the range of log10(P) thresholds and other input parameters used for the given GWAS boosting. AUC (<5% FPR) scores that surpass the deviations of randomized networks indicate a significant GWAS boosting. Users can then download reprioritized genes with the final araGWAB scores for the optimal P-value threshold.

Table 1. Nine phenotypes that were efficiently boosted by araGWAB.

| Phenotype                       | Description                                                                 |
|---------------------------------|-----------------------------------------------------------------------------|
| As2CFU2                         | In planta bacterial growth (number of CFU/leaf area) of the P. viridiflava strains were individually measured |
| At1CFU2                         | In planta bacterial growth (number of CFU/leaf area) of the P. viridiflava strains were individually measured |
| Bacterial titer                 | Bacterial titer of Pseudomonas syringae pv. tomato DC3000                   |
| FLC                             | FLC gene expression level                                                  |
| FRI                             | FRI gene expression level                                                  |
| LD                              | Days to flowering time under 16 h daylight, 18°C                           |
| LDV                             | Days to flowering time under 16 h daylight, 18°C, vernalized (5 wks, 4°C)   |
| Leafser10                       | Level of leaf serration of plants grown in 10°C                            |
| Trichomeavg JA                  | Trichome density measured after jasmonic acid treatment                    |

\[
\log O(i \in D|D_{GWAS}) = S_i + \log O(i \in D|D_{GWAS})
\]

where, logO(i \in D|D_{GWAS}) is the log odds of association with the phenotype obtained from the GWAS data.

The shortcoming of network-based boosting is that the final araGWAB score for hub genes that connect to many other genes with low significance can be greatly boosted, potentially resulting in false positives. To reduce this type of artifact, araGWAB uses a P-value threshold to restrict the genes that contribute to the boosting process. For a given P-value threshold, araGWAB evaluates prediction quality for the given phenotype by calculating the retrieval rate of “reference phenotype-associated genes,” which are known to be involved in the phenotype (positives) and the other genes (negatives), resulting in receiver operating characteristic (ROC) curves. We utilized the area under the ROC curve (AUC) as a function of prediction quality for the given P-value threshold. Because only high-ranked candidates were considered for follow-up studies in general, we determined the prediction quality based on AUC before the 5% false-positive rate (AUC (<5% FPR)). For each phenotype, predictions were made by the optimal P-value threshold that achieved the maximum AUC (<5% FPR) score.
Results

We conducted GWAS boosting for 64 of the 107 phenotypes for which we could compile appropriate reference phenotype-associated genes from GOBP annotations. Among the analyzed 64 phenotypes, GWAS signals for 9 phenotypes (Table 1) involved in the defense against bacterial pathogens (As2CFU2, At1CFU2, and Bacterial titer; Fig. 2A–C), flowering time regulation (Flowering Locus C [FLC], FRI, LD, and LDV; Fig. 2D–G), and organ development (Leafserr10 and Trichomeavg JA; Fig. 2H–I) in A. thaliana were effectively boosted by araGWAB. Network boosting for all other phenotypes showed no significant improvement in retrieving reference phenotype-associated genes, indicated as AUC (<5% FPR) scores by araGWAB stayed within two standard deviations of 100 randomized network boosting for the entire range of the log10(P)-value threshold. In all nine phenotypes, network boosting retrieved reference phenotype-associated genes most efficiently (indicated by highest AUC (<5% FPR) score by araGWAB) using only SNPs that passed the optimal log10(P) threshold: −3 for As2CFU2, −3.6 for At1CFU2, −3.6 for Bacterial titer, −2.4 for FLC, −2.1 for FRI, −3.9 for LD, −4.2 for LDV, −3.3 for Leafserr10, and −3.9 for Trichomeavg JA. The araGWAB server returned reprioritized candidate genes for each phenotype by network boosting with the optimal P-value threshold. Pre-calculated results of network boosting for the nine phenotypes are available from the web site.

Next, we sought for supporting evidence from the literature for the top 20 candidate genes (excluding reference phenotype-associated genes) by network boosting for each of the 9 phenotypes. For all 9 phenotypes, a total of 40 network-boosted candidates (40/180 = 22.2%) were supported by direct evidence (e.g., mutant phenotype assay) or indirect evidence (e.g., expression analysis and protein-protein interactions) in the literature (Supplemental Table S1). For example, among the top 20 network-boosted candidates for "the days to flowering..."
time under 16 h daylight, 18 °C” (LD) phenotype, six genes were supported by evidence in the literature (Fig. 3, nodes with red borderlines). We found that only two of the genes (AT5G10140 and AT1G22770) could be identified with the original GWAS signal alone. In fact, AT5G10140 is a well-known transcription factor involved in flowering time regulation, i.e., FLC. We predicted FLC to be a new candidate gene because it was used for training AraNet, and thus was excluded from the reference phenotype-associated genes. Nevertheless, this also demonstrated that araGWAS effectively retrieved known phenotype-associated genes. As expected, four other literature-supported candidate genes (AT1G27360, AT3G28730, AT3G09270, and AT5G15020) were largely boosted by network integration, as indicated by the large size of the nodes in the network.

Discussion
Network-based boosting of GWAS signals provides advantages for the discovery of phenotype-associated genes. First, it can effectively integrate information from population-based and molecular profiling studies. The complementarity of these data was demonstrated by the fact that many phenotype-associated Arabidopsis genes were retrieved not by GWAS alone but by network-based boosting. Second, the integration enables utilization of the inherent value of SNPs with subthreshold significance, thus substantially increasing the information usage of GWAS data. Currently, the majority of the published GWAS for plants do not provide summary statistics for all SNPs. As clearly demonstrated in the present study, sharing the entire summary statistics data will potentiate GWAS for the genetic dissection of complex phenotypes in plants.

We obtained different optimal P-value thresholds for the best efficiency of network boosting in different GWAS. We reasoned that several factors affected the optimal P-value thresholds for the given GWAS. First, as mentioned above, hub genes that have many connected genes in the network are highly likely to be boosted by the network. If the given phenotype has many reference phenotype-associated genes that are network hubs, accounting for only SNPs with relatively high significance (i.e., SNPs with relatively low P-values) may retrieve many true positives by network boosting while minimizing the chances of introducing false positives. Second, phenotypes differ in their degree of genetic heterogeneity. If many genes with small effects contribute to the phenotype, including SNPs with low significance (i.e., SNPs with relatively high P-values) might improve the network boosting. Third, the quality of GWAS data varies. High-quality GWAS data may allow the use of SNPs with relatively low significance for network boosting with minimal probability of noise introduction.

Given that only 9 of the 64 analyzed phenotypes were boosted significantly, the current araGWAS requires improvement. The boosting effect relies on the original GWAS signals. Unless there are many genes significantly associated with the phenotype in the original GWAS, we cannot expect a considerable boosting effect by GBA. This issue can be partially resolved using restructured population and regional sampling in plant GWAS. The quality of co-functional gene networks also influences boosting efficiency. Although AraNet is one of the most comprehensive networks of Arabidopsis genes (covers >84% of the coding genome), it still falls short in the complete reconstruction of biological processes. We might be able to boost more phenotypes by improving the co-functional gene network of *A. thaliana* in the future.

Since high-quality co-functional gene networks are available for non-model crop species, we will be able to apply the same strategy of network-based boosting for GWAS on phenotypes of economic interest in crops. However, the majority of GWAS in crop species have not released raw genotype and phenotype data to the public to date. Therefore, we highly recommend reporting summary statistics data of GWAS in crop species for follow-up research.

Figure 3. A network of candidate genes by araGWAS for the LD phenotype. Only the genes with an araGWAS score > 5.25 are shown. The intensity of the node color represents the significance by the original GWAS and node size represents the degree of network boosting by araGWAS. The nodes with red borderlines are candidate genes with literature support.
References
1. MacArthur, J. et al. The new NHGRI-EBI Catalog of published genome-wide association studies (GWAS Catalog). *Nucleic acids research* **45**, D896–D901, https://doi.org/10.1093/nar/gkw1133 (2017).
2. Korte, A. & Farlow, A. The advantages and limitations of trait analysis with GWAS: a review. *Plant Methods* **9**, 29, https://doi.org/10.1186/1746-4811-9-29 (2013).
3. Atwell, S. et al. Genome-wide association study of 107 phenotypes in Arabidopsis thaliana inbred lines. *Nature* **465**, 627–631, https://doi.org/10.1038/nature08800 (2010).
4. Huang, X. & Han, B. Natural variations and genome-wide association studies in crop plants. *Annu Rev Plant Biol* **65**, 531–551, https://doi.org/10.1146/annurev-plant-052013-035715 (2014).
5. Manolio, T. A. et al. Finding the missing heritability of complex diseases. *Nature* **461**, 747–753, https://doi.org/10.1038/nature08494 (2009).
6. McLellan, J. & King, M. C. Genetic heterogeneity in human disease. *Cell* **141**, 210–217, https://doi.org/10.1016/j.cell.2010.03.032 (2010).
7. Lee, I., Blom, U. M., Wang, P. I., Shim, J. E. & Marcotte, E. M. Prioritizing candidate disease genes by network-based boosting of genome-wide association data. *Genome research* **21**, 1109–1121, https://doi.org/10.1101/gr.118992.110 (2011).
8. Shim, J. E. et al. GWAR: a web server for the network-based boosting of human genome-wide association data. *Nucleic acids research* **45**, W154–161, https://doi.org/10.1093/nar/gkx284 (2017).
9. Shim, J. E., Lee, T. & Lee, I. From sequencing data to gene functions: co-functional gene network approaches. *Anim Cells Syst* **21**, 77–83, https://doi.org/10.1090/19783534.2017.1284156 (2017).
10. Lee, T. & Lee, I. AraNet: A Network Biology Server for Arabidopsis thaliana and Other Non-Model Plant Species. *Methods in molecular biology* **1629**, 225–238, https://doi.org/10.1007/978-1-4939-7125-1_15 (2017).
11. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **23**, 1719–1720, https://doi.org/10.1093/bioinformatics/btm170 (2007).
12. Gene Ontology, C. Gene Ontology Consortium: going forward. *Nucleic acids research* **41**, D372–D381, https://doi.org/10.1093/nar/gkr1053 (2013).
13. Gene Ontology, C. Gene Ontology Consortium: going forward. *Nucleic acids research* **41**, D1049–1056, https://doi.org/10.1093/nar/gku1179 (2015).
14. Kim, S. et al. Recombination and linkage disequilibrium in Arabidopsis thaliana. *Nat Genet* **39**, 1151–1155, https://doi.org/10.1038/ng1115 (2007).
15. Lamesch, P. et al. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. *Nucleic acids research* **40**, D1202–1210, https://doi.org/10.1093/nar/gkr1090 (2012).
16. Michaels, S. D. & Amasino, R. M. FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. *The Plant cell* **11**, 949–956 (1999).
17. Mizoguchi, T. et al. Distinct roles of GIGANTEA in promoting flowering and regulating circadian rhythms in Arabidopsis. *The Plant cell* **17**, 2225–2270, https://doi.org/10.1101/tpc.033464 (2005).
18. Cao, D., Cheng, H., Wu, W., Soo, H. M. & Peng, J. Gibberellin mobilizes distinct DELLA-dependent transcriptional regulators to seed germination and floral development in Arabidopsis. *Plant physiology* **142**, 509–525, https://doi.org/10.1104/pp.106.082889 (2006).
19. Schmid, M. et al. Dissection of floral induction pathways using global expression analysis. *Development* **130**, 6001–6012, https://doi.org/10.1242/dev.008842 (2003).
20. Van Lijsebettens, M. & Grasser, K. D. The role of the transcript elongation factors FACT and HUB1 in leaf growth and the induction of flowering. *Plant signaling & behavior* **5**, 713–717 (2010).
21. Gu, X., Wang, Y. & He, Y. Photoperiodic regulation of flowering time through periodic histone deacetylation of the florigen gene FT. *PLoS Biology* **11**, e1001649, https://doi.org/10.1371/journal.pbio.1001649 (2013).
22. Brach, B., Morris, G. P. & Borevitz, J. O. Genome-wide association studies in plants: the missing heritability is in the field. *Genome Biol* **12**, 232, https://doi.org/10.1186/gb-2011-12-10-232 (2011).
23. Lee, T. et al. RiceNetv2: an improved network prioritization server for rice genes. *Nucleic acids research* **43**, W122–127, https://doi.org/10.1093/nar/gkw233 (2015).
24. Korn, H. et al. TomatoNet: A Genome-wide Co-functional gene Network for Unveiling Complex Traits of Tomato, a Model Crop for Fleshy Fruits. *Molecular plant* **10**, 652–655, https://doi.org/10.1093/mp/mtv063 (2017).
25. Lee, T. et al. WheatNet: a Genome-Canvas Functional Network for Hexaploid Bread Wheat, Triticum aestivum. *Molecular plant* **10**, 1133–1136, https://doi.org/10.1093/mp/mtv063 (2017).
26. Lee, T., Kim, H. & Lee, I. Network-assisted crop systems genetics: network inference and integrative analysis. *Current opinion in plant biology* **24**, 61–70, https://doi.org/10.1016/j.pbi.2015.02.001 (2015).

Author Contributions
T.L. and I.L. conceived the project. T.L. developed software under supervision of I.L. T.L. and I.L. wrote the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-21301-4.

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.