Defining the functional significance of intergenic transcribed regions based on heterogeneous features of phenotype genes and pseudogenes

John P. Lloyd[1], Zing Tsung-Yeh Tsai[2], Rosalie P. Sowers[3], Nicholas L. Panchy[4], Shin-Han Shiu[1,4,5*]

[1] Department of Plant Biology, Michigan State University, East Lansing, MI 48824, USA

[2] Department of Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI 48109, USA

[3] Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802, USA

[4] Genetics Program, Michigan State University, East Lansing, MI 48824, USA

[5] Ecology, Evolutionary Biology, and Behavior Program, Michigan State University, East Lansing, MI 48824, USA

* To whom correspondence should be addressed.

Correspondence to:

Shin-Han Shiu
Michigan State University
E-mail: shius@msu.edu
Telephone number: 517-353-7196

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ABSTRACT

With advances in transcript profiling, the presence of transcriptional activities in intergenic regions has been well established in multiple model systems. However, whether intergenic expression reflects transcriptional noise or the activity of novel genes remains unclear. We identified intergenic transcribed regions (ITRs) in 15 diverse flowering plant species and found that the amount of intergenic expression correlates with genome size, a pattern that could be expected if intergenic expression is largely nonfunctional. To further assess the functionality of ITRs, we first built machine learning classifiers using *Arabidopsis thaliana* as a model that can accurately distinguish functional sequences (phenotype genes) and nonfunctional ones (pseudogenes and unexpressed intergenic regions) by integrating 93 biochemical, evolutionary, and sequence-structure features. Next, by applying the models genome-wide, we found that 4,427 ITRs (38%) and 796 annotated ncRNAs (44%) had features significantly similar to benchmark protein-coding or RNA genes and thus were likely parts of functional genes. However, ~60% of ITRs and ncRNAs were more similar to nonfunctional sequences and should be considered transcriptional noise unless falsified with experiments. The predictive framework established here provides not only a comprehensive look at how functional, genic sequences are distinct from likely nonfunctional ones, but also a new way to differentiate novel genes from genomic regions with noisy transcriptional activities.

INTRODUCTION

Advances in sequencing technology have helped to identify pervasive transcription in intergenic regions with no annotated genes. These intergenic transcripts have been found in metazoa and fungi, including *Homo sapiens* (human; ENCODE Project Consortium 2012), *Drosophila melanogaster* (Brown et al. 2014), *Caenorhabditis elegans* (Boeck et al. 2016), and *Saccharomyces cerevisiae* (Nagalakshmi et al. 2008). In plants, ~7,000 and ~15,000 intergenic transcripts have also been reported in *Arabidopsis thaliana* (Yamada et al. 2003; Stolc et al. 2005; Moghe et al. 2013; Krishnakumar et al. 2015) and *Oryza sativa* (Nobuta et al. 2007), respectively. The presence of intergenic transcripts indicates that there may be additional genes in genomes that have escaped gene finding efforts thus far. Knowledge of the complete suite of functional elements present in a genome is an important goal for large-scale functional genomics efforts and the quest to connect genotype to phenotype. Thus the identification of functional intergenic transcribed regions (ITRs) represents a fundamental task that is critical to our understanding of the gene space in a genome.

Loss-of-function study represents the gold standard by which the functional significance of genomic regions, including ITRs, can be confirmed (Niu and Jiang 2013). In *Mus musculus* (mouse), at least 25 ITRs with loss-of-function mutant phenotypes have been identified (Sauvageau et al. 2013; Lai et al. 2015), indicating that they are *bona fide* genes. In addition, loss-of-function mutants have been used to confirm ITR functionality in mouse embryonic stem cell proliferation (Ivanova et al. 2006; Guttman et al. 2009) and male reproductive development (Heinen et al. 2009), as well as brain and eye development in *Danio rerio* (Ulitsky et al. 2011). In human, 162 long intergenic non-coding RNAs harbor phenotype-associated SNPs, suggesting that these expressed intergenic regions may be functional (Ning et al. 2013). In addition to intergenic expression, most model organisms feature an abundance of annotated non-coding RNA (ncRNA) sequences (Zhao et al. 2016), which are mostly identified through the presence of expression occurring outside of annotated genes. Thus, the only difference between ITRs and most ncRNA sequences is whether or not they have been annotated. Similar to the ITR examples above, a small number of ncRNAs have been confirmed as functional through loss-of-function experimental characterization, including but not limited to *Xist* in mouse (Penny et al. 1996; Marahrens et al. 1997), *Malat1* in human (Bernard et al. 2010), *bereft* in *D. melanogaster* (Hardiman et al. 2002), and *At4* in *A. thaliana* (Shin et al. 2006). However, despite the presence of a few notable examples, the number of ITRs and ncRNAs with well-established functions is dwarfed by those with no known function.

While some ITRs and ncRNAs are likely novel genes, intergenic transcription may also be the byproduct of noisy expression that can occur due to nonspecific landing of RNA Polymerase II (RNA Pol II) or spurious regulatory signals that drive expression in random genomic regions (Struhl 2007). Thus, whether an intergenic transcript is considered functional cannot depend solely on the fact that it is expressed. In addition to being biochemically active, the genomic region must be under selection. This line of logic has revived the classical ideas on differentiating “causal role” and “selected effect” functionality (Doolittle et al. 2014). A “causal role” definition requires a definable activity to consider a genomic region as functional (Cummins 1975; Amundson and Lauder 1994), which is adopted by the ENCODE Consortium (2012) to classify ~80% of the human genome as having biochemical functions. This finding has been used as evidence to disprove the presence of junk DNA that is not under natural selection (see Eddy 2013). This has drawn considerable critique because biochemical activity itself is not an indication of selection (Graur et al., 2013; Niu and Jiang, 2013). Instead, selected effect functionality is advocated to be a more suitable definition for a genomic region with discernible activity (Amundson and Lauder 1994; Graur et al., 2013; Doolittle et al. 2014). Under the selected effect functionality definition, ITRs and most annotated ncRNA genes remain functionally ambiguous.

Functional ITRs represent genic sequences that have not been identified with conventional gene finding programs. Such programs incorporate sequence characteristics, transcriptional evidence, and conservation information to define genic regions that are expected to be functional. Thus, genes that lack the features typically associated with genic regions remain unidentified. Due to the debate on the definitions of function post-ENCODE, Kellis et al. (2014) suggested that evolutionary, biochemical, and genetic evidences provide complementary information to define functional genomic regions. Integration of chromatin accessibility, transcriptome, and conservation evidence was successful in identifying regions in the human genome that are under selection (Gulko et al. 2014). Moreover, a comprehensive integration of biochemical, evolutionary, and genetic evidence resulted in highly accurate identification of human disease genes and pseudogenes (Tsai et al. 2017). However, it is not known if such predictions are possible or if the features that define functional genomic regions in human are applicable in other species. In plants, even though many biochemical signatures are known to be associated with genic regions, these signatures have not been incorporated to assist in identifying the functional genomic regions.

To investigate the functionality of intergenic transcription, we first identified ITRs in 15 flowering plant species with 17-fold genome size differences and evaluated the relationship between the prevalence of intergenic expression and genome size. Next, we determined whether 93 evolutionary, biochemical, and sequence-structure features could distinguish functional sequences (phenotype genes) and nonfunctional ones (pseudogenes) using *A. thaliana* as a model. We then jointly considered all 93 features to establish functional gene prediction models using machine learning methods. Finally, we applied the models to ITRs and annotated ncRNAs to determine whether these functionally ambiguous sequences are more similar to known functional or likely nonfunctional sequences.

RESULTS & DISCUSSION

Relationship between genome size and intergenic expression indicates that intergenic transcripts may generally be nonfunctional

Transcription of an unannotated, intergenic region could be due to nonfunctional transcriptional noise or the activity of a novel gene. If noisy transcription occurs due to random landing of RNA Pol II or spurious regulatory signals, a naïve expectation is that, as genome size increases, the amount of intergenic expression would increase accordingly. By contrast, we expect that the extent of expression for genic sequences will not be significantly correlated with genome size because larger plant genomes do not necessarily have more genes (r^2=0.01; p=0.56; see Methods). Thus, to gauge if intergenic transcribed regions (ITRs) generally behave more like what we expect of noisy or genic transcription, we assessed the correlation between genome size and the amount of intergenic expression occurring within 15 flowering plant species.

We first identified genic and intergenic transcribed regions using leaf transcriptome data from 15 flowering plants with 17-fold differences in genome size (Supplemental Table 1). Identical numbers of RNA-sequencing (RNA-seq) reads (30 million) and the same mapping procedures were used in all species to facilitate cross-species comparisons (see Methods). Transcribed regions were considered as ITRs if they did not overlap with any gene annotation and had no significant translated sequence similarity to plant protein sequences. As expected, the amount of expression originating from annotated genic regions had no significant correlation with genomes size (r^2=0.03; p=0.53; **Fig. 1A**). In contrast, the amount of intergenic expression occurring was significantly and positively correlated (r^2=0.30; p=0.04; **Fig. 1B**). Because more intergenic expression is occurring in species with more genome space, this is consistent with the interpretation that a significant proportion of intergenic expression represents transcriptional noise. However, the correlation between genome size and intergenic expression explained ~30% of the variation (**Fig. 1B**), suggesting that other factors also affect ITR content, including the possibility that some ITRs are truly functional, novel genes. To further evaluate the functionality of intergenic transcripts, we next identified the biochemical and evolutionary features of functional genic regions and tested whether intergenic transcripts in *A. thaliana* were more similar to functional or nonfunctional sequences.

Expression, conservation, and epigenetic features are significantly distinct between benchmark functional and nonfunctional genomic sequences

To determine whether intergenic transcripts resemble functional sequences, we first asked what features allow benchmark functional and nonfunctional genomic regions to be distinguished. For benchmark functional sequences, we used genes with visible loss-of-function phenotypes when mutated (referred to as phenotype genes, n=1,876; see Methods). These phenotype genes were considered functional based on the selected effect functionality criterion (Neander 1991) because their mutations have significant growth and/or developmental impact and likely contribute to reduced fitness. For benchmark nonfunctional genomic regions, we utilized pseudogene sequences (n=761; see Methods). These pseudogenes exhibit sequence similarity to known genes, but harbor disabling mutations, including frame shifts and/or in-frame stop codons, that result in the production of presumably nonfunctional protein products. Considering that only 2% of pseudogenes are maintained over 90 million years of divergence between human and mouse (Svensson et al. 2006), it is expected that the majority of pseudogenes are no longer under selection (Li et al. 1981).

We evaluated 93 gene or gene product features for their ability to distinguish between phenotype genes and pseudogenes. These features were grouped into seven categories, including chromatin accessibility, DNA methylation, histone 3 (H3) marks, sequence conservation, sequence-structure, transcription factor (TF) binding, and transcription activity. Feature values

(Supplemental Table 2) were calculated for a randomly-selected 500 base pair (bp) window inside a phenotype gene or pseudogene. We used Area Under the Curve - Receiver Operating Characteristic (AUC-ROC) as a metric to measure how well a feature distinguishes between phenotype genes and pseudogenes. AUC-ROC values range between 0.5 (random guessing) and 1 (perfect separation of functional and nonfunctional sequences), with AUC-ROC values of 0.7, 0.8, and 0.9 considered fair, good, and excellent performance, respectively. Among the seven feature categories, transcription activity features were highly informative (median AUC-ROC=0.88; **Fig. 2A**). Sequence conservation, DNA methylation, TF binding, and H3 mark features were also fairly distinct between phenotype genes and pseudogenes (median AUC-ROC ~ 0.7 for each category; **Fig. 2B-E**). By contrast, chromatin accessibility and sequence-structure features were largely uninformative (median AUC-ROC=0.51 and 0.55, respectively; **Fig. 2F,G**). The poor performance of chromatin accessibility features is likely because the DNase I hypersensitive site (DHS) datasets were sparse, as only 2-6% of phenotype gene and pseudogene sequences overlapped a DHS peak. Further, median nucleosome occupancy of phenotype genes (median normalized nucleosome occupancy = 1.22) is only slightly lower than that of pseudogenes (median = 1.31; Mann Whitney U test, $p < 2e-4$). For sequence-structure features based on dinucleotide structures (see Methods), we found that poor performance was likely due to phenotype genes and pseudogenes sharing similar dinucleotide sequence compositions (r^2=0.99, p<3e-16).

Error rates for functional region predictions are high when only single features are considered

Within each feature category, there was a wide range of performance between features (**Fig. 2**, Supplemental Table 3) and there were clear biological or technical explanations for features that perform poorly. For the transcription activity category, 17 out of 24 features had an AUC-ROC performance >0.8, including the best-performing feature, expression breadth (AUC-ROC=0.95; **Fig. 2A**). However, five transcription activity-related features performed poorly (AUC-ROC<0.65), including the presence of expression (transcript) evidence (AUC-ROC=0.58; **Fig. 2A**). This is because 80% of pseudogenes were considered expressed in ≥1 of 51 RNA-seq datasets, demonstrating that presence of transcripts should not be used by itself as evidence of functionality. For the sequence conservation category, maximum and average phastCons conservation scores were highly distinct between phenotype genes and pseudogenes (AUC-ROC=0.83 and 0.82, respectively; **Fig. 2B**). On the other hand, identity to best matching nucleotide sequences found in *Brassicaceae* and algal species were not informative (AUC-ROC=0.55 and 0.51, respectively; **Fig. 2B**). This was because 99.8% and 95% of phenotype genes and pseudogenes, respectively, had a potentially homologous sequence within the *Brassicaceae* family, and only 3% and 1%, respectively, in algal species. Thus, *Brassicaceae* genomes were too similar and algal genomes too dissimilar to *A. thaliana* to provide meaningful information. H3 mark features also displayed high variability. The most informative H3 mark features were based on the number and coverage of activation-related marks (AUC-ROC=0.87 and 0.85, respectively; **Fig. 2E**), consistent with the notion that histone marks are often jointly associated with active genomic sequences to provide a robust regulatory signal (Schreiber and Bernstein 2002; Wang et al. 2008). By comparison, the coverage and intensity of H3 lysine 27 trimethylation (H3K27me3) and H3 threonine 3 phosphorylation (H3T3ph) were largely indistinct between phenotype genes and pseudogenes (AUC-ROC range: 0.55-0.59; **Fig. 2E**).

Despite this high variability in performance, some features and feature categories had high AUC-ROCs, suggesting that these features may individually provide sufficient information for distinguishing between functional and nonfunctional genomic regions. To assess this possibility, we next evaluated the error rates of function predictions based on single features. We first considered expression breadth of a sequence, the best predicting feature of functionality. Despite high AUC-ROC (0.95), the false positive rate (FPR; % of pseudogenes predicted as phenotype genes) was 21% when only expression breadth was used, while the false negative rate (FNR; % of phenotype genes predicted as pseudogenes) was 4%. Similarly, the best-performing H3 mark- and sequence conservation-related features had FPRs of 26% and 32%, respectively, and also incorrectly classified at least 10% of phenotype genes as pseudogenes. Thus, error rates are high even when considering well-performing single features, indicating the need to jointly consider multiple features for distinguishing phenotype genes and pseudogenes.

Consideration of multiple features in combination produces accurate predictions of functional genomic regions

To consider multiple features in combination, we first conducted principle component (PC) analysis to investigate how well phenotype genes and pseudogenes could be separated. Between the first two PCs, which jointly explain 40% of the variance in the feature dataset, phenotype genes (**Fig. 3A**) and pseudogenes (**Fig. 3B**) were distributed in largely distinct space. However, there remains substantial overlap, indicating that standard parametric approaches are not well suited to distinguishing between benchmark functional and nonfunctional sequences. Thus, we instead considered all 93 features for phenotype gene and pseudogenes in combination using random forest (referred to as the full model; see Methods). The phenotype gene and pseudogene sequences and associated conservation, biochemical, and sequence-structure features were separated into distinct training and testing sets and the full model was generated and validated using independent data subsets (cross-validation). The full model provided more accurate predictions (AUC-ROC=0.98; FNR=4%; FPR=10%; **Fig. 3C**) than any individual feature (**Fig. 2**; Supplemental Table 3). An alternative measure of performance based on the precision (proportion of predicted functional sequences that are truly functional) and recall (proportion of truly functional sequences predicted correctly) values among predictions generated by the full model also indicated that the model was performing well (**Fig. 3D**). When compared to the best-performing single feature (expression breadth), the full model had a similar FNR but half the FPR (10% compared to 21%). Thus, the full model is highly capable of distinguishing between phenotype genes and pseudogenes.

We next determined the relative contributions of different feature categories in predicting phenotype genes and pseudogenes and whether models based on a subset of features would perform similarly as the full model. Seven prediction models were established, each using only the subset of features from a single category (**Fig. 2**). Although none of these category-specific models had performance as high as the full model, the models based on transcription activity, sequence conservation, and H3 mark features scored highly (AUC-ROC=0.97, 0.92, and 0.91, respectively; **Fig. 3C**). Particularly, the transcription activity feature category model performed almost as well as the full model (FNR=6%, FPR=12%). We emphasize that the breadth and level of transcription are the causes of the strong performance of the transcription activity-only model, not the presence of expression evidence.

To evaluate whether the strong performance of the full model is being driven solely by transcription activity-related features, we also built a function prediction model did not consider these features (full (-TX), **Fig. 3C,D**). We found that the model excluding transcription activity features performed almost as well as the full model and similarly to the transcription activity-feature-only model, but with an increased FPR (AUC-ROC=0.96; FNR=3%; FPR=20%). This indicates that predictions of functional regions are not reliant solely on transcription data. Instead, a diverse array of features can be considered to make highly accurate predictions of the functionality of a genomic sequence. Meanwhile, our finding of the high performance of the transcription activity-only model highlights the possibility of establishing an accurate model for distinguishing functional genic and nonfunctional genomic sequences in plant species with only a modest amount of transcriptome data.

Functional likelihood allows the prediction of functional and nonfunctional genomic regions

To provide a measure of the potential functionality of any sequence in the *A. thaliana* genome, including ITRs and ncRNAs, we utilized the confidence score from the full model as a "functional likelihood" value (Tsai et al. 2017; see Methods). The functional likelihood (FL) score ranges between 0 and 1, with high values indicating that a sequence is more similar to phenotype genes (functional) and low values indicating a sequence more closely resembles pseudogenes (nonfunctional). FL values for all genomic regions examined in this study are available in Supplemental Table 4. As expected, phenotype genes had high FL values (median=0.97; **Fig. 4A**) and pseudogenes had low values (median=0.01; **Fig. 4B**). To call sequences as functional or not, we defined a threshold FL value of 0.35 (see Methods). Using this threshold, 96% of phenotype genes (**Fig. 4A**) and 90% of pseudogenes (**Fig. 4B**) are correctly classified as functional and nonfunctional, respectively, demonstrating that the full model is highly capable of distinguishing functional and nonfunctional sequences.

We next applied our model to predict the functionality of annotated protein-coding genes, transposable elements, and unexpressed intergenic regions. Most annotated protein-coding genes not included in the phenotype gene dataset had high FL scores (median=0.86; **Fig. 4C**) and 80% were predicted as functional. Of the 20% of protein-coding genes that were predicted as nonfunctional, we expect that at least 4% represent false negatives based on the FNR of the full model. The actual FNR among protein-coding genes may be higher, however, as phenotype genes represent a highly active and well conserved subset of all genes. However, a subset of the low-scoring protein-coding genes may also represent gene sequences undergoing functional decay and *en route* to pseudogene status. To assess this possibility, we examined 1,940 *A.*

thaliana "decaying" genes that may be experiencing pseudogenization due to promoter disablement (Yang et al. 2011) and found that, while these decaying genes represented only 7% of all *A. thaliana* annotated protein-coding genes, they made up 45% of protein-coding genes predicted as nonfunctional (Fisher's Exact Test (FET), p < 1E-11). In addition to protein-coding genes, we evaluated the FLs of transposable elements (TEs) and randomly-selected, unexpressed intergenic regions that are most likely nonfunctional. As expected, the FLs were low for both TEs (median=0.03, **Fig. 4D**) and unexpressed intergenic regions (median=0.07; **Fig. 4E**), and 99% of TEs and all unexpressed intergenic sequences were predicted as nonfunctional, further demonstrating the utility of the function prediction model. Overall, the FL measure provides a useful metric to distinguish between phenotype genes and pseudogenes. In addition, the FLs of annotated protein-coding genes, TEs, and unexpressed intergenic sequences agree with *a priori* expectations regarding the functionality of these sequences.

Exclusion of features from multiple tissues increases prediction performance for narrowly-expressed sequences

Although the full model performs exceedingly well, there remain false predictions. There are 76 phenotype genes (4%) predicted as nonfunctional (referred to as low-FL phenotype genes). We assessed why these phenotype genes were not correctly identified by first asking what category of features were particularly distinct between low-FL and the remaining phenotype genes. We found that the major category that led to the misclassification of phenotype genes was transcription activity, as only 7% of low-scoring phenotype genes were predicted as functional in the transcription activity-only model, compared to 98% of high FL phenotype genes (**Fig. 5**). By contrast, >65% of low-FL phenotype genes were predicted as functional when sequence conservation, H3 mark, or DNA methylation features were used. This could suggest that the full model is less effective in predicting functional sequences that are weakly or narrowly expressed. While sequence conservation features are distinct between functional and nonfunctional sequences when considered in combination, a significantly higher proportion of low-FL phenotype genes were specific to the *Brassicaceae* family, with only 33% present in dicotyledonous species outside of the *Brassicaceae*, compared to 78% of high-scoring phenotype genes (FET, p < 4e-12), thus our model likely has reduced power in detecting lineage-specific genes.

Given the association between transcription activity features and functional predictions, we next investigated how functional predictions performed for conditionally-functional and narrowly-expressed sequences. We found that genes with conditional phenotypes (see Methods) had no significant differences in FLs (median=0.96) as those with phenotypes under standard growth conditions (median=0.97; U test, p=0.38, Supplemental Fig. 1A). Thus, our model can capture conditionally functional sequences. Next, we evaluated FL distributions among sequences with different breadths of gene expression. For this comparison, we focused on non-stress, single-tissue expression datasets (Supplemental Table 5), which was distinct from the expression breadth feature in the prediction model that considered all datasets. While phenotype genes were better predicted than pseudogenes among sequences with the same number of tissues with expression evidence (U tests, all p < 1.7E-06; Supplemental Fig. 1B), 65% of the 62 phenotype genes expressed in ≤3 tissues were predicted as nonfunctional. Further, there was a significant correlation between the number of tissues with expression evidence and FL values of all sequences in our analysis (r^2=0.77; p < 2E-16). Thus, the function prediction model is biased against narrowly-expressed phenotype genes.

We also found that 80 pseudogenes (10%) were defined as functional (high-FL pseudogenes). Consistent with misclassifications among phenotype genes, a key difference between high-FL pseudogenes and those that were correctly predicted as nonfunctional was that high-FL pseudogenes were more highly and broadly expressed (**Fig. 5**). A significantly higher proportion of high-FL pseudogenes came from existing genome annotation as 19% of annotated pseudogenes were classified as functional, compared to 4% of pseudogenes identified through a computational pipeline (Zou et al 2009) (FET, p < 1.5E-10). We found that high-FL pseudogenes might be more recently pseudogenized and thus have not yet lost many genic signatures, as the mean number of disabling mutations (premature stop or frameshift) per kb in high-scoring pseudogenes (1.9) were significantly lower than that of low-scoring pseudogenes (4.0; U test, p < 0.02). Lastly, we cannot rule out the possibility that a small subset of high-scoring pseudogenes represent truly functional sequences, rather than false positives (e.g. Poliseno et al. 2010; Karreth et al. 2015). Overall, the misclassification of both narrowly-expressed phenotype genes and broadly-expressed pseudogenes highlights the need for an updated prediction model that is less influenced by expression breadth.

To tailor functional predictions to narrowly-expressed sequences, we generated a "tissue-agnostic" model that attempts to minimize the contribution of biochemical activities occurring in many tissues by excluding expression breadth and features that were available across multiple tissues (see Methods). The tissue-agnostic model performed similarly to the full model (AUC-ROC=0.97; FNR=4%; FPR=15%; Supplemental Fig. 2; Supplemental Table 4). Importantly, the proportion of phenotype genes expressed in ≤3 tissues predicted as functional increased by 23% (35% in the full model to 58% in the tissue-agnostic model, Supplemental Fig. 1C), indicating that the tissue-agnostic model is more suitable for predicting the functionality of narrowly-expressed sequences than the full model, although there was an increase in FPR (from 10% to 15%). We next sought to evaluate the FL of ITR and annotated ncRNA sequences utilizing both the full model and the tissue-agnostic model, as these sequences were often narrowly-expressed (Supplemental Fig. 3A).

Intergenic transcribed regions and annotated ncRNAs are mostly predicted as nonfunctional

A subset of ITRs and ncRNAs likely represent novel genes or unannotated exon extensions of known genes (Johnson et al. 2005; Moghe et al. 2013). Nevertheless, most ITRs and ncRNAs are functionally ambiguous, as they are predominantly identified by the presence of expression evidence and few have been characterized genetically. To evaluate the functionality of ITRs and ncRNAs, we applied both the full and tissue-agnostic models to 895 ITRs, 136 TAIR ncRNAs, and 252 Araport long ncRNAs (referred to as Araport ncRNAs; see Methods). The median FLs based on the full model were low (0.09) for both ITRs (**Fig. 4F**) and Araport ncRNAs (**Fig. 4G**), and only 15% and 9% of these sequences were predicted as functional, respectively. By contrast, TAIR ncRNAs had a significantly higher median FL value (0.53; U tests, both *p*<5e-31; **Fig. 4H**) and 68% were predicted as functional. The higher proportion of functional TAIR ncRNA predictions compared to ITRs and Araport ncRNAs could be best explained by differences in features from the transcription activity category (**Fig. 5**). We also note that a greater proportion of ITRs and Araport ncRNAs are predicted as functional when considering only DNA methylation or H3 mark features (**Fig. 5**). However, these two category-specific models also had higher false positive rates (unexpressed intergenic sequences and pseudogenes, **Fig. 5**). Thus, single feature models do not provide additional support for the functionality of most Araport ncRNAs and ITRs.

We next applied the tissue-agnostic model that is less biased against narrowly-expressed sequences (Supplemental Fig. 1C) to ITRs and TAIR/Araport ncRNAs that were generally narrowly-expressed (Supplemental Fig. 3A). Compared to the full model, around twice as many ITRs (30%) and Araport ncRNAs (19%) but a similar number of TAIR ncRNA (67%) were predicted as functional. Considering the union of the full and tissue-agnostic model predictions, 268 ITRs (32%), 57 Araport ncRNAs (23%), and 105 TAIR ncRNAs (77%) were likely functional. ITRs and annotated ncRNAs closer to annotated genes tended to be predicted as functional (Supplemental Fig. 4A). Using the 95th percentile of intron lengths for all genes as a threshold to call ITRs and annotated ncRNAs as proximal or distal to neighboring genes, 57% of likely functional and 35% of likely nonfunctional ITRs and ncRNAs were proximal to neighboring genes, respectively (FET, p < 2E-09). To assess if a subset these likely functional, proximal ITRs/ncRNAs may be unannotated exons of known genes, we assessed whether they tended to have similar features with their neighbors. Compared to feature similarities between neighboring and random gene pairs (Supplemental Fig. 4B-D), likely functional ITRs/ncRNAs were less similar to their neighbors, regardless of proximity (Supplemental Fig. 4C,D). Thus, despite their proximity to annotated genes, it remains unclear if some ITRs or annotated ncRNAs represent unannotated exon extensions of known genes or not. In addition, for proximal functional ITRs/annotated ncRNAs, we cannot rule out the possibility that they represent false-positive functional predictions due to the accessible and active chromatin states of nearby genes. Given the challenge in ascertaining the origin of likely functional, proximal ITRs/ncRNAs, we instead conservatively estimate that 116 distal, functional ITRs and annotated ncRNAs may represent fragments of novel genes.

Intergenic transcribed regions and annotated ncRNAs do not resemble benchmark RNA genes

Thus far, we predicted the majority of ITR and annotated ncRNA sequences as nonfunctional. We demonstrated that the full model was able to predict conditional phenotype genes (Supplemental Fig. 1A) and the tissue-agnostic model was more effective than the full model in predicting narrowly expressed phenotype genes (Supplemental Fig. 1B,C). Thus, conditional or tissue-specific functionality do not fully explain why the majority of ITRs and ncRNAs are predicted as nonfunctional. However, the function prediction models so far were built by contrasting protein-coding genes with pseudogenes and it remains possible that these protein-coding gene-based models can not accurately predict RNA genes. To evaluate this possibility, we generated a tissue-agnostic model using features calculated from a randomly-selected 100 bp sequence within a phenotype protein-coding gene or pseudogene body (for features, see Supplemental Table 6). The reason for using 100 bp sequence is that most RNA genes are too short to be considered by earlier models, which were based on 500 bp sequences. In addition, features from the tissue agnostic model are more suitable for RNA gene prediction as annotated RNA genes tend to be more narrowly expressed than phenotype genes (U tests, all p < 2e-05; Supplemental Fig. 3B). The 100 bp tissue-agnostic model performed similarly to the full 500 bp model in distinguishing between phenotype protein-coding genes and pseudogenes, except with higher FNR (AUC-ROC=0.97; FNR=13%; FPR=5%; Supplemental Fig. 5), but only predicted three out of six RNA genes with documented mutant phenotypes (phenotype RNA genes) as functional (Supplemental Fig. 5I). Further, other RNA Pol II-transcribed RNA genes exhibited mixed predictions from the 100 bp tissue-agnostic model, as 15% of microRNA (miRNA) primary transcripts (Supplemental Fig. 5J), 73% of small nucleolar RNAs (snRNAs; Supplemental Fig. 5K), and 50% of small nuclear RNAs (snRNAs; Supplemental Fig. 5L) were predicted as functional. Although the proportion of phenotype RNA genes predicted as functional (50%) is significantly higher than the proportion of pseudogenes predicted as functional (5%, FET, p < 0.004), this finding suggests that a model built with protein-coding genes has a substantial FNR for detecting RNA genes.

To determine whether the suboptimal predictions by the phenotype protein-coding gene-based models are because RNA genes belong to a class of their own, we next built a multi-class function prediction model aimed at distinguishing four classes of sequences: benchmark RNA genes (n=46), phenotype protein-coding genes (1,882), pseudogenes (3,916), and randomly-selected, unexpressed intergenic regions (4,000). Benchmark RNA genes include six phenotype RNA genes and 40 high-confidence miRNA primary transcript sequences (see Methods). Unexpressed intergenic sequences were included to provide another set of likely nonfunctional sequences distinct from pseudogenes. Expression breadth and tissue-specific features were excluded from the four-class model and 100 bp sequences were used. In the four-class model, 87% of benchmark RNA genes, including all six phenotype RNA genes, were predicted as functional sequences (65% RNA gene-like and 22% phenotype protein-coding gene-like; **Fig. 6A**). In addition, 95% of phenotype genes were predicted as functional (**Fig. 6B**), including 80% of narrowly expressed genes, an increase of 22% over the 500 bp tissue-agnostic model (Supplemental Fig. 1B). For the remaining two sequence classes, 70% of pseudogenes (**Fig. 6C**) and 100% of unexpressed intergenic regions (**Fig. 6D**) were predicted as nonfunctional (either pseudogenes or unexpressed intergenic sequences). Thus, the four-class model improves prediction accuracy of RNA genes and narrowly expressed genes. However, the inclusion of RNA genes in the model has significantly increased the ambiguity in pseudogene classification.

Since the four-class model was able to distinguish benchmark RNA genes from nonfunctional sequence classes, we next evaluated whether ITRs and annotated ncRNAs resemble functional sequences with the four-class model. Note that the 100 bp model used here allowed us to evaluate an additional 10,938 ITRs and 1,406 annotated ncRNAs. We find that 34% of ITR, 38% of Araport ncRNA, and of 65% TAIR ncRNAs were predicted as functional sequences (**Fig. 6E-G**). To provide an overall estimate of the proportion of likely-functional ITRs and annotated ncRNAs, we considered the predictions from the four-class model (**Fig. 6**), the full model (**Fig. 3,4**), and the tissue-agnostic models (Supplemental Fig. 2,5). Based on support from at least one of the four models, we classified 4,437 ITRs (38%) and 796 annotated ncRNAs (44%) as functional, as they resembled either phenotype protein-coding or RNA genes. Our findings lend support that they are likely parts of novel or annotated genes. Meanwhile, we find that a substantial number of ITRs (62%) and annotated ncRNAs (56%) are predicted as nonfunctional. Moreover, at least a third of ITRs (**Fig. 6E**) and Araport ncRNAs (**Fig. 6F**) most closely resemble unexpressed intergenic regions. Thus, we show that the majority of ITRs and annotated ncRNA regions resemble nonfunctional genomic regions, and therefore could represent regions of noisy transcription.

CONCLUSION

Discerning the location of functional regions within a genome represents a key goal in genomic biology. Despite advances in computational gene finding, it remains challenging to determine whether intergenic transcribed regions (ITRs) represent functional or noisy biochemical activity. We established robust function prediction models based on the evolutionary, biochemical, and structural characteristics of phenotype genes and pseudogenes. The prediction models accurately define functional and nonfunctional regions and are applicable genome-wide. These results echo recent findings that human phenotype genes could be distinguished from pseudogenes (Tsai et al. 2017). Given that function predictions were successful in both plant and metazoan model systems, integrating the evolutionary and biochemical features of known genes will likely be applicable to any species. The next step will be to test whether function prediction models can be applied across species, which could ultimately allow the phenotype data and omics resources available in model systems to effectively guide the identification of functional regions in non-models.

Expression data was highly informative to functional predictions. We found that the prediction model based on only 24 transcription activity-related features performs nearly as well as the full model that integrates additional information including conservation, H3 mark, methylation, and TF binding data. In human, use of transcription data from cell lines also produced highly accurate predictions of functional genomic regions (AUC-ROC=0.96; Tsai et al. 2017). Despite the importance of transcription data, we emphasize that the presence of expression evidence is an extremely poor predictor. Taken together, these results indicate that function prediction models can be established in any species, model or not, with a modest number of transcriptome datasets (e.g. 51 in this study and 19 in human). One caveat of the current model is that narrowly-expressed phenotype genes are frequently predicted as pseudogene and broadly-expressed pseudogenes tend to be called functional. To improve the function prediction model, it will be important to explore additional features unrelated to transcription. Because few phenotype genes are narrowly-expressed (5%) in the *A. thaliana* training data, more phenotyping data for narrowly expressed genes will be crucial as well.

Upon application of the function prediction models genome-wide, we found that 4,427 ITRs and 796 annotated ncRNAs in *A. thaliana* are likely functional. Assuming each entry equals a novel gene, this estimate represents a 19% increase in annotated gene space (excluding annotated ncRNAs) for the model plant. However, considering the high false positive rates (e.g. 10% for the full and 31% for the four-class model), this is most likely an overestimate of the number of novel genes contributed by functional ITRs and annotated ncRNAs. In addition, we emphasize that the majority of ITRs and ncRNAs resemble pseudogenes and random unexpressed intergenic regions. Similarly, most human ncRNAs are more similar to nonfunctional sequences than they are to protein coding and RNA genes (Tsai et al. 2017). Furthermore, the significant relationship between the amount of intergenic expression occurring in a species and the size of a genome is consistent with the interpretation that intergenic transcripts are generally nonfunctional. Thus, instead of assuming any expressed sequence must be functionally significant, we advocate that the null hypothesis should be that it is not, particularly considering that most ITRs and annotated ncRNAs have not been experimentally characterized. The machine learning framework we have described provides an approach for distinguishing between functional and noisy biochemical activity, and will help defining the gene space in a genome.

METHODS

Identification of transcribed regions in leaf tissue of 15 flowering plants

RNA-sequencing (RNA-seq) datasets were retrieved from the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/sra/) for 15 flowering plant species (Supplemental Table 1). All datasets were generated from leaf tissue and sequenced on Illumina HiSeq 2000 or 2500 platforms. Genome sequences and gene annotation files were downloaded from Phytozome v.11 (www.phytozome.net; Goodstein et al. 2011) or Oropetium Base v.01 (www.sviridis.org; VanBuren et al. 2015). Genome sequences were repeat masked using RepeatMasker v4.0.5 (www.repeatmasker.org) if a repeat-masked version was not available. Only one end from paired-end read datasets were utilized in downstream processing. Reads were trimmed to be rid of low scoring ends and residual adaptor sequences using Trimmomatic v0.33 (LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:20, Bolger et al. 2014) and mapped to genome sequences using TopHat v2.0.13 (default parameters except as noted below; Kim et al. 2013). Reads ≥20 nucleotides in length that mapped uniquely within a genome were used in further analysis.

For each species, thirty million mapped reads were randomly selected from among all datasets and assembled into transcript fragments using Cufflinks v2.2.1 (default parameters except as noted below, Trapnell et al. 2010), while correcting for sequence-specific biases during the sequencing process by providing an associated genome sequence with the -b flag. The expected mean fragment length for assembled transcript fragments in Cufflinks was set to 150 from the default of 200 so that expression levels in short fragments would not be overestimated.

The 1st and 99th percentile of intron lengths for each species were used as the minimum and maximum intron lengths, respectively, for both the TopHat2 and Cufflinks steps. Intergenic transcribed regions (ITRs) were defined by transcript fragments that did not overlap with gene annotation and did not have significant six-frame translated similarity to plant protein sequences in Phytozome v.10 (BLASTX E-value < 1E-05). The correlation between assembled genome size and gene counts was determined with data from the first 50 published plant genomes (Michael and Jackson, 2013).

Phenotype data sources

Mutant phenotype data for *A. thaliana* protein-coding genes was collected from a published dataset (Lloyd and Meinke 2012), the Chloroplast 2010 database (Ajjawi et al. 2010; Savage et al. 2013), and the RIKEN phenome database (Kuromori et al. 2006) as described by Lloyd et al. (2015). Phenotype genes used in our analyses were those whose disruption resulted in lethal or visible defects under standard laboratory growth conditions. Genes with documented mutant phenotypes under standard conditions were considered as a distinct and non-overlapping category from other annotated protein-coding genes. We identified six RNA genes with documented loss-of-function phenotypes through literature searches (Supplemental Table 7): *At4* (AT5G03545; Shin et al. 2006), *MIR164A* and *MIR164D* (AT2G47585 and AT5G01747, respectively; Guo et al. 2005), *MIR168A* (AT4G19395; Li et al. 2012b), and *MIR828A* and *TAS4* (AT4G27765 and AT3G25795, respectively; Hsieh et al. 2009). Conditional phenotype genes were those belonging to the Conditional phenotype group as described by Lloyd and Meinke (2012). Loss-of-function mutants of these genes exhibited phenotype only under stress conditions.

Arabidopsis thaliana genome annotation

A. thaliana protein-coding gene, miRNA gene, snoRNA gene, snRNA gene, ncRNA region, pseudogene, and transposable element annotations were retrieved from The Arabidopsis Information Resource v.10 (TAIR10; www.arabidopsis.org; Berardini et al. 2015). Additional miRNA gene and lncRNA region annotations were retrieved from Araport v.11 (www.araport.org; Krishnakumar et al. 2015). A pseudogene-finding pipeline (Zou et al. 2009) was used to identify additional pseudogene fragments and count the number of disabling mutations (premature stop or frameshift mutations). Genes, pseudogenes, and transposons with overlapping annotation were excluded from further analysis. Overlapping lncRNA annotations were merged for further analysis. When pseudogenes from TAIR10 and the pseudogene-finding pipeline overlapped, the longer pseudogene annotation was used.

A. thaliana ITRs analyzed include: (1) the Set 2 ITRs in Moghe et al. (2013), (2) the novel transcribed regions from Araport v.11, and (3) additional ITRs from 206 RNA-seq datasets (Supplemental Table 5). Reads were trimmed, mapped, and assembled into transcript fragments as described above, except that overlapping transcript fragments from across datasets were merged. ITRs analyzed did not overlap with any TAIR10, Araport11, or pseudogene annotation. Overlapping ITRs from different annotated subsets were kept based on a priority system: Araport11 > Set 2 ITRs from Moghe et al. (2013) > ITRs identified in this study. For each sequence entry (gene, ncRNA, pseudogene, transposable element, or ITR), a 100 and 500 base pair (bp) window was randomly chosen for calculating feature values and subsequent model building steps. Feature descriptions are provided in the following sections. The feature values for randomly selected 500 and 100 bp windows are provided in Supplemental Table 2 and 6, respectively. Additionally, non-expressed intergenic sequences were randomly-sampled from genome regions that did not overlap with annotated genes, pseudogenes, transposable elements, or regions with genic or intergenic transcript fragments (100 bp, n=4,000; 500 bp, n=3,716). All 100 and 500 bp windows described above are referred to as sequence windows throughout the Methods section.

Sequence conservation and structure features

There were 10 sequence conservation features examined. The first two were derived from comparisons between *A. thaliana* accessions including nucleotide diversity and Tajima's D among 81 accessions (Cao et al. 2011) using a genome matrix file from the 1,001 genomes database (www.1001genomes.org). The python scripts are available through GitHub (https://github.com/ShiuLab/GenomeMatrixProcessing). The remaining eight features were derived from cross-species comparisons, three based on multiple sequence and five based on pairwise alignments. Three multiple sequence alignment-based features were established using aligned genomic regions between *A. thaliana* and six other plant species (*Glycine max*, *Medicago truncatula*, *Populus trichocarpa*, *Vitis vinifera*, *Sorghum bicolor*, and *Oryza sativa*) (referred to as conserved blocks). For each conserved block, the first feature was the proportion of a sequence window that overlapped a conserved block (referred to as coverage), and the two other features were the maximum and average phastCons scores within each sequence window. The phastCons score was determined for each nucleotide within conserved blocks (Li et al. 2012a). Nucleotides in a sequence window that did not overlap with a conserved block were assigned a phastCons score of 0. For each sequence window, five pairwise alignment-based cross-species conservation features were the percent identities to the most significant BLASTN match (if E-value<1E-05) in each of five taxonomic groups. The five taxonomic groups included the *Brassicaceae* family ($n_{species}$=7), other dicotyledonous plants (22), monocotyledonous plants (7), other embryophytes (3), and green algae (5). If no sequence with significant similarity was present, percent identity was scored as zero.

For sequence-structure features, we used 125 conformational and thermodynamic dinucleotide properties collected from DiProDB database (Friedel et al. 2009). Because the number of dinucleotide properties was high and dependent, we reduced the dimensionality by utilizing principal component (PC) analysis as described previously (Tsai et al. 2015). Sequence-structure values corresponding to the first five PCs were calculated for all dinucleotides in and averaged across the length of a sequence window and used as features when building function prediction models.

Transcription activity features

We generated four multi-dataset and 20 individual dataset transcription activity features. To identify a set of RNA-seq datasets to calculate multi-dataset features, we focused on the 72 of 206 RNA-seq datasets each with ≥20 million reads (see above; Supplemental Table 5). Transcribed regions were identified with TopHat2 and Cufflinks as described in the RNA-seq analysis section except that the 72 *A. thaliana* RNA-seq datasets were used. Following transcript assembly, we excluded 21 RNA-seq datasets because they had unusually high RPKM (Reads Per Kilobase of transcript per Million mapped reads) values (median RPKM value range=272~2,504,294) compared to the rest (2~252). The remaining 51 RNA-seq datasets were used to generate four multi-dataset transcription activity features including: expression breadth, 95th percentile expression level, maximum transcript coverage, and presence of expression evidence (for values see Supplemental Table 5). Expression breadth was the number of RNA-seq datasets that have ≥1 transcribed region that overlapped with a sequence window. The 95th percentile expression level was the 95th percentile of RPKM values across 51 RNA-seq datasets where RPKM values were set to 0 if there was no transcribed region for a sequence window. Maximum transcript coverage was the maximum proportion of a sequence window that overlapped with a transcribed region across 51 RNA-seq datasets. Presence of expression evidence was determined by overlap between a sequence window and any transcribed region in the 51 RNA-seq datasets.

In addition to features based on multiple datasets, 20 individual dataset features were derived from 10 datasets: seven tissue/organ-specific RNA-seq datasets including pollen (SRR847501), seedling (SRR1020621), leaf (SRR953400), root (SRR578947), inflorescence (SRR953399), flower, (SRR505745) and silique (SRR953401), and three datasets from non-standard growth conditions, including dark-grown seedlings (SRR974751) and leaf tissue under drought (SRR921316) and fungal infection (SRR391052). For each of these 10 RNA-seq datasets, we defined two features for each sequence window: the maximum transcript coverage (as described above) and the maximum RPKM value of overlapping transcribed regions (referred to as Level in **Fig. 2**). If no transcribed regions overlapped a sequence window, the maximum RPKM value was set as 0. For the analysis of narrowly- and broadly-expressed phenotype genes and pseudogenes (Supplemental Fig. 1B,C), we used 28 out of 51 RNA-seq datasets generated from a single tissue and in standard growth conditions to calculate the number of tissues with evidence of expression (tissue expression breadth). In total, seven tissues were represented among the 28 selected RNA-seq datasets (see above; Supplemental Table 5), and thus tissue expression breadth ranges from 0 to 7 (note that only 1 through 7 are shown in Supplemental Fig. 1B,C due to low sample size of phenotype genes in the 0 bin). The tissue breadth value is distinct from the expression breadth feature used in model building that was generated using all 51 datasets and considered multiple RNA-seq datasets from the same tissue separately (range: 0-51).

Histone 3 mark features

Twenty histone 3 (H3) mark features were calculated based on eight H3 chromatin immunoprecipitation sequencing (ChIP-seq) datasets from SRA. The H3 marks examined include four associated with activation (H3K4me1: SRR2001269, H3K4me3: SRR1964977, H3K9ac: SRR1964985, and H3K23ac: SRR1005405) and four associated with repression (H3K9me1: SRR1005422, H3K9me2: SRR493052, H3K27me3: SRR3087685, and H3T3ph: SRR2001289). Reads were trimmed as described in the RNA-seq section and mapped to the TAIR10 genome with Bowtie v2.2.5 (default parameters; Langmead et al. 2009). Spatial Clustering for Identification of ChIP-Enriched Regions (SICER) v.1.1 (Xu et al. 2014) was used to identify ChIP-seq peaks with a false discover rate ≤ 0.05 with a non-overlapping window size of 200, a gap parameter of 600, and an effective genome size of 0.92 according to Koehler et al. (2011). For each H3 mark, two features were calculated for each sequence window: the maximum intensity among overlapping peaks and peak coverage (proportion of overlap with the peak that overlaps maximally with the sequence window). In addition, four multi-mark features were generated. Two of the multi-mark features were the number of activating marks (0-4) overlapping a sequence window and the proportion of a sequence window overlapping any peak from any of the four activating marks (activating mark peak coverage). The remaining two multi-mark features were the same as the two activating multi-mark features except focused on the four repressive marks.

DNA methylation features

Twenty-one DNA methylation features were calculated from bisulfite-sequencing (BS-seq) datasets from seven tissues (pollen: SRR516176, embryo: SRR1039895, endosperm: SRR1039896, seedling: SRR520367, leaf: SRR1264996, root: SRR1188584, and inflorescence: SRR2155684). BS-seq reads were trimmed as described above and processed with Bismark v.3 (default parameters; Krueger and Andrews 2011) to identify methylated and unmethylated cytosines in CG, CHH, and CHG (H = A, C, or T). Methylated cytosines were defined as those with ≥5 mapped reads and with >50% of mapped reads indicating that the position was methylated. For each BS-seq dataset, the percentage of methylated cytosines in each sequence window for CG, CHG, and CHH contexts were calculated if the sequence window had ≥5 cytosines with ≥5 reads mapping to the position. To determine whether the above parameters where reasonable, we assessed the false positive rate of DNA methylation calls by evaluating the proportion of cytosines in the chloroplast genome that are called as methylated, as the chloroplast genome has few DNA methylation events (Ngernprasirtsiri et al. 1988; Zhang et al. 2006). Based on the above parameters, 0-1.5% of cytosines in CG, CHG, or CHH contexts in the chloroplast genome were considered methylated in any of the seven BS-seq datasets. This indicated that the false positive rates for DNA methylation calls were low and the parameters were reasonable.

Chromatin accessibility and transcription factor binding features

Chromatin accessibility features consisted of ten DHS-related features and one micrococcal nuclease sequencing (MNase-seq)-derived feature. DHS peaks from five tissues (seed coat, seedling, root, unopened flowers, and opened flowers) were retrieved from the Gene Expression Omnibus (GSE53322 and GSE53324; Sullivan et al. 2014). For each of the five tissues, the maximum DHS peak intensity and DHS peak coverage were calculated for each sequence window. Normalized nucleosome occupancy per bp based on MNase-seq was obtained from Liu et al. (2015). The average nucleosome occupancy value was calculated across each sequence window. Transcription factor (TF) binding site features were based on *in vitro* DNA affinity purification sequencing data of 529 TFs (O'Malley et al. 2016). Two features were generated for each sequence window: the total number of TF binding sites and the number of distinct TFs bound.

Single-feature prediction performance

The ability for each single feature to distinguish between functional and nonfunctional regions was evaluated by calculating AUC-ROC value with the Python scikit-learn package (Pedregosa et al. 2011). AUC-ROC values range between 0.5 (equivalent to random guessing) and 1 (perfect predictions) and values above 0.7, 0.8, and 0.9 are considered to be fair, good, and excellent, respectively. Thresholds to predict sequences as functional or nonfunctional using a single feature were defined by the feature value that produced the highest F-measure, the harmonic mean of precision (proportion of sequences predicted as functional that are truly functional) and recall (proportion of truly functional sequences predicted as functional). The F-measure allows consideration of both false positives and false negatives at a given threshold. FPR were calculated as the percentage of negative (nonfunctional) cases with values above or equal to the threshold and thus falsely predicted as functional. FNR were calculated as the percentage of positive (functional) cases with values below the threshold and thus falsely predicted as nonfunctional.

Binary classification with machine learning

For binary classification (two-class) models that contrasted phenotype genes and pseudogenes, the random forest (RF) implementation in the Waikato Environment for Knowledge Analysis software (WEKA; Hall et al. 2009) was utilized. Three types of two-class models were established, including the full model (500 bp sequence window, **Fig. 3C**,**D** and **Fig. 4**), tissue-agnostic models (500 bp, Supplemental Fig. 2; 100 bp, Supplemental Fig. 5), and single feature category models (**Fig. 3C,D**). For each model type, we first generated 100 balanced datasets by randomly selecting equal numbers of phenotype genes (positive examples) and pseudogenes (negative examples). For each of these 100 datasets, 10-fold stratified cross-validation was utilized, where the model was trained using 90% of sequences and tested on the remaining 10%. Thus, for each model type, a sequence window had 100 prediction scores, where each score was the proportion of 500 random forest trees that predicted a sequence as a phenotype gene in a balanced dataset. The median of 100 prediction scores was used as the functional likelihood (FL) value (Supplemental Table 4). The FL threshold to predict a sequence as functional or nonfunctional was defined based on maximum F-measure as described in the previous section. We tested multiple -K parameters (2 to 25) in the WEKA-RF implementation, which alters the number of randomly-selected features included in each RF tree (Supplemental Table 8), and found that 15 randomly-selected features provided the highest performance based on AUC-ROC (calculated and visualized using the ROCR package; Sing et al. 2005). Binary classification models were also built using all features from 500 bp sequences (equivalent to the full model) with the Sequential Minimal Optimization - Support Vector Machine (SMO-SVM) implementation in WEKA (Hall et al. 2009). The results of SMO-SVM models were highly similar to the full RF results: *PCC* between the FL values generated by RF and SMO-SVM=0.97; AUC-ROC of SMO-SVM=0.97; FPR=12%; FNR=3%. By comparison, the full RF model had AUC-ROC=0.98, FPR=10%, FNR=4%.

Tissue-agnostic models were generated by excluding the expression breadth feature and 95th percentile expression level and replacing all features from RNA-seq, BS-seq, and DHS datasets that were available in multiple tissues. For multiple-tissue RNA-seq data, the maximum expression level across 51 RNA-seq datasets (in RPKM) and maximum coverage (as described in the transcription activity section) of a sequence window in any of 51 RNA-seq datasets were used. For multi-tissue DNA methylation features, minimum proportions of methylated cytosines in any tissue in CG, CHG, and CHH contexts were used. For DHS data, the maximum peak intensity and peak coverage was used instead. In single feature category predictions, fewer total features were used and therefore lower –K values (i.e. the number of random features selected when building random forests) were considered in parameter searches (Supplemental Table 8).

Multi-class machine learning model

For the four-class model, benchmark RNA gene, phenotype protein-coding gene, pseudogene, and random unexpressed intergenic sequences were used as the four training classes. Benchmark RNA genes consisted of six RNA genes with documented loss-of-function phenotypes and 40 high-confidence miRNA genes from miRBase (www.mirbase.org; Kozomara and Griffiths-Jones 2014). We generated 250 datasets with equal proportions (larger classes randomly sampled) of training sequences. Two-fold stratified cross-validation was utilized due to the low number of benchmark RNA genes. The features included those described for the tissue-agnostic model and focused on 100 bp sequence windows. The RF implementation, *cforest*, in the *party* package of R (Strobl et al. 2008) was used to build the classifiers. The four-class predictions provide prediction scores for each sequence type: an RNA gene, phenotype protein-coding gene, pseudogene, and unexpressed intergenic score (Supplemental Table 4). The prediction scores indicate the proportion of random forest trees that classify a sequence as a particular class. Median prediction scores from across 100 balanced runs were used as final prediction scores. Scores from a single balanced dataset models sum to 1, but not the median from 100 balanced runs. Thus, the median scores were scaled to sum to 1. For each sequence window, the maximum prediction score among the four classes was used to classify a sequence as phenotype gene, pseudogene, unexpressed intergenic, or RNA gene.

FIGURE LEGENDS

Figure 1. Relationship between genome size and number of nucleotides covered by RNA-seq reads (expression) in 15 flowering plant species. (*A*) annotated genic regions. (*B*) intergenic regions excluding any annotated features. Mb: megabase. Gb: gigabase. Dotted lines: linear model fits. r^2: square of Pearson's correlation coefficient.

Figure 2. Predictions of functional (phenotype gene) and non-functional (pseudogene) sequences based on each individual feature. Prediction performance is measured using Area Under the

Curve - Receiver Operating Characteristic (AUC-ROC). Features include those in the categories of (*A*) transcription activity, (*B*) sequence conservation, (*C*) DNA methylation, (*D*) transcription factor (TF) binding, (*E*) histone 3 (H3) marks, (*F*) sequence structure, and (*G*) chromatin accessibility. AUC-ROC ranges in value from 0.5 (equivalent to random guessing) to 1 (perfect predictions). Dotted lines: median AUC-ROC of features in a category.

Figure 3. Predictions of functional and nonfunctional sequences based on multiple features. Smoothed scatterplots of the first two principle components (PCs) of (*A*) phenotype gene and (*B*) pseudogene features. The percentages on the axes in *(A)* indicate the feature value variation explained by the associated PC. (*C*) AUC-ROC values of function prediction models built when considering all features (Full), all except transcription activity (TX)-related features (Full (-TX)), and all features from each category. The category abbreviations follow those in **Fig. 2**. (*C*) Precision-recall curves of the models with matching colors from (*B*). The models were built using feature values calculated from 500 bp sequence windows.

Figure 4. Functional likelihood distributions of various sequence classes based on the full model. (*A*) Phenotype genes. (*B*) Pseudogenes. (*C*) Annotated protein-coding genes. (*D*) Transposable elements. (*E*) Random unexpressed intergenic sequences. (*F*) Intergenic transcribed regions (ITR). (*G*) Araport11 ncRNAs. (*H*) TAIR10 ncRNAs. The full model was established using 500 bp sequence windows. Higher and lower functional likelihood values indicate greater similarity to phenotype genes and pseudogenes, respectively. Vertical dashed lines indicate the threshold for calling a sequence as functional or nonfunctional. The percentages to the left and right of the dashed line indicate the percent of sequences predicted as functional or nonfunctional, respectively.

Figure 5. Proportion of phenotype genes, pseudogenes, ITRs, and ncRNAs predicted as functional in the full and single-category models. Percentages of sequence classes that are predicted as functional in models based on all features and the single category models, each using all features from a category (abbreviated according to **Figure 2**. The models are sorted from left to right based on performance (AUC-ROC). The colors of and numbers within the blocks indicate the proportion sequences predicted as functional by a given model. Phenotype gene and pseudogene sequences are shown in three sub-groups: all sequences (All), and those

predicted as functional (high functional likelihood (FL)) and nonfunctional (low FL) in the full model. ITR: intergenic transcribed regions.

Figure 6. Function predictions based on a four-class prediction model. (*A*) Stacked bar plots indicate the prediction scores of benchmark RNA genes for each of the four classes: dark blue - phenotype protein-coding gene (Ph), cyan - RNA gene (RNA), red - pseudogene (Ps), yellow – random intergenic sequence (Ig). A benchmark RNA gene is classified as one of the four classes according to the highest prediction score. The color bars below the chart indicate the predicted class, with the same color scheme as the prediction score. Sequences classified as Ph or RNA were considered functional, while those classified as Ps or Ig were considered nonfunctional. Percentages below a classification region indicate the proportion of sequences classified as that class. (*B*) Phenotype protein-coding gene prediction scores. (*C*) Pseudogene prediction scores. (*D*) Random unexpressed intergenic region prediction scores. Note that no sequence was predicted as functional. (*E*) Intergenic transcribed region (ITR), (*F*) Araport11 ncRNA regions. (*G*) TAIR10 ncRNA regions.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Impacts of conditional phenotypes and expression breadth on the function prediction model. (*A*) Functional likelihood distributions of phenotype genes with mutant phenotypes under standard growth conditions (non-conditional) and non-standard growth conditions such as stressful environments (conditional) based on the 500 bp full model. Feature values were calculated from a random 500 bp region from within the sequence body. Higher and lower functional likelihood values indicate a greater similarity to phenotype genes and pseudogenes, respectively. (*B*,*C*) Distributions of functional likelihood scores for phenotype genes (blue) and pseudogenes (red) for sequences with various breadths of expression for (*B*) the 500 bp full model and (*C*) the 500 bp tissue-agnostic model generated by excluding the expression breadth and features available from multiple tissues. The tissue-agnostic model is aimed toward minimizing the effects of biochemical activity occurring across multiple tissues and predicts a greater proportion of narrowly-expressed phenotype genes as functional compared to the full model.

Supplemental Figure 2. Distributions of functional likelihood scores based on the 500 bp tissue-agnostic model. (*A*) Phenotype genes. (*B*) Pseudogenes. (*C*) Annotated protein-coding genes. (*D*) Transposable elements. (*E*) Random unexpressed intergenic sequences. (*F*) Intergenic transcribed regions (ITR). (*G*) Araport11 ncRNAs. (*H*) TAIR10 ncRNAs. Vertical dashed lines display the threshold to define a sequence as functional or nonfunctional. The numbers to the left and right of the dashed line show the percentage of sequences predicted as functional or nonfunctional, respectively.

Supplemental Figure 3. Distributions of expression breadth of different sequence classes. (*A*) Based on 500 bp feature regions. (*B*) Based on 100 bp feature regions.

Supplemental Figure 4. Distance of ITRs and annotated ncRNA regions to and feature similarity with neighboring genes. (*A*) Distance from intergenic transcribed regions (ITRs) and annotated ncRNAs to the closest neighboring gene. ITR and ncRNA sequences are separated by whether they are predicted as functional (F) or nonfunctional (NF) by the 500 bp full model. (*B*) Feature similarity based on Pearson's Correlation Coefficients (PCC) between random pairs of ITRs, Araport11 ncRNAs, TAIR10 ncRNAs, or annotated genes. (*C*) Feature similarity between proximal neighbors (within 95th percentile (456 bp) of intron lengths), and (*D*) Feature similarity between distal neighbors (>456 bp). Pairs involving ITRs and annotated ncRNAs were divided by whether the ITR or ncRNA sequence was predicted as functional (F) or nonfunctional (NF) by the full model. Feature values were quantile normalized prior to calculating correlations.

Supplemental Figure 5. Distributions of functional likelihood scores based on the 100 bp tissue-agnostic model. (*A*) Phenotype genes. (*B*) Pseudogenes. (*C*) Protein-coding gene. (*D*) transposable elements. (*E*) Random unexpressed intergenic sequences. (*F*) Intergenic transcribed regions (ITR). (*G*) Araport11 ncRNAs. (*H*) TAIR10 ncRNAs. (*I*) RNA genes with loss-of-function mutant phenotypes. (J) MicroRNAs, (*K*) Small nucleolar RNAs, (*L*) Small nuclear RNAs. The tissue-agnostic model was built with 100 bp features and while excluding the expression breadth and tissue-specific features. Higher functional likelihood values indicate greater similarity to phenotype genes while lower values indicate similarity to pseudogenes. Vertical dashed lines display the threshold to define a sequence as functional or nonfunctional.

The numbers to the left and right of the dashed line show the percentage of sequences predicted as functional or nonfunctional, respectively.

SUPPLEMENTAL TABLES

Supplemental Table 1. Leaf tissue RNA-sequencing datasets for 15 flowering plant species

Supplemental Table 2. Conservation, biochemical, and sequence-structure feature values calculated from 500 bp sequences.

Supplemental Table 3. False positive and false negative rates for single feature classifications.

Supplemental Table 4. Function predictions for all models generated in this study.

Supplemental Table 5. RNA-sequencing datasets for identifying intergenic transcribed regions, calculating transcription activity features, and assessing tissue-specific predictions.

Supplemental Table 6. Conservation, biochemical, and sequence-structure feature values calculated from 100 bp sequences.

Supplemental Table 7. RNA genes with documented loss-of-function phenotypes.

Supplemental Table 8. K parameters tested for random forest runs.

DATA ACCESS

All data are available in the text of this article or in the supplemental material.

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AUTHOR CONTRIBUTIONS

J.P.L., Z.T.-Y.T., and S.-H.S. designed the research. J.P.L., Z.T.-Y.T., R.P.S., and N.L.P. performed the research. J.P.L., Z.T-Y.T., R.P.S., N.L.P., and S.-H.S. wrote the article.

DISCLOSURE DECLARATION

The authors have no conflicts of interest to disclose.

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FIGURES

Figure 1.

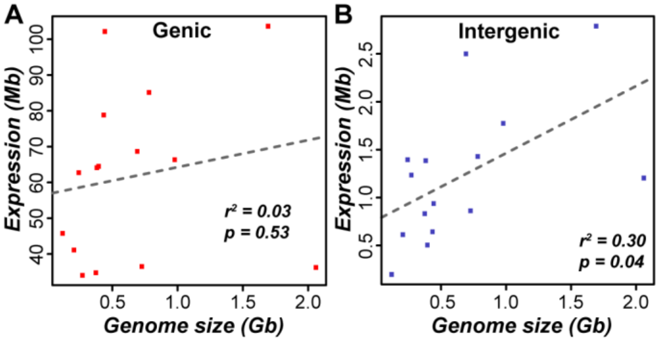


Figure 2.

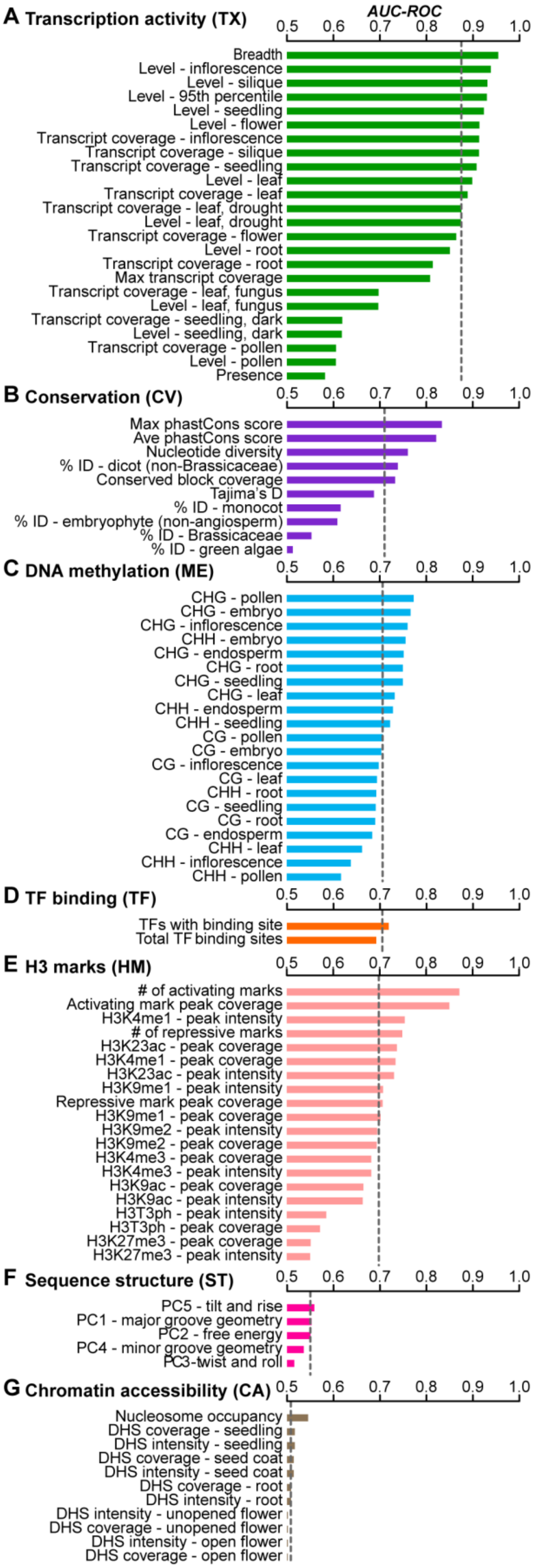

AUC-ROC
A Transcription activity (TX)
0.5
0.6
0.7
0.8
0.9
1.0
Breadth
Level - inflorescence
Level - silique
Level - 95th percentile
Level - seedling
Level - flower
Transcript coverage - inflorescence
Transcript coverage - silique
Transcript coverage - seedling
Level - leaf
Transcript coverage - leaf
Transcript coverage - leaf, drought
Level - leaf, drought
Transcript coverage - flower
Level - root
Transcript coverage - root
Max transcript coverage
Transcript coverage - leaf, fungus
Level - leaf, fungus
Transcript coverage - seedling, dark
Level - seedling, dark
Transcript coverage - pollen
Level - pollen
Presence
B Conservation (CV)
Max phastCons score
Ave phastCons score
Nucleotide diversity
% ID - dicot (non-Brassicaceae)
Conserved block coverage
Tajima's D
% ID - monocot
% ID - embryophyte (non-angiosperm)
% ID - Brassicaceae
% ID - green algae
C DNA methylation (ME)
CHG - pollen
CHG - embryo
CHG - inflorescence
CHH - embryo
CHG - endosperm
CHG - root
CHG - seedling
CHG - leaf
CHH - endosperm
CHH - seedling
CG - pollen
CG - embryo
CG - inflorescence
CG - leaf
CHH - root
CG - seedling
CG - root
CG - endosperm
CHH - leaf
CHH - inflorescence
CHH - pollen
D TF binding (TF)
TFs with binding site
Total TF binding sites
E H3 marks (HM)
# of activating marks
Activating mark peak coverage
H3K4me1 - peak intensity
# of repressive marks
H3K23ac - peak coverage
H3K4me1 - peak coverage
H3K23ac - peak intensity
H3K9me1 - peak intensity
Repressive mark peak coverage
H3K9me1 - peak coverage
H3K9me2 - peak intensity
H3K9me2 - peak coverage
H3K4me3 - peak coverage
H3K4me3 - peak intensity
H3K9ac - peak coverage
H3K9ac - peak intensity
H3T3ph - peak intensity
H3T3ph - peak coverage
H3K27me3 - peak coverage
H3K27me3 - peak intensity
F Sequence structure (ST)
PC5 - tilt and rise
PC1 - major groove geometry
PC2 - free energy
PC4 - minor groove geometry
PC3-twist and roll
G Chromatin accessibility (CA)
Nucleosome occupancy
DHS coverage - seedling
DHS intensity - seedling
DHS coverage - seed coat
DHS intensity - seed coat
DHS coverage - root
DHS intensity - root
DHS intensity - unopened flower
DHS coverage - unopened flower
DHS intensity - open flower
DHS coverage - open flower

Figure 3.

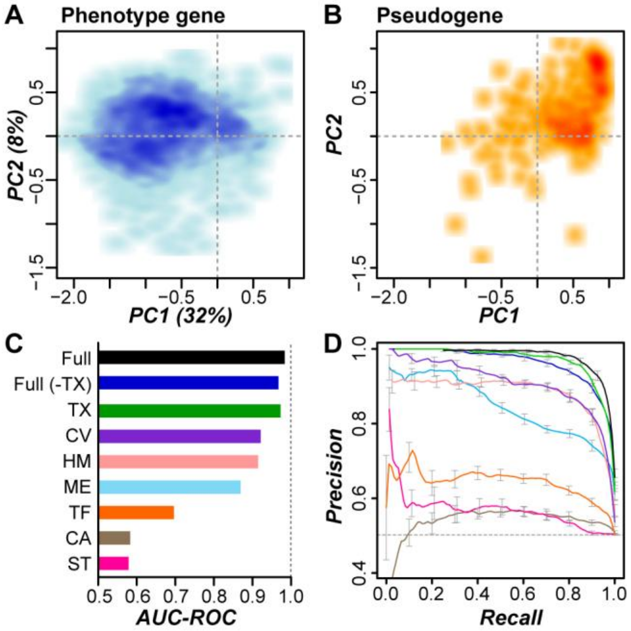

A
Phenotype gene
PC2 (8%)
PC1 (32%)
−1.5
−0.5
0.5
−2.0
−0.5
0.5
B
Pseudogene
PC2
PC1
C
Full
Full (-TX)
TX
CV
HM
ME
TF
CA
ST
0.5 0.6 0.7 0.8 0.9 1.0
AUC-ROC
D
Precision
Recall
0.4
0.6
0.8
1.0
0.0 0.2 0.4 0.6 0.8 1.0

Figure 4.

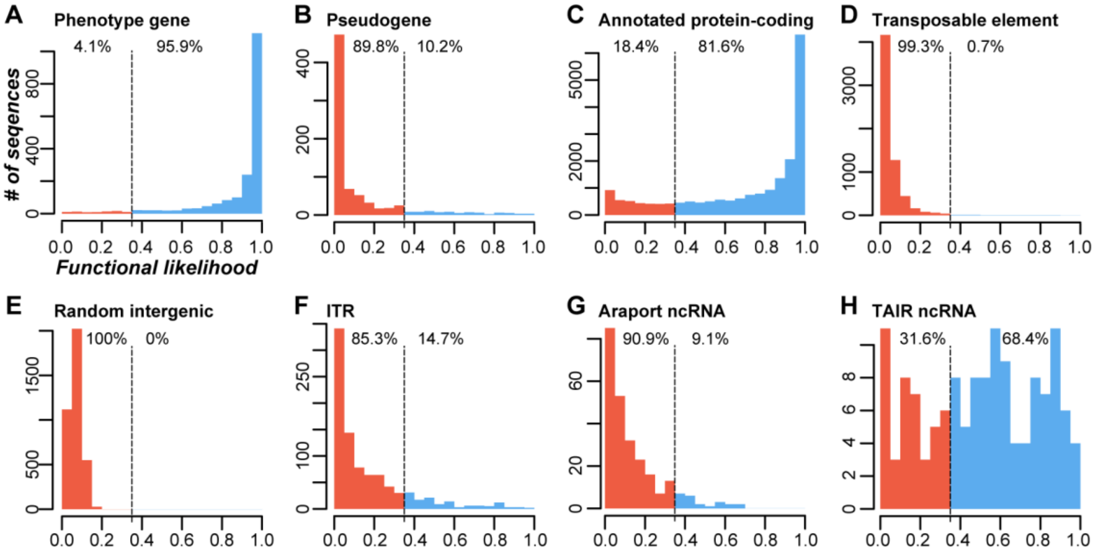
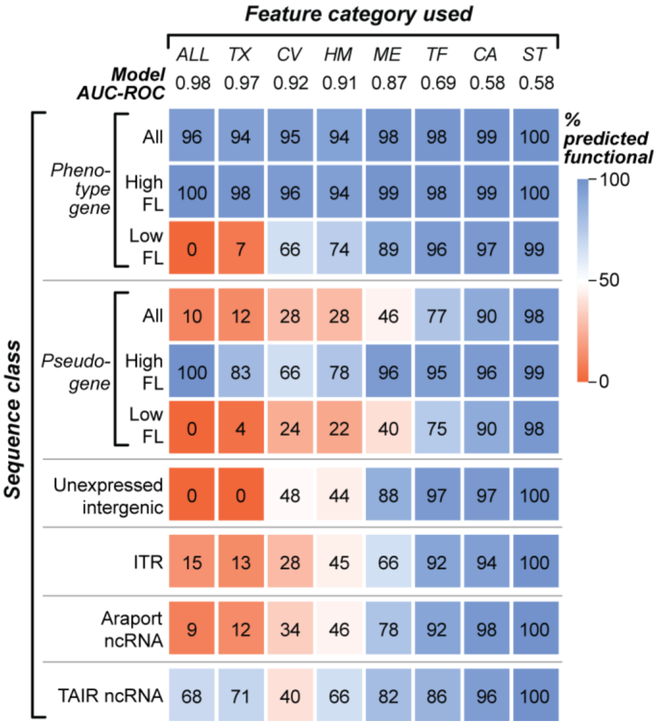
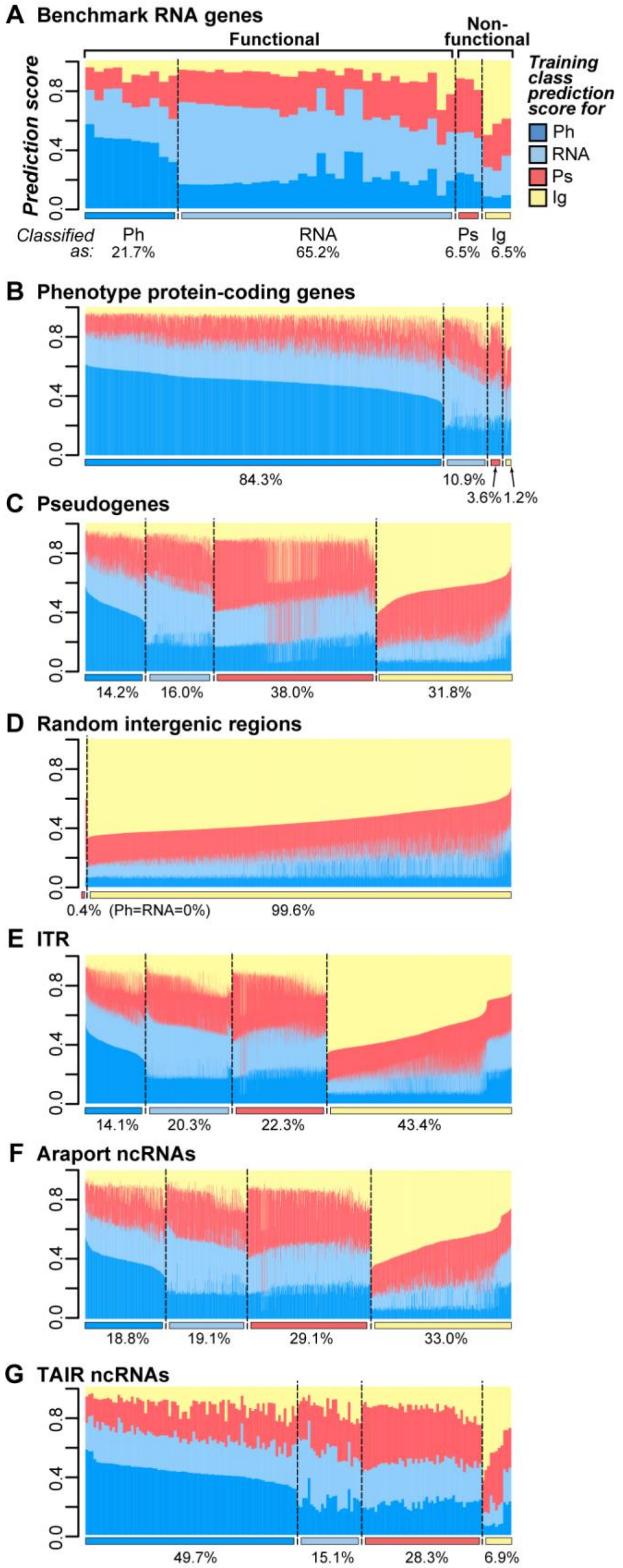

A
Phenotype gene
4.1%
95.9%
# of segences
0
400
800
0.0 0.2 0.4 0.6 0.8 1.0
Functional likelihood
B
Pseudogene
89.8%
10.2%
0
200
400
0.0 0.2 0.4 0.6 0.8 1.0
C
Annotated protein-coding
18.4%
81.6%
0
2000
5000
0.0 0.2 0.4 0.6 0.8 1.0
D
Transposable element
99.3%
0.7%
0
1000
3000
0.0 0.2 0.4 0.6 0.8 1.0
E
Random intergenic
100%
0%
0
500
1500
0.0 0.2 0.4 0.6 0.8 1.0
F
ITR
85.3%
14.7%
0
100
250
0.0 0.2 0.4 0.6 0.8 1.0
G
Araport ncRNA
90.9%
9.1%
0
20
60
0.0 0.2 0.4 0.6 0.8 1.0
H
TAIR ncRNA
31.6%
68.4%
0
2
4
6
8
0.0 0.2 0.4 0.6 0.8 1.0

Figure 5.



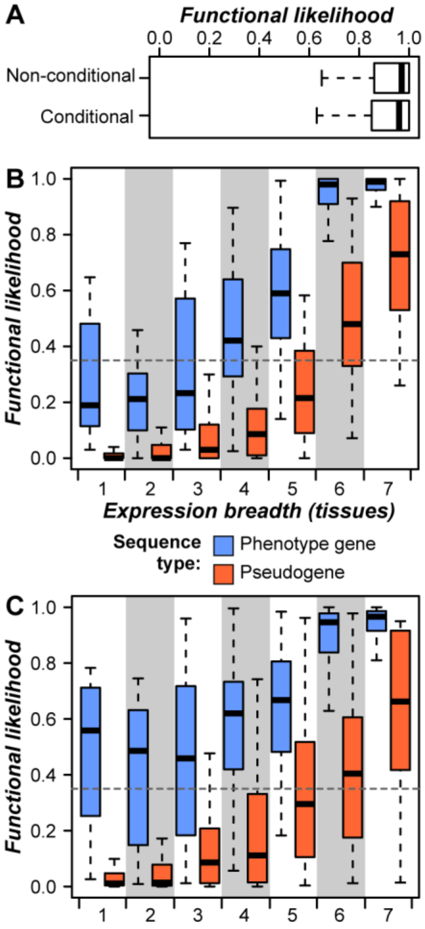
Sequence class		ALL	TX	CV	HM	ME	TF	CA	ST
Model AUC-ROC		0.98	0.97	0.92	0.91	0.87	0.69	0.58	0.58
Phenotype gene	All	96	94	95	94	98	98	99	100
	High FL	100	98	96	94	99	98	99	100
	Low FL	0	7	66	74	89	96	97	99
Pseudogene	All	10	12	28	28	46	77	90	98
	High FL	100	83	66	78	96	95	96	99
	Low FL	0	4	24	22	40	75	90	98
Unexpressed intergenic		0	0	48	44	88	97	97	100
ITR		15	13	28	45	66	92	94	100
Araport ncRNA		9	12	34	46	78	92	98	100
TAIR ncRNA		68	71	40	66	82	86	96	100

Figure 6.


A Benchmark RNA genes
Functional
Non-functional
Prediction score
0.0
0.4
0.8
Training class prediction score for
Ph
RNA
Ps
Ig
Classified as:
Ph 21.7%
RNA 65.2%
Ps 6.5%
Ig 6.5%
B Phenotype protein-coding genes
84.3%
10.9%
3.6%
1.2%
C Pseudogenes
14.2%
16.0%
38.0%
31.8%
D Random intergenic regions
0.4% (Ph=RNA=0%)
99.6%
E ITR
14.1%
20.3%
22.3%
43.4%
F Araport ncRNAs
18.8%
19.1%
29.1%
33.0%
G TAIR ncRNAs
49.7%
15.1%
28.3%
6.9%

1137 SUPPLEMENTAL FIGURES

Supplemental Figure 1.



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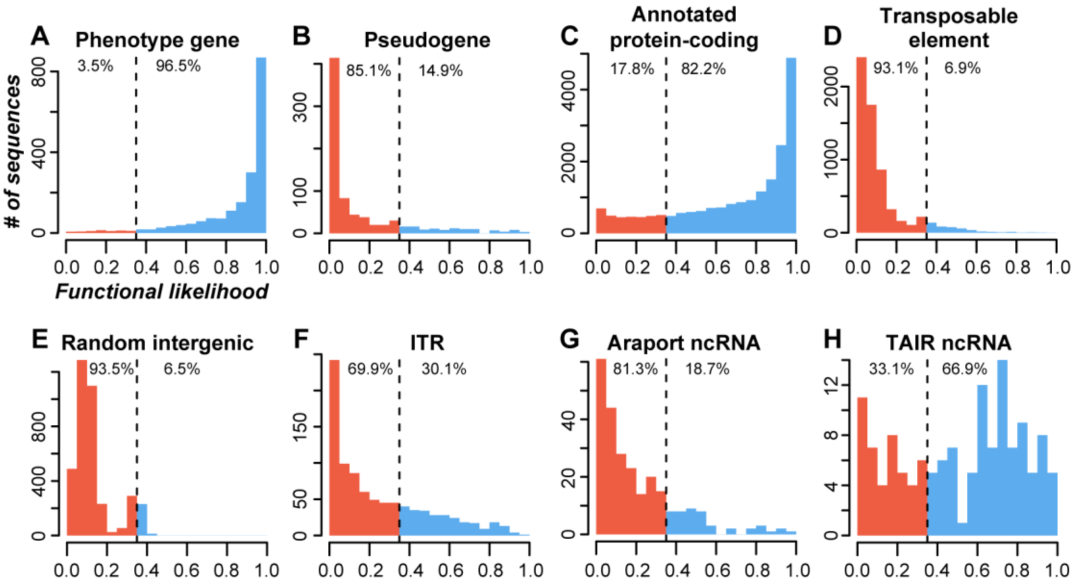
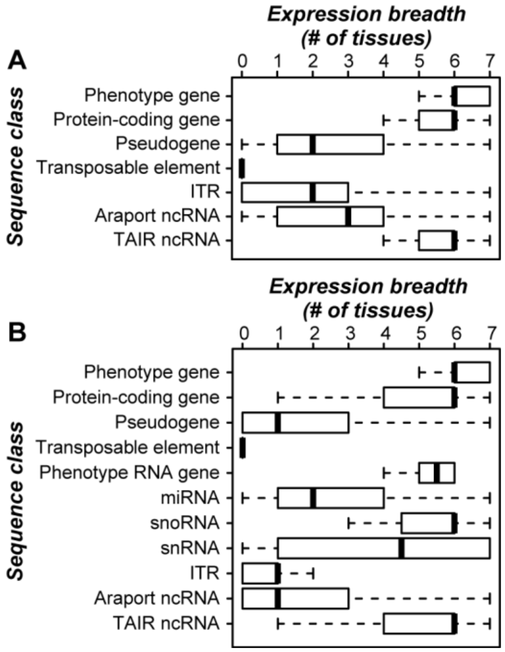
Supplemental Figure 2.



Supplemental Figure 3.



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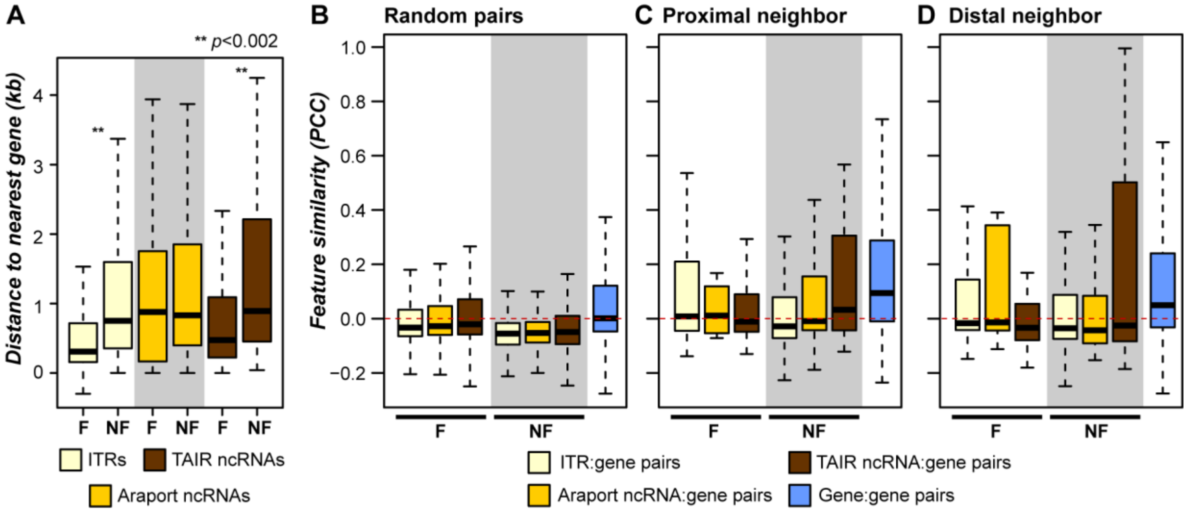
Supplemental Figure 4.



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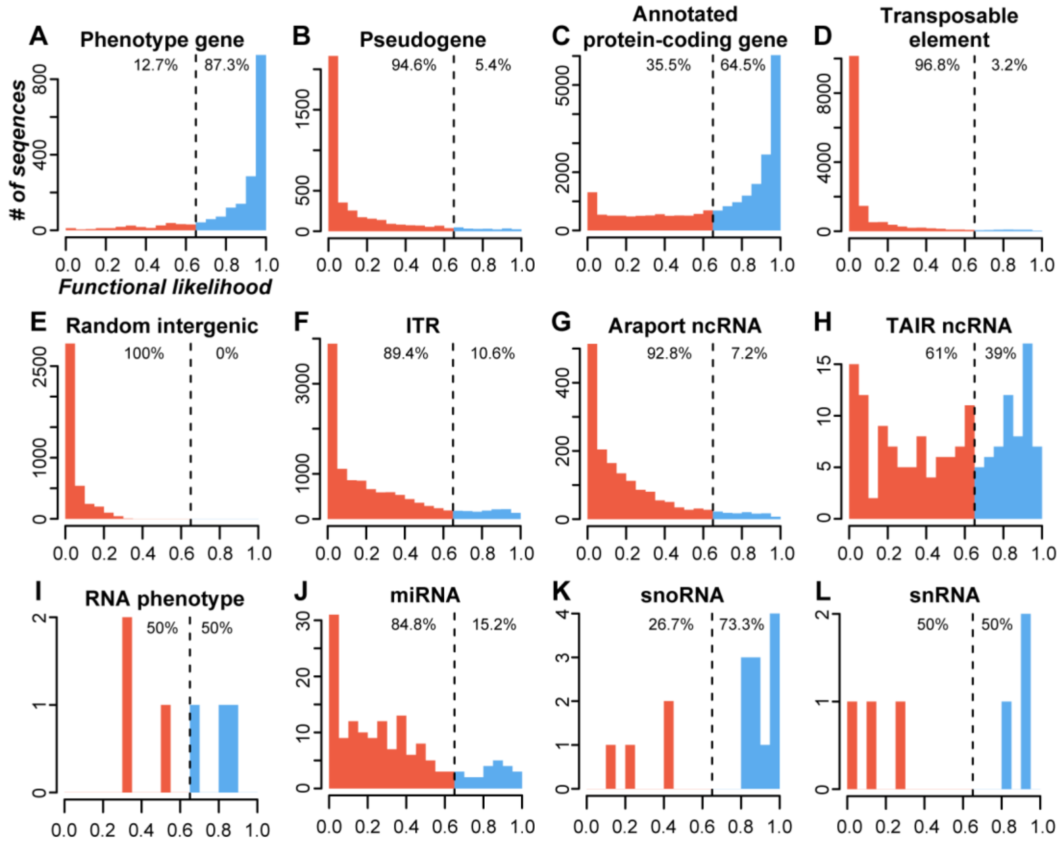
Supplemental Figure 5.


A Phenotype gene
# of seqences
Functional likelihood
B Pseudogene
C Annotated protein-coding gene
D Transposable element
E Random intergenic
F ITR
G Araport ncRNA
H TAIR ncRNA
I RNA phenotype
J miRNA
K snoRNA
L snRNA