Gene co-expression network connectivity is an important determinant of selective constraint

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Abstract

Several studies have investigated general properties of the genetic architecture of gene expression variation. Most of these used controlled crosses and it is unclear whether their findings extend to natural populations. Furthermore, systems biology has established that biological networks are buffered against large effect mutations, but there remains little data resolving this with natural variation of gene expression. Here we utilise RNA-Sequencing to assay gene expression in winter

buds undergoing bud flush in a natural population of *Populus tremula*. We performed expression

Quantitative Trait Locus (eQTL) mapping and identified 164,290 significant eQTLs associating 6,241

unique genes (eGenes) with 147,419 unique SNPs (eSNPs). We found approximately four times as

many local as distant eQTLs, with local eQTLs having significantly higher effect sizes. eQTLs were

primarily located in regulatory regions of genes (UTRs or flanking regions), regardless of whether

they were local or distant. We used the gene expression data to infer a co-expression network and

investigated the relationship between network characteristics, gene expression genetic architecture

and population genetics parameters: eGenes were underrepresented in network module cores (hubs)

and overrepresented in the periphery of the network, with a negative correlation between eQTL

effect size and network connectivity. Module core genes have experienced stronger selective

constraint on coding and non-coding sequence, with connectivity being more strongly associated

with selection signatures than expression level. Our integrated genetics and genomics results suggest

that purifying selection is the primary mechanism underlying the genetic architecture of natural

variation in gene expression assayed in flushing leaf buds of *P. tremula* and that connectivity within

the co-expression network is linked to the strength of purifying selection.

34 Author summary

Numerous studies have shown that many genomic polymorphisms contributing to phenotypic

variation are located outside of protein coding regions, suggesting that they act by modulating gene

expression. Furthermore, phenotypes are seldom explained by individual genes, but rather emerge

from networks of interacting genes. The effect of regulatory variants and the interaction of genes can

be described by co-expression networks, which are known to contain a small number of highly

connected nodes and many more lowly connected nodes, making them robust to random mutation.

While previous studies have examined the genetic architecture of gene expression variation, few were

performed in natural populations with fewer still integrating the co-expression network.

We undertook a study using a natural population of European aspen (*Populus tremula*), showing that

highly connected genes within the co-expression network are associated with polymorphisms of

lower than average effect size, suggesting purifying selection. Furthermore, the most highly

connected genes within co-expression network hubs were underrepresented for identified expression

quantitative trait loci, suggesting stabilizing selection. In contrast, genes in the periphery of the

network displayed signatures of relaxed selective constraint. Highly connected genes are therefore

buffered against large expression modulation, providing a mechanistic explanation of how network

robustness is established and maintained at the population level.

Introduction

A central aim of biology is to understand how emergent phenotypes are encoded in the genome and

how genetic variation engenders phenotypic variation within populations. While the biological

function of many genes can be ascertained through use of mutant screens, such approaches tend to

identify genes essential to a biological process. There is no clear consensus as to whether variation in

these genes underlies natural variation in phenotypic traits, how this relates to the evolutionary

history of the traits or to the properties of the associated transcriptional networks. Additionally,

many traits are complex, being controlled by the action and interaction of numerous genes, often

rendering mutational approaches inadequate or infeasible. In such cases, turning to nature and

employing population genetics and systems biology approaches (systems genetics) to uncover genetic

and genomic co-variation between assayed traits (genomic markers, gene expression, protein

abundance, metabolites etc.) and 'classical' phenotypes (physiognomy, morphology, physiology etc.)

represents an appealing alternative.

The availability of massively parallel sequencing technologies affords new possibilities for addressing

biological questions, for example enabling the generation of *de novo* genome assemblies and

population-wide resequencing data that can be used to perform genome-wide association studies

(GWAS), even in species with large genomes that harbour high levels of polymorphism or that

display rapid linkage disequilibrium (LD) decay [1]. The use of genome-wide resequencing data

allows the discovery of, in theory, all genetic polymorphisms within an individual. These genetic

markers, of which single nucleotide polymorphisms (SNPs) are currently the most commonly

considered, can then be used to perform association or linkage mapping to identify the subset of

polymorphisms engendering phenotypic variation among individuals. While the majority of SNPs are

expected to have no, or insignificant, consequence for a given trait, an implication of the infinitesimal

model [2] is the expectation that the control of variation for any given trait will be contributed by a

large number of loci, each of small effect size. It is not clear how this holds across the scale of

molecular to complex, integrative phenotypes (*e.g.* from transcription to morphology) or whether this

pattern contrasts between adaptive and selectively neutral phenotypic trait variation.

Advances in sequencing technologies have concordantly revolutionised transcriptomics studies,

particularly in non-model organisms. Following seminal work [3,4], numerous early studies in a range

of species established that there is a significant heritable component underlying natural variation of

gene expression levels among individuals within populations [5–17] and that this variation underlies a

number of phenotypes [18–25]. Given these findings, it became apparent that gene expression values

could be considered in the same way as any other quantitative phenotype and be subjected to linkage

or association mapping to identify polymorphisms contributing to expression level variation among

individuals [26], as first reported in [20], with the identified loci termed expression Quantitative Trait

Locus (eQTL; [7]) or, less commonly, expression level polymorphisms (ELPs; [27]). eQTLs are

classified as either local or distant acting depending on the physical location of the associated

polymorphism in relation to the gene that the eQTL is mapped for: local eQTLs are usually defined

as being located within a specified physical distance of the gene location (typically up to 2 Mbp,

although this varies depending on species) while distant eQTLs represent polymorphisms that are

located beyond that threshold distance or on another chromosome. eQTLs can be further classified

as acting in *cis* or *trans*: *cis* eQTLs act in an allele-specific manner and are usually considered to be

local, although long-range *cis* interactions can occur, for example when a polymorphism is located in

an enhancer that is physically distant from the gene of interest; *trans* acting eQTLs affect both alleles

of a gene and are most commonly located distant to that gene. There continues to be strong interest

in eQTLs as they can identify mechanistic links between phenotype and genotype [28,29].

Importantly, the majority of polymorphisms that have been associated to phenotypes using GWAS

in a wide range of species are located outside of protein coding or transcribed regions [30–33],

suggesting that they influence expression rather than altering protein or transcript function.

A number of previous eQTL studies have been conducted using plant species including *Arabidopsis*

thaliana [34–39], *Zea mays* [40–42] and *Oryza sativa* [43,44], and in forest tree species [8,45–47]. These,

together with studies in other eukaryotic systems, have yielded generalities concerning the genetic

architecture of gene expression variation, including that a greater number of local eQTLs are

typically identified and that these individually explain a larger proportion of gene expression variance

than do distant eQTLs [35,48–51]. With the exception of human studies, the majority of previous

work was conducted using controlled, often inter-specific, crosses and it is not clear how generally

applicable their conclusions are for natural populations of unrelated individuals. Few studies have

considered whether observed, heritable variation is adaptive [52,53] or whether signatures of

population differentiation are observed at the transcriptome level [53–58], and there is a lack of

consensus as to the extent to which selection acting on gene expression underlies adaptive

phenotypic trait variation [53]. There has also been little work to date examining eQTLs in the

context of the co-expression network that they induce. Co-expression networks are known to be

scale-free, having few highly connected nodes (genes) and many nodes with few connections. This

property imparts an inherent ability to buffer against single mutations of large negative effect [59–61]

as random mutation of an expression pattern (*i.e.* an eQTL) or coding sequence will more often

affect a network node of low connectivity. However, there has been little exploration of how

network properties relate the selection.

Species in the *Populus* genus have been established as a powerful model system for forest tree

genomics due to their relatively small genome, rapid growth, propensity for clonal propagation and

ease of genetic transformation [62]. *P. tremula* (European aspen) has many features that render it a

particularly useful model for population genetics and speciation studies [63,64], studies of which are

facilitated by availability of a draft *de novo* genome assembly [65] and population resequencing data

[66].

Here, we generated population-wide RNA-Seq data in a natural collection of *P. tremula*, assaying gene

expression in winter buds at the point of bud break. We performed eQTL mapping and constructed

a co-expression network, which was scale free and modular, with highly connected genes in the

module cores being under-represented for eQTLs and with eQTL effect size being negatively

correlated with gene connectivity. Patterns of polymorphism and divergence within genes in module

cores demonstrate that they are likely experiencing stronger selective constraint relative to genes in

the network periphery. Our results suggest that purifying selection plays an important role in

buffering the transcriptional network against large perturbations and that natural variation in gene

expression is more prevalent in genes of low network connectivity as a result of relaxed selective

constraint.

Results

Population level gene expression similarity

We utilised the northern common garden (located at 63.9° N, near Umeå, Sweden) of the Swedish

Aspen (SwAsp) collection [67], which comprises 116 *P. tremula* genotypes sampled across the species

distribution range in Sweden (56.2° to 66.4° N, Fig 1A). The SwAsp collection was previously shown

to contain abundant genetic variation, with low linkage disequilibrium (LD) [64,68] and minimal

population structure [69]. Based on whole genome re-sequencing data aligned to a *de novo* assembly

of the *P. tremula* genome (available at PopGenIE.org; [65]), we called 4,509,654 SNPs after stringent

filtering, which we utilised to perform eQTL mapping.

To examine the genetic architecture of natural variation in gene expression within the SwAsp

collection, we generated paired-end RNA-Seq expression data from winter buds at the point of

spring bud break/flush for 219 individuals (clonal replicates), representing 86 genotypes, and first

examined the distribution of broad-sense heritability (H^2) and population differentiation (Q_{ST}) (Fig

1B,C respectively) for all expressed, annotated genes. H^2 ranged from 0.0 to 1.0 with a mean (± s.d)

of 0.30 (0.22) and with 5,924 genes (17%) having H^2 > 0.5. Permutation testing showed that 21,219

genes had significantly higher heritability than expected by chance (p < 0.01). There was a weak

positive correlation between H^2 and median expression level (Pearson r = 0.09; df = 32,767; p <

2.2×10^{-16}), and a relatively strong positive correlation to expression variance (Pearson r = 0.43; df =

32,767; p < 2.2×10^{-16}). Q_{ST} ranged from 0.0 to 1.0 with a mean (± s.d) of 0.06 (0.12) and had a weak

negative correlation with expression variance (Pearson r = -0.02; df = 29,670; p < 4.5×10^{-4}) and a

positive correlation with median expression (Pearson r = 0.18; df = 29,670; p < 2.2×10^{-16}). To

further examine whether population structure was apparent on the basis of expression variation

among genotypes, we performed hierarchical clustering of all individuals (Fig 1D) or genotypes (S1

Fig). We observed some evidence of clustering among genotypes (regions of blue in Fig 1D, S1 Fig)

but as genotypes (or individuals) did not cluster according to population of origin (as indicated by the

y axis color bars), this did not appear to be explained by population structure. We tested whether the

clustering could be used to predict the population of origin for genotypes by cutting the dendrogram

to produce 12 clusters that were used as the response in a multinomial logistic regression. The mean

accuracy for a 10-fold cross validation was 0.09. We performed permutation tests, which showed that

the mean Q_{ST} for all genes was significantly lower than expected by chance (p < 0.001).

We selected the 500 genes with the highest H^2 (0.88-1.0, 0.93 ± 0.03) and 500 genes with the highest

Q_{ST} (0.54-1.0, 0.71 ± 0.13) and subjected these to GO enrichment analysis to identify whether these

gene sets contained characteristic functional signatures (see S1 File for all results). Genes with high

H^2 were enriched for categories including protein phosphorylation (GO:0006468; p = 3.7×10^{-6}),

while high Q_{ST} genes were enriched in terms including translation (GO:0006412; p = 4.2×10^{-20}) and

gene expression (GO:0010467; p = 4.6×10^{-12}). Likewise, we considered the genes with the lowest

values, which revealed enrichment of terms including cell wall modification (GO:0042545; p =

2.6×10^{-7}) for the 2,289 genes with an H^2 of zero and enrichment of terms including amino acid

activation (GO:0043038; p = 0.0012) among the 11,895 genes with a Q_{ST} of zero.

We performed a regression analysis to ascertain whether a set of geographic (latitude, longitude,

elevation), climatic (temperature, precipitation) or other (time since sample collection) factors

significantly explained the global patterns of gene expression similarity among genotypes (S2 Fig), as

identified by performing a PCA of the expression data. None of the gene expression principal

components (PCs) were significantly explained by these environmental factors, with the only

significant results found between PCs 2, 5 and 7 and the number of hours from collecting branches

from the field until bud sampling in the greenhouse for RNA extraction, which explained 6.6%,

3.2%, and total 2.1% expression variance, respectively.

We subsequently filtered expression values to remove unexpressed genes and uninformative

expression profiles with low variance. Of 35,154 annotated genes, 20,835 were expressed in all

samples, including biological replicates, and 23,183 were expressed in all genotypes when considering

genotype means. Filtering to remove uninformative expression retained 22,306 genes with the 12,848

removed genes representing those that were either not expressed in our bud samples (6,736 genes

with median expression of zero of which 2,385 had no detectable expression at all), or that were

weakly expressed (1,762 genes with variance < 0.05 and median expression < 2), together with genes

that had stable expression among genotypes (4,350 genes with expression variance < 0.05 and

median expression >= 2). The latter potentially represent genes with canalised gene expression.

Analysis of this set of stably expressed genes identified enrichment for GO categories including

protein transport (GO:0015031, $p = 6.8 \times 10^{-11}$) and protein localisation (GO:0008104, $p = 2.2 \times 10^{-10}$).

In contrast, the 500 genes with the highest variance across all samples were enriched for GO

categories related to protein phosphorylation (GO:0006468, $p < 10^{-6}$), chitin metabolic process

(GO:0006030, $p < 10^{-4}$), and cell wall macromolecule catabolic process (GO:0016998, $p < 10^{-4}$).

Comparing the variance of these 500 genes with mean F_{ST} calculated using SNPs within those genes

revealed no apparent relationship.

A recent reanalysis [70] of two existing datasets assaying gene expression among natural accessions

of *A. thaliana* [71,72] observed that thousands of genes displayed clear present/absent expression

among accessions. In contrast, we did not find any genes displaying such a pattern of expression

variation (S3 Fig), an observation that we also confirmed in an independent *P. tremula* dataset [73],

albeit containing substantially fewer genotypes.

eQTL mapping

We performed eQTL mapping, defining an eQTL as a significant association between a SNP

(termed an eSNP) and the expression of a gene (termed an eGene). Furthermore, we classified an

eQTL as *local* if the eSNP was located on the same chromosome and not more than 100 kbp from

the associated eGene, and as *distant* otherwise. This distance threshold was determined based on the

distribution of distances between eSNPs and their associated genes and the assumption that most

detectable eQTLs located within one chromosome were local (S4 Fig). We did not consider whether

eQTLs acted in *cis* or *trans*. Prior to mapping eQTLs, hidden confounders were removed from the

gene expression data by removing variance attributable to the first nine principal components of the

expression data, removal of which maximised the number of eQTLs identified (S5 Fig). After

removing these hidden confounders, we repeated the gene expression clustering analysis and

observed that the previous sample clustering was no longer apparent (S6 Fig).

In total 164,290 eQTLs were identified at 5% empirical FDR: 131,103 local and 33,187 distant.

These eQTLs represented pairwise associations between 6,241 unique genes (eGenes; 28% of genes

considered) and 147,419 unique SNPs (eSNPs), with a mean of 21.0 local and 5.3 distant eSNPs per

eGene, respectively. 4,091 genes had only local eQTLs, 1,050 had only distant eQTLS while 1,100

had both. The genomic context of eSNPs was determined by intersecting eSNP positions with gene

annotations. After normalising for feature length, the majority of local eSNPs were located within

untranslated regions (UTRs) and up- or down-stream (regulatory) regions of genes, with distinctly

lower representation within exons than introns (Fig 2D). The genomic context distribution of local

and distant eSNPs was largely similar, although there were distinctly more eSNPs located within
intergenic regions for distant eQTLs. A local eSNP/eQTL can be located within the region (5'/3' 2
kbp flanking, 5'/3' UTR, exon, intron) of the associated eGene, within the region of a gene other
than the associated eGene or within an intergenic region. We found that approximately half of the
local eSNPs were located within the region of the eGene itself and half within another gene, with
relatively few local eSNPs located in intergenic regions. There was a clear tendency for a local eSNP
to be located proximal to the transcription start site (TSS) or the stop codon (S7 Fig). eGenes had
significantly higher heritability than non eGenes (median heritability difference was 0.16, permutation
test $p < 0.0001$) (Fig 2B), with this trend being slightly higher for local than distant eQTLs (S8 Fig).
eGenes similarly had higher expression variance than non-eGenes, both before (mean variance of
0.32 versus 0.24; permutation test $p < 0.0001$) and after (mean variance of 0.18 versus 0.084;
permutation test $p < 0.0001$) hidden confounder removal. In particular, eGenes represented 60 of
the 75 genes with variance > 2 (hypergeometric test, $p < 2.2\times10^{-16}$) and 178 of the 250 genes with
variance > 1 ($p < 2.2\times10^{-16}$) (S9 Fig). Before hidden confounder removal eGenes had marginally
higher mean expression than non-eGenes (mean expression 3.5 and 3.3, respectively; permutation
test p-value < 0.0001). There were no significant differences after hiden confounder removal,
regardless of whether the eQTLwas local or distant. eGenes with at least one local eQTL were
enriched for GO categories related to tRNA metabolic process (GO:0006399, $p = 1.5\times10^{-5}$), ncRNA
metabolic process (GO:0034660, $p = 2.6\times10^{-5}$) and organonitrogen compound biosynthetic process
(GO:1901566, $p = 2.2\times10^{-5}$), among others, while eGenes with at least one distant eQTL were
enriched for categories including protein phosphorylation (GO:0006468, $p = 0.0064$; see S1 File for
all results).

In contrast to a number of previous studies [20,35,37,48,51], we did not find evidence for any

distantly acting hotspots (Fig 2C, S10 Fig), which represent loci where large numbers of *trans*-acting

variants are co-located. The vast majority of eSNPs were associated with a single eGene (132,258

eSNPs) with a maximum of six eGenes associated with a single eSNP (S11A Fig). In contrast, only

1,248 of the 6,241 eGenes were associated with a single eSNP, with eGenes associated with up to

1,547 eSNPs (S11B Fig). To partially account for linkage, we fitted linear models between the

expression of each eGene and all the significant eSNPs for that gene, both local and distant. The use

of a linear model masks eSNPs that contain identical/redundant information and thus effectively

identifies haplotype blocks present in all individuals (which we refer to as 'unique eSNPs'), while also

producing a measure of how well the combination of eSNPs explains the expression of the

corresponding eGene (in terms of percentage variance explained, %VE). Of the 4,993 eGenes

associated with more than one eSNP, 4,703 were also associated to more than one unique eSNP of

which 4,210 genes were associated with at least one local eSNP and 1,203 were associated with at

least one distant eSNP. The adjusted %VE for the combination of eQTLs was, in general, higher

(mean %VE 51.1) than for single eSNPs (mean %VE 44.3). Local eSNPs explained significantly

more of the variance than distant eSNPs (local mean adjusted %VE = 51, distant mean adjusted

%VE = 47, Mann-Whitney $p < 2.2 \times 10^{-16}$, Fig 2A) and also had higher statistical significance (Mann-

Whitney $p = 6.9 \times 10^{-12}$, Fig 2E). As expected, there was a positive correlation between the maximum

%VE of the eSNPs associated with an eGene and gene expression H^2 (Pearson r = 0.47, df = 6,232,

$p < 2.2 \times 10^{-16}$). Of note, there were 2,780 non-eGenes with $H^2 > 0.5$, representing a population of

genes of relatively high expression heritability but with no identified eSNP associated with that

heritable variation.

 Co-expression network

We used genotype mean gene expression values, after removal of hidden confounders, to calculate a

co-expression network and subsequently considered the network characteristics for genes with and

without a mapped eQTL (eGenes). In common with other biological networks, the network was

scale-free ($R^2 = 0.97$). We compared the correlation and variance properties of our dataset to that of

the *P. tremula* expression atlas (exAtlas; [65]), which represents different tissues collected from a

single genotype. The correlation distribution for the exAtlas samples was much wider (mean

correlation $0.01 \pm$ s.d. 0.36) than that of our population expression data (mean correlation $0.00 \pm$ s.d.

0.12; Kolmogorov-Smirnov D = 0.14, $p < 2.2 \times 10^{-16}$). The expression variance for the SwAsp

expression data was also significantly lower than in the exAtlas data (Wilcoxon signed rank test, V =

18274602, $p < 2.2 \times 10^{-16}$; Fig 3A).

Clustering analysis of the co-expression network identified 38 co-expression modules (S12 Fig)

containing a total of 20,686 genes (min=86 genes, max=1591 genes). These were enriched for a

number of Gene Ontology (GO) categories including translation (modules 9, 10, and 14),

photosynthesis (module 22) and oxidation-reduction process (module 29; for all results see S1 File).

Despite the narrow distribution of correlation values, the modules were reasonably well defined, as

indicated by the normalised connectivity difference (k_{diff}), *i.e.* the difference between intra- and inter-

modular connectivity. All modules exhibited a positive mean k_{diff}, with only 157 genes (0.7%) having a

negative k_{diff}. This was in stark contrast to genes assigned to the 'junk' module (*i.e.* all genes not

assigned to any well-defined module), where there were 480 genes with negative k_{diff} (29%).

We examined the relationship of eGenes and eQTLs to network connectivity, determining

significance of these results using permutations tests shuffling gene assignments while maintaining

the network structure (*i.e.* node gene IDs were shuffled while edges remained constant). In general,

eGenes had lower connectivity and betweenness centrality than non-eGenes (permutation test, p <

0.0001 for both, Fig 3B). Moreover, genes with a positive k_{diff} were significantly under-represented

for eGenes (permutation test, p < 0.0001). We defined the core of each module as the 10% of genes

in a module with the highest normalised k_{diff} while also having an intra-modular connectivity >1.

Using this definition, all 38 modules contained at least one core gene, with the percentage of core

genes ranging from 2-10% (S2 File, S12 Fig). Before removal of hidden confounders, core genes had

both higher mean expression and variance than non-core genes (difference of 1.1 and 0.16,

respectively; permutation test, p < 0.0001). However, the co-expression network was inferred from

the expression data after the removal of hidden confounds, where these difference were virtually

non-existent (no difference in mean expression: permutation test p = 0.39, and marginal difference

in variance: mean variance of 0.12 and 0.11, permutation test p = 0.0184, S13 Fig). Additionally,

there was a weak negative relationship between network connectivity and gene expression variance in

the network as a whole (Pearson r = -0.08; df = 22,304; p < 2.2×10^{-16}; S9 Fig) and eGenes of the

highest connectivity had lower effect size (based on the maximum effect size across all eQTLs

associated to each eGene, Pearson r = -0.15; df = 6,239; p < 2.2×10^{-16}, S14 Fig). Among the module

cores, 28 contained at least one eGene, with 25 module cores being significantly under-represented

for eGenes (permutation test, p < 0.05). This further emphasised that, in general, eGenes were not

central in the network. There was at least one eSNP located within the region of a core gene (5'/3'

flanking, 5'/3' UTR, exon, intron) for 32 of the 38 modules, with module cores also being under-

represented for eSNPs (permutation test, p < 0.0001). For a number of metrics, module 23 had

notable differences to the general pattern: It contained few core genes, all of which were eGenes; the

core genes had high variance compared to non-core genes; the module in general was enriched for

eGenes.

Subsequently, we tested whether the periphery of the network was over-represented for eGenes.

Sixty-four of 145 peripheral genes were eGenes, representing a significant enrichment (permutation

test, p < 0.0001). On the other hand, network module cores were enriched for both transcription

factors (permutation test, p < 0.0001), which had higher connectivity than non-transcription factors

(permutation test, p < 0.0001), and phylogenetically conserved genes (permutation test, p < 0.0001),

defined as genes with orthologs in *P. tremuloides*, *P. trichocarpa* and *A. thaliana* (20,318 genes; [74]).

Furthermore, *P. tremula*-specific genes, *i.e.* genes without orthologs in *P. tremuloides*, *P. trichocarpa* or *A.*

thaliana (1,614 genes), were slightly under-represented in network module cores (permutation test, p

= 0.009) while being slightly over-represented among eGenes (permutation test, p = 0.0076).

In addition to eGenes having generally lower connectivity, there was also a negative relationship

between eQTL effect size and co-expression connectivity for both local (Pearson r = -0.15; df =

5,189; p < 2.2×10^{-16}) and distant eQTL (Pearson r = -0.12; df = 2,148; p < 6.2×10^{-8}). eGenes had

higher heritability than non eGenes regardless of whether they were located within a module core or

not (mean difference of 0.10 and 0.15, respectively, permutation p < 0.0001; Fig 3C), and identified

eSNPs explained a substantial proportion of the heritable variance (blue boxes in Fig 3C). However,

the heritability of eGenes within the core was lower than for eGenes outside the core (mean

difference of 0.10, permutation test p < 0.0001, Fig 3C) and H^2 correlated negatively with

connectivity in the network as a whole (Pearson r = -0.30; df = 22,304; p < 2.2×10^{-16}).

We examined the distribution of the mode of action (Fig 3D) and genomic context (Fig 3E) of

eSNPs. Module cores were underrepresented for eGenes, which is apparent by the low proportion of

eGenes in cores compared to non-cores (Fig 3D). There were distinctly more distantly acting eSNPs

within the core than the non-core (Fig 3D) and of these there were more distantly acting eSNPs

located within exons of core eGenes compared to core non-eGenes, which had higher representation

of distantly acting eSNPs located in UTRs (Fig 3E). The genomic context distribution of local acting

eSNPs was similar in all cases (core/non-core and eGene/non-eGene; Fig 3E), however it is clearly

apparent that non-core eGenes contained by far the greatest density of eSNPs.

339 Paralogs with diverged expression are more likely to be eGenes

In *P. tremula* 3,910 paralog pairs were detected [74], with 2,140 of these (4,185 unique genes) passing

our gene expression and variance filtering criteria. These paralogs were significantly under-

represented for eGenes, with 1,078 of the 4,185 genes having at least one associated eSNP

(hypergeometric test, p = 0.0004). Comparing the expression correlation of paralog pairs to that of

random gene pairs showed that paralogs exhibited conserved regulation (permutation test, p <

0.001). We compared the expression correlation distributions of paralog pairs containing 0, 1, and 2

eGenes (Fig 4) and found that a higher number of eGenes in a pair was associated with lower

expression correlation (linear model $\hat{\beta} = -10.52$, p < 2.2×10^{-16}). Excluding paralogs did not alter

the fact that eGenes had significantly lower network connectivity than non-eGenes.

349 Gene expression evolution is associated with rate of sequence evolution

To assess whether genes with and without eQTLs (eGenes vs. non-eGenes) are experiencing

different levels of selective constraint, we assessed nucleotide diversity (θ_π) and the site frequency

spectrum of segregating mutations using Tajima's D in different genomic contexts. We found that,

irrespective of whether eQTLs were local or distant, eGenes exhibited significantly higher genetic

variation than non-eGenes (Fig 5A). Furthermore, although Tajima's D values are overall negative in

P. tremula, likely reflecting a historical range expansion [64], eGenes exhibited significantly higher

Tajima's D values compared to non-eGenes (Fig 5C), again regardless of whether the associated

eQTL was local or distant. These patterns were consistent for the different genetic contexts

considered, most likely reflecting local patterns of linkage (S15 Fig). One explanation for the lower

diversity at non-eGenes is that existing variation is segregating at lower frequencies and that non-

eGenes are experiencing stronger selective constraint compared to eGenes. To test this hypothesis

we looked for differences in selection efficacy on protein sequence evolution between eGenes and

non-eGenes by examining the ratios of intraspecific nonsynonymous to synonymous polymorphisms

($\theta_{0\text{-fold}}/\theta_{4\text{-fold}}$) and interspecific nonsynonymous to synonymous substitutions (d_N/d_S) across these

genes (using *P. trichocarpa* as an outgroup). Both $\theta_{0\text{-fold}}/\theta_{4\text{-fold}}$ and d_N/d_S estimates were significantly

lower in non-eGenes compared to eGenes (Fig 5E,G), likely reflecting stronger purifying selection

acting on non-eGenes. As has been reported previously for *Capsella grandiflora* [28] and a wild

population of baboons [75], we observed a negative correlation between minor allele frequency and

eQTL effect size (S16 Fig), however permutation testing revealed that the effect of the winner's

curse was more prevalent in the permuted than in the original data (S17 Fig), which is in contrast to

the results in *Capsella.*

Likewise, we contrasted patterns of sequence diversity between genes located in module cores and

those not (core vs. non-core). Independent of whether a gene was an eGene or not, core genes had

significantly lower levels of genetic diversity and Tajima's D values (Fig 5B,D). In addition, core-

genes also had significantly reduced ratios of non-synonymous to synonymous polymorphisms ($\theta_{0\text{-}}$

$_{\text{fold}}/\theta_{4\text{-fold}}$) and substitutions ($d_N/d_S$) (Fig 5F,H). Again, these patterns were consistent across different

genomics contexts (S15 Fig). Taken together, these results suggest that genes in network module

cores experience reduced rates of molecular evolution due to stronger purifying selection and are

evolving under stronger selective constraint compared to non-core genes.

In order to examine and account for the non-independence among different measures of gene

expression, we performed PCA and regression analyses on representative gene expression measures

in addition to exploring the pairwise correlation among these factors (S18 Fig). The PCA considered

co-expression network connectivity, core vs. non-core, gene expression level and variance and eGene

vs. non-eGene and revealed that PC1, which explained 37.03% of the variation in these five

measures, was mainly dominated by the connectivity of genes in the co-expression network and

whether they are in network module cores or not (Fig 6A, S19A Fig), with a notably smaller

contribution of expression level. Gene expression variance and eGene status showed a strong

influence on PC2 (Fig 6A, S19A Fig) while expression level contributed largely to PC3 (Fig 6A, S19B

Fig). Logistic regression analyses for eGene status (S3 File) identified H^2, co-expression connectivity,

Tajima's D, expression level and core gene status, respectively, as significant predictors. In the case

of core gene status, whether a gene had an associated eQTL (eGene status), expression variance,

expression level and H^2 were the most predictive variables. Similarly, the regression model for gene

expression level identified eGene status, co-expression connectivity, core gene status, nucleotide

diversity and Tajima's D as the most predictive factors, respectively (S3 File).

We then tested for significant (Spearman's rank) correlations between sequence evolution rates and

PCs. The rates of sequence evolution over both short (represented by within-species diversity: θ_π,

Tajima's D and $\theta_{0\text{-fold}}/\theta_{4\text{-fold}}$) and long (represented by between-species divergence, d_N/d_S) timescales

showed significantly negative correlations with the connectivity and core status of genes in the co-

expression network (PC1) (Fig 6B-E). This indicates that the connectivity of genes within the

network both globally and within the local context of expression modules (core status) are key factor

associated with the rates of sequence evolution. PC2, which largely reflected the variance of gene

expression, showed significant positive correlations with patterns of genetic diversity within species

(θ_π and Tajima's D), and significant, although relatively weak, negative correlation with the rate of

protein sequence evolution (Fig 6B-E). In addition, gene expression level (largely represented by

PC3) showed significantly negative correlations with the rates of sequence evolution (Fig 6B-E).

Discussion

There have, to date, been relatively few studies assaying gene expression in natural, outbreeding

populations. Here, we used a collection of individuals of the outbreeding forest tree species *Populus*

tremula sampled across the species range in Sweden (the SwAsp collection), and grown in a clonally

replicated common garden experiment, to assay gene expression using RNA-Sequencing. We

identified prevalent heritability in gene expression levels, with 17% of the genes (5,924) having $H^2 >$

0.5, which is in line with observations in a number of species [5–17,53]. While many previous eQTL

studies were conducted using controlled, inter-specific crosses, these results attest the substantial

genetic variation contributing to gene expression in natural populations, highlighting the potential for

natural selection to modulate expression levels.

We identified more local than distant eQTLs, with local eQTLs explaining significantly more of the

variance in gene expression than distant eQTLs (Fig 2A), which is in agreement with previous studies

[35,48–51]. Although each eSNP was typically associated with only a single gene, many genes were

associated with more than one unique eSNP, with the combined variance explained by these eSNPs

being higher than for the individual eSNPs, both for local and distant eQTLs, and even when

accounting for potential linkage. For a large proportion of eGenes, the identified set of eSNPs

explained a relatively high proportion of the heritable expression variation (median 1.0 and s.d. 2.3;

S20 Fig). While it appeared that a single eSNP often accounted for a large proportion of the

explained heritable variance, due to linkage it is not possible to determine the relative contribution of

each eSNP within a haplotype block, with the apparent contribution of individual eSNPs largely

resulting from the order in which they are entered into the statistical model. While eGenes had

higher H^2 than non-eGenes, as is expected, there were, nonetheless, 2,780 non-eGenes with $H^2 > 0.5$,

potentially reflecting that the expression variance of these genes is controlled by many eSNPs of

small effect size that we lacked the power to detect.

Local eQTLs were most frequently located in regulatory regions (Fig 2D), with UTRs having the

highest density of local eSNPs (~1.5 eSNP per kbp) followed by flanking regions (2 kbp up- and

downstream, ~1 per kbp) and introns (~0.75 per kbp), which is consistent with previous findings in

natural populations of humans [76,77], *Drosophila* [78] and *Capsella grandiflora* [28]. As UTRs are

currently not well annotated in *P. tremula* it should be noted that many SNPs currently classified as

being located in flanking regions may actually reside within UTRs. The global distribution of

genomic contexts for all investigated SNPs (regardless of whether they were eSNPs or not) was

similar to that of both local and distant eSNPs, suggesting no notable ascertainment bias for eSNPs.

We used the gene expression values to constructed a co-expression network. Compared to networks

more typical of systems biology studies, for example the *P. tremula* exAtlas network [65], where

samples originated from different tissues of a single genotype, the pairwise expression correlations

underlying our co-expression network were low. Despite this, the network displayed typical

characteristics, being scale-free with hubs and distinct modules [79]. The simplest explanation for the

presence of natural variation in gene expression would be that each unique expression pattern is

caused by a single eSNP: In network modules, where many genes share the same expression pattern,

a single eSNP modulating the expression of a central regulator would be sufficient to induce similar

expression variation (i.e. co-expression) in the set of connected genes within that network module. In

our data, we observed at least one eGene in 28 of the 38 module cores and one eSNP in 32 module

cores, thus potentially accounting for the co-expression structure of a majority of the modules. In

contrast, genes located in the network periphery have low connectivity and relatively unique

expression profiles. An eSNP modulating expression of such a gene would likely affect only the

peripheral eGene that it is associated with. Consistent with this, genes in module cores (the set of the

most highly connected genes within a module) were under-represented for eGenes and, respectively,

those in the network periphery were enriched for eGenes (Fig 3B). Furthermore, eGene connectivity

was negatively correlated with variance explained by the associated eSNPs (S14 Fig). It has similarly

been reported that hubs in human protein-protein interaction networks are less likely to be

associated with a detectable eQTL and that the effect size of eQTLs is negatively correlated with

connectivity in the protein-protein interaction network [80].

The Salicaceae lineage underwent a relatively recent (58 million years ago) whole-genome duplication

shared by all member species and that remains represented by a large number of paralogous gene

pairs in the genomes of *Populus* species [81]. If many of these duplicated genes are functionally

redundant or in the process of diverging, one would expect them to be overrepresented for eGenes

as sub- or neo-functionalisation requires derived SNPs to drive expression or coding divergence.

However, we saw an under-representation of eGenes in paralog pairs, suggesting that the SNPs that

initially induced expression divergence have reached fixation. Interestingly, we found progressively

lower expression correlation between paralog pairs containing zero, one or two eGenes (Fig 4),

indicating that a subset of paralogs are still undergoing sub- or neo-functionalisation, with their

associated eSNPs driving expression divergence.

A primary aim of our study was to integrate the results of eQTL mapping within the context of the

corresponding gene co-expression network. Regulatory networks are known to be redundant, scale-

free and, therefore, to be inherently buffered against single mutations of large negative effect [59–61].

In our case, genes with the potential to cause such large negative effects would be observed as hubs

in the co-expression network globally or as core genes within modules at the local scale. We would

therefore expect hub/core genes to be buffered against single polymorphisms of large effect size

and, in particular, the persistence of large negative effect mutations. As such, we could expect the

expression of highly connected genes to be subjected to stabilizing selection and the associated

eSNPs to be under purifying selection. In support of these expectations, our results indicate that

connectivity within the co-expression network is associated with the rate of sequence evolution and

that connectivity and core status are more influential than expression level (Fig 6, S3 File).

Connectivity and core status contributed primarily to independent PCs than expression level (Fig

6A), with connectivity being correlated negatively with signatures of sequence evolution at both

short (Fig 6B-C) and long (Fig 6D-E) timescales. In contrast, expression level exhibited low

correlation to sequence evolution at short timescales, but higher negative correlations over long

timescales (Fig 6B-E). Genes with high co-expression connectivity, mostly those within network

module cores, have significantly reduced genetic diversity and exhibit reduced rates of protein

evolution compared to more peripheral genes (Fig 5). This suggests that core genes, which represent

genes of high potential effect, have evolved under stronger evolutionary constraint than genes in the

periphery of the network. As genes in module cores generally have higher levels of pleiotropy and

lower levels of dispensability [59], they are consequently more constrained against changes in both

gene expression and protein sequence compared to genes in the periphery of the network [82].

Furthermore, we show that eGenes are overrepresented among non-core genes of the network and

that they have experienced lower levels of purifying selection relative to non-eGenes, corroborating a

relaxation of both expression and coding constraints for these genes. Relaxed selection of peripheral eGenes is thus expected to result in an accumulation of weakly deleterious mutations that will segregate as intra-specific polymorphisms. Consistent with these observations, we found that conserved genes (*i.e.* old genes) were significantly over-represented in network cores while *P. tremula* specific genes (*i.e.* young genes) were under-represented. This is in agreement with previous studies that have shown that evolutionarily ancient genes tend to be more central in regulatory networks, with increased constraints on expression and fewer associated eQTLs [83,84]. Taken together, these results indicate how the co-expression network can be buffered against large perturbations via constraint of core genes while enabling flexibility and adaptation by tolerating an accumulation of mutations within the network periphery.

Conclusion

We identified substantial heritability of gene expression within a natural, out-breeding population, of *P. tremula*. Polymorphisms associated with expression variance were most frequently located within regulatory regions, suggesting that they act by inducing expression variance. The gene co-expression network displayed typical characteristics, being scale-free and containing a small number of highly connected genes, which were underrepresented by eQTLs and with a negative correlation between eQTL effect size and network connectivity. Integration of the eQTL and population genetics analyses with characteristics of the associated gene co-expression network highlight that the context of a gene within the co-expression network appears to be an important determinant of the evolutionary dynamics of transcribed loci. Our results point towards stronger selection acting on network core genes compared to genes in the periphery of the co-expression network, with a negative correlation between rates of sequence evolution and gene connectivity, suggesting that the majority of segregating variation associated with gene expression is under purifying selection.

Materials and Methods

Samples

We collected branches form the SwAsp collection common garden in north of Sweden on 27th May 2012, before natural bud break but as close to the point of natural spring bud break as possible. Branches were placed in the greenhouse facility at the Umeå Plant Science Centre under conditions selected to induce rapid bud break (24 h light, temperature of 20 °C and humidity 50-70 %). At a defined point of emergence (S21 Fig) buds were harvested, flash frozen in liquid nitrogen and stored at -80 ° C until used for RNA isolation. Only terminal buds were sampled (i.e. no lateral buds were included). The time from the day branches were placed in the greenhouse until bud flush sampling ranged from one day to eight days (S22A Fig) and there was a high, positive correlation to bud flush date recorded in the field for the same year (S22B Fig; r=0.776, p < 2.2×10^{-16}). As has previously been reported [85], there was very low Q_{ST} for bud flush, either in the field or for the greenhouse material (Q_{ST} 0.13 and 0.07 respectively), however H^2 was high (H^2 = 0.82 and 0.71 respectively).

RNA isolation

One to two buds per clonal replicate were ground using one 3 mm stainless steel bead (Qiagen, Redwood city, USA) in Corning® 96 well PP 1.2 ml cluster tubes (Sigma-Aldrich, St. Louis, USA) using a Mixer Mill MM400 (Retsch, Haan, Germany) at 20 Hz for 2 x 15 sec. Total RNA was extracted from all samples according to [86] with the omission of the L spermidine. Buffer volumes were adjusted according to starting material (70 - 130 mg). RNA isolation was performed using one extraction with CTAB buffer followed by one chloroform : isoamyl alcohol IAA (24:1) extraction. All other steps were performed as in [86]. DNA contamination was removed using DNA-free™

535   DNA removal Kit (Life Technologies, Carlsbad, USA). RNA purity was measured using a

536   NanoDrop 2000 (Thermo Scientific, Wilmington, USA) and RNA integrity was assessed using the

537   Plant RNA Nano Kit for the Bioanalyzer (Agilent Technologies, Santa Clara, USA).

RNA-Sequencing and analysis

RNA-Sequencing was performed as in [73]. Briefly, paired-end (2×100 bp) RNA-Seq data were

generated using standard Illumina protocols and kits (TruSeq SBS KIT-HS v3, FC-401-3001; TruSeq

PE Cluster Kit v3, PE-401-3001) and all sequencing was performed using the Illumina HiSeq 2000

platform at the Science for Life Laboratory, Stockholm, Sweden. Raw data is available at the

European Nucleotide Archive (ENA, [87]) with accession number ERP014886.

RNA-Seq FASTQ-files were pre-processed and aligned to v1.0 of the *P. tremula* reference genome

[74] as in [88]. In short, reads were quality and adapter trimmed using Trimmomatic v0.32 [89],

rRNA matching reads were filtered using SortMeRNA v1.9 [90], reads were aligned to the v1.0 *P.*

tremula reference genome using STAR 2.4.0f1 [91] and read counts were obtained using htseq-count

from HTSeq [92]. FastQC [93] was used to track read quality throughout the process. Normalised

gene expression values were obtained by applying a variance stabilising transformation (VST) to the

raw counts from HTSeq, as implemented in the DESeq2 R package [94].

Gene expression H^2 and Q_{ST}

552   We calculated repeatability as an assumed upper bound estimate of broad sense heritability of gene

553   expression (see [95] for discussion) from the variance estimates in our data according to the equation

554
$$H^2 = \frac{V_G}{V_P}$$

where V_G is the genetic component of the variance calculated as the expression variance between

genotypes for a particular gene (*i.e.* variance among individual means) and V_P is the total phenotypic

variance calculated as the sum of V_G and V_E, where V_E is the environment component of the

variance calculated as the expression variance within genotypes for a particular gene (i.e. the mean

variance among clonal replicates). Point estimates of H^2 were obtained using the repeatability

function from the heritability v1.1 R package [96]. The significance of the broad sense heritability

was estimated by using an empirical null model where heritabilities were based on random genotype

assignments. For each gene, 1,000 permutations were performed and empirical p-values were

calculated using the empPvals function in the qvalue 2.6.0 R package.

Population differentiation (Q_{ST}; [97]) was calculated as

$$565 \qquad Q_{ST} = \frac{V_{between}}{V_{between} + 2V_{genetic}}$$

where $V_{between}$ is the variance among populations and $V_{genetic}$ is the genetic variance among genotypes

as computed using the lmer function from the lme4 v1.1.12 R package [98] using the formula

`expression ~ 1 + (1|population) + (1|clone)`

where *expression* is the expression of a gene, *population* is a factor representing the population of each

sample, and *clone* is a factor representing genotype replicates. As we use repeatability as an upper

bound estimate of H^2, our Q_{ST} estimates are conservative [99]. Similar to the broad sense heritability,

the significance of the Q_{ST} was estimated using permutation testing where genotype labels were

shuffled among the populations 1,000 times. Due to the characteristics of the data and the method,

many of the permutations resulted in undefined QST estimates (an average of 511 missing values per

gene). Thus, the empirical p-values for the genes were based on different number of null values.

Hidden confounder removal

Genotype mean gene expression data was adjusted for hidden confounders before mapping eQTLs and constructing the co-expression network. Hidden confounders in the gene expression data was accounted for by regressing out the 9 first principal components (PCs) of the gene expression data [77,100,101]. The number of components to remove was determined by running the eQTL mapping with 0 to 20 PCs removed and selecting the number of components that yielded the largest number of significant eQTLs (Benjamini-Hochberg $p < 0.05$) (S5 Fig). This approach was based on the assumption that the number of identified eQTLs would increase if the removed PCs were removing unwanted, systematic variation (i.e. noise) rather than informative biological variation [77,100–103].

eQTL mapping

eQTL mapping was performed by associating genotype mean gene expression levels with biallelic SNPs using the R package Matrix eQTL v2.1.1 [104]. The corresponding raw resequencing data for all SwAsp genotypes is available at the NCBI SRA resource as BioProject PRJNA297202 (SRA: SRP065057) and the resultant Variant Call Format (VCF) file used for association mapping is available from [66]. Before performing association mapping, genes were filtered on variance so that only genes with a gene expression variance above 0.05 were included. SNPs were also filtered on minor allele frequency (MAF) and major genotype frequency (MGF); any SNPs with MAF < 0.1 or MGF > 0.9 were excluded to avoid spurious associations. A motivating example for the MGF filtering can be seen in S23 Fig. We also generated an LD-trimmed SNP set by removing one SNP from each pair of SNPs with a between SNP correlation coefficient (r^2) >0.2 in blocks of 50 SNPs using PLINK v1.9 [105], yielding 217,489 independent SNPs that were retained for analyses of population structure. The first genotype principal component based on the set of independent SNPs was used as a covariate in the linear model used by Matrix eQTL to account for the weak signature

of population structure. Permutation testing was used to determine eQTL significance whereby

genotype sample labels were permuted 1,000 times and the maximum absolute t-statistic from Matrix

eQTL was recorded for each expressed gene across all SNPs for each permutation, resulting in 1,000

random t-statistics being collected for each gene. Empirical p-values were calculated for each eQTL

in the observed data using the permuted t-statistics for the observed eGene with the empPvals

function in the qvalue v2.4.2 R package [106], and q-values (empirical FDR) were calculated with the

qvalue function in the same package.

When determining the genomic context of eSNPs, there were some cases where introns overlapped

exons as a result of overlapping gene model being present on the same strand. These 41 eSNPs were

discarded from the counting. Another type of overlap that was discarded were cases where an eSNP

overlapped a gene feature, but no sub-feature inside that gene (e.g. UTR, exon or intron). These

1961 eSNPs were excluded from the counting. Since many of the features overlap (e.g. exon and

untranslated regions), the priority for counting was untranslated region, exon/intron,

upstream/downstream and intergenic.

Permutation tests involving eGenes and non-eGenes were performed by shuffling eGene

assignments among the 22,306 genes that were considered for eQTL mapping. This was repeated

10,000 times and empirical p-values were calculated using the empPvals function in the qvalue v2.6.0

R package. The minor allele frequency (MAF) was compared between eSNPs (both local and distant)

and all SNPs throughout the genome. To test for the signature of selection on eQTLs, we also

estimated the correlation between minor allele frequency and eQTL effect size.

 Co-expression

The R package WGCNA v1.51 [107] was used for constructing a co-expression network. The input

gene expression values were per-gene genotype means corrected for hidden confounders (the same

data used for eQTL mapping). We chose to use the unsigned network type for this study with the

motivation that we did not want to discard negative relationships. By looking at this from an eQTL

perspective, an eSNP can be positively associated with one gene while negatively associated with

another. The relationship between these two genes would be missed if we used the signed or the

signed-hybrid approaches. Using the unsigned approach, we assure that genes with strong negative

correlation end up in the same network modules. A soft thresholding power of 5 was used to

calculate adjacencies. The topological overlap matrix (TOM) was generated using the TOMsimilarity

function with the signed approach in order to take negative edges into account (see [107] for details).

In order to identify network modules, hierarchical clustering was applied to the TOM dissimilarity

matrix (1 – TOM) and the resulting dendrogram was divided into modules using the cutreedynamic

function. The connectivity of the network was then defined as the adjacency sum for each node, i.e.

the weights of the edges that are connected to this node. This concept was applied to modules as

well to obtain measures of intra- and inter-modular connectivity, i.e. the connectivity based on edges

connecting the gene with other genes inside the same module, and connectivity based on edges

connecting the gene with genes outside of the module.

To define the periphery of the network we applied a hard edge-threshold to the network where only

gene-pairs with an absolute Pearson correlation > 0.4 were linked, which corresponded to the top

0.1% most correlated gene pairs. Genes were then classed as peripheral if they linked to only one

other gene.

Conserved genes and *P. tremula*-specific genes were inferred from gene family analysis based on

protein sequence similarity available on the PopGenIE FTP server [108]. In short, two rounds of

TribeMCL were run using BLASTP with an E-value threshold of 10^{-5} where the first round was all-

vs-all while the second was all-vs-all inside each gene family.

Transcription factors were identified by BLASTing the the *P. tremula* protein sequences against the

protein sequences of transcription factors in *P. trichocarpa* with an E-value threshold of 10^{-10} and

taking the best hit for each *P. trichocarapa* transcription factor. The *P. trichocarpa* transcription factor

annotations were taken from Plant Transcription Factor Database v3.0 [109].

All permutation tests based on the network were performed by computing the measure of interest on

the original network, and then performing the same computation on 10,000 networks were the gene

labels were shuffled. Empirical p-values were calculated using the empPvals function in the qvalue

v2.6.0 R package [106].

Population genetic analysis

To estimate nucleotide diversity (θ_π) and Tajima's D [110] among genes, we only used the reads with

mapping quality above 30 and the bases with quality score higher than 20 for the estimation of θ_π

and Tajima's D by ANGSD [111]. Genes with less than 50 covered sites left from previous quality

filtering steps (Wang et al. In prep) were excluded. In addition, θ_π and Tajima's D were also

calculated for seven site categories of each gene: 1kbp upstream of genes, 1kbp downstream of

genes, 3' UTR, 5' UTR, intron, 0-fold non-synonymous and 4-fold synonymous sites within genes.

We further compared the estimates of $\theta_{0\text{-fold}}/\theta_{4\text{-fold}}$ between different categories of genes. We used the

transcript with the highest content of protein-coding sites to categorize the seven genomic features

within each gene. Finally, we estimated the ratio of non-synonymous substitution and synonymous

substitution rate (d_N/d_S) using gKaKs pipeline [112] with codeml method [113] for a total of 33,039

orthologous genes that were determined by best BLASTp sequence homology searches between *P. tremula* and *P. trichocarpa* [74,108]. Significance for the above statistical measurements between each pair of gene categories was evaluated using Mann-Whitney tests.

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Figures

Fig 1. Population location and gene expression overview. (A) Map of the original locations of the SwAsp populations. The red arrow points to the location of the common garden used in this study. (B) Distribution of gene expression heritability for all genes and for the subset of genes after filtering to remove uninformative expression. (C) Distribution of gene expression Q_{ST} for all genes and for the subset of genes after filtering to remove uninformative expression. (D) Sample clustering based on all samples, including biological replicates. The heatmap represents the sample correlation matrix based on the 500 genes with the highest expression variance. Darker colour indicates higher correlation. The coloured bar represents the populations the samples belong to. The small clusters on the diagonal correspond to biological replicates of each genotype.

Fig 2. eQTL overview. (A) Expression variance explained (R^2) for local and distant eQTLs. Box plots show the maximum variance explained by a single eQTL for each gene and the total variance explained by all eQTLs for each gene. The widths of the boxes are proportional to the number of genes represented. The pairwise significiance of a Mann-Whitney test is indicated by asterisks: ***$p <$ 0.001, **$p <$ 0.01. (B) Genomic context of local and distant eSNPs. Context categories are normalized for feature length. When an eSNP overlapped with features on both strands, both of them were counted. For both local and distant eQTLs the features are based on the gene that is closest to the eSNP, and furthermore, for local eQTLs, the features are divided into whether the eSNP is located in or near the same gene that it is associated to ("associated gene") or not ("other gene"). Flanking regions represent 2 kbp upstream and downstream from the gene. (C) Scatter plot showing the positions of all significant eQTLs in this study. No evidence of eQTL hotspots can be observed. Numbers indicate chromosome. (D) Broad sense heritability distributions for eGenes and

non-eGenes. (E) Manhattan plots for local eQTLs (upper) and distant eQTLs (lower). Each point

represents an eQTL.

Fig 3. Co-expression characteristics of eGenes and eSNPs. (A) Gene expression variance

distribution for all genes in the SwAsp data (before removal of hidden confounders) and the exAtlas

data. (B) Distribution of co-expression connectivity for eGenes and non-eGenes. (C) Distributions

of the proportion of total variance explained and heritability for eGenes and non-eGenes divided

into core and non-core genes. (D) Genes having distantly, locally, or both distantly and locally acting

eSNPs located within 2 kbp (or inside) the gene divided into core and non-core genes. (E) Genomic

context of distantly, locally, or both distantly and locally acting eSNPs located within 2 kbp of an

eGene divided into core and non-core eGenes. The eSNP counts are normalised for total feature

length.

Fig 4. Correlation within paralog pairs as a function of the number of eGenes in the paralog

pair. The widths of the boxes are proportional to the number of genes in each set. The mean

correlations for paralog pairs with 0, 1, or 2 eGenes were 0.17, 0.10, and 0.06, respectively. The

pairwise significance of a Mann-Whitney test is indicated by asterisks: ***p < 0.001, **p < 0.01.

Fig 5. Measures of sequence diversity and divergence. Nucleotide diversity (A,B), Tajima's D

(C, D), $\theta_{0\text{-fold}}/\theta_{4\text{-fold}}$ (E,F) and d_N/d_S (G,H) are compared between eGenes (with local eQTLs or with

only distant eQTLs) and non-eGenes, as well as core and non-core genes from the gene expression

network. Significance between each pair of gene categories was evaluated using Mann-Whitney tests

and significance is indicated by asterisks above the boxplot: ***p < 0.001, **p < 0.01, *p < 0.05, n.s.

p >0.05.

Fig 6. Associations between metrics of gene expression and sequence evolution in *Populus*

tremula. (A) Percentage variance explained by five principal components comprising co-expression

network connectivity, genes in network cores or not (Core vs. non-core genes), gene expression

levels, gene expression variance and genes with eQTLs or not (eGenes vs. non-eGenes). Colour

shadings depict the proportion contribution of each gene expression measure to each principal

component. (B-E) Spearman's rank correlations between PCs and four metrics of sequence

evolution: nucleotide diversity (θ_π), Tajima's D, $\theta_{0\text{-fold}}/\theta_{4\text{-fold}}$ and d_N/d_S. Stacking of the barplots

shows the relative contribution of each gene expression measure to each PC. Asterisks indicate

significant correlations, ****P* < 1e-5, ***P* < 0.001,**P*<0.05, ns=not significant (*P* >0.05).

Supporting information

1028

S1 File. GO enrichment analysis results. Contains GO enrichment results for local and distant

eGenes, network module cores, and subsets of genes with extreme H^2 and Q_{ST} values.

S2 File. Various gene statistics. Contains statistics for all genes, as well as all genes included in the

generation of the co-expression network and the eQTL mapping.

S3 File. HTML document outlining the regression models constructed. Regression models

were constructed in R using a number of starting variables. Uninformative variables were then

removed by backward elimination using the stepAIC function from the MASS package resulting in a

reduced model.

S1 Fig. Clustering of genotypes. (Left) Heatmap of the sample correlation matrix based on the 500

most variably expressed genes. Darker colour indicates higher correlation. The coloured bar

represents the populations the genotypes belong to. (Right) The two first principal components from

a principal component analysis (PCA) based on all genes. Again, colours represent the genotype

population. The percentages in the axis labels indicate the amount of variance explained by each

component.

S2 Fig. Correlations between gene expression principal components (PCs) and

environmental variables. The values in each tile represent the Pearson correlation between the

gene expression PC (x-axis) and environmental variable (y-axis). Small asterisks represent a nominal

p-value < 0.05 while large asterisks represent Benjamini-Hochberg (BH) adjusted p-values < 0.05.

The only factor with significant correlations to expression PCs was "Hours to harvest", which is the

number of hours into the sampling period that the buds were harvested. It was significantly

associated with PC4 (BH-adjusted $p = 4.6 \times 10^{-6}$), PC7 (BH-adjusted $p = 0.030$) and PC2 (BH-

adjusted $p = 0.033$).

S3 Fig. Relationship between gene expression ranks and the number of genotypes with

detectable expression of the genes. The number of genotypes that a gene was expressed in was

determined by counting the number of genotypes with non-zero expression for each gene. The gene

expression ranks were calculated by ranking the mean gene expression values where the mean was

calculated only considering samples with non-zero expression.

S4 Fig. Physical distance between eSNPs and their associated genes for each of the 19

chromosomes.

S5 Fig. The number of eQTLs detected for different levels of hidden confounder removal.

The y-axis shows the number of eQTLs detected at 5% FDR (prior to the empirical FDR

calculation). The x-axis shows the number of principal components regressed out of the gene

expression data. Distant eQTLs are defined as eQTLs where the gene and the SNP are located on

different scaffolds.

S6 Fig. Heatmap representing the sample clustering based on gene expression values after

hidden confounder removal. The 500 most variable genes in the original expression data were used

for calculating the sample correlations (i.e. the same genes as in Fig 1D). The colour bar represents

the population of the samples with the same colour scheme as in Fig 1D. The small clusters on the

diagonal represent genotype replicates.

S7 Fig. Distribution of local eSNPs relative to the gene that they are associated to. Up and

downstream regions were divided into 1 kbp bins while the intergenic region was normalised to 20

bins. In other words, up and downstream counts are not directly comparable to intragenic counts.

S8 Fig. Heritability distributions for four non-overlapping sets of genes: genes with both local

and distant eQTLs, genes with only distant eQTLs, genes with only local eQTLs, and genes with no

significant eQTLs. The box widths are proportional to the number of genes in each set.

S9 Fig. Gene expression variance plotted against co-expression network connectivity. eGenes

are indicated by red points.

S10 Fig. Sliding window approach to detect eQTL hotspots. The upper panel shows the

number of eSNPs in genomic windows of 100 kb for each of the 19 chromosomes. The lower panel

shows the number of unique genes that are associated to each genomic window (nominal eQTL p-

value < 1×10^{-6}).

S11 Fig. eSNPs per eGene and eGenes per eSNP. (A) Bar plot of the number of associated

eGenes per eSNP. (B) Histogram of the number of associated eSNPs per eGene. (C) The mean co-

expression for genes associated with the same eSNP. The numbers on the x-axis represents the

number of eGenes that a single eSNP is associated to, and the numbers above the boxes shows the

total number of eSNPs that are represented, and in parentheses are the number of unique genes that

they are associated with.

S12 Fig. The number of genes and the number of core genes in each network module. Each

bar is divided into eGene and non-eGene assignments. Module 0 contains genes not assigned to any

module.

S13 Fig. Gene expression variance in module cores and non-cores for each of the 38

modules.

S14 Fig. Relationship between total variance explained by eQTL and co-expression

connectivity. The variance explained is the adjusted variance explained based on all eQTL

associated with each gene.

S15 Fig. Comparisons of nucleotide diversity and Tajima's D for different genomic features.

Upstream and downstream are 1 kbp away from the gene start and end, respectively. 0-fold and 4-

fold refers to 0-fold and 4-fold degenerate sites, respectively.

S16 Fig. Minor allele frequency of eSNPs. (A) Comparison of minor allele frequency between all

SNPs, distant eSNPs and local eSNPs. (B) The relationship between minor allele frequency and

effect size (absolute value of beta) of eQTLs.

S17 Fig. Relationship between MAF and effect size in permuted eQTLs. eQTL permutations

were done by selecting 20 random subsets of 150,000 SNPs each. Each of these sets were associated

with gene expression levels, and in addition, 50 permutations were performed for each set by

shuffling the genotype labels of the SNP data, resulting in a total of 1,000 permuted eQTL sets. (A)

The number of SNPs for the nominal p-value threshold 10^{-6} (the same initial threshold that was

used for the original associations). (B) The Spearman correlation between minor allele frequency and

absolute effect size for the 20 sets and their corresponding permutations. (C) Allele frequency

spectra for the 20 subsets and their permutations.

S18 Fig. Association between metrics of gene expression and sequence evolution. Scatter

plots (lower off-diagnal, the red-to-yellow-to-blue gradient indicates decreased density of observed

events at a give location in the graph) and correlations with probability values (upper off-diagonal,

spearman's rank correlations) for measures of gene expression and sequence evolution: kTotal, the

total (global) connectivity in the network; is_core_gene, gene is part of module core or not;

orig_expr_mean, mean expression before hidden confounder removal; orig_expr_var, expression

variance before hidden confounder removal; is_egene, gene with eQTLs or not; tP, pairwise

nucleotide diversity; tajD, Tajima's D; tP_0_4, the ratio of pairwise nucleotide diversity at zero-fold

non-synonymous and four-fold synonymous sites; kaks, dN/dS.

S19 Fig. Loadings of five gene expression measures on PC1 to PC5. It comprises co-expression

network connectivity, genes in network cores or not (Core vs. non-core genes), gene expression

levels, gene expression variance and genes with eQTLs or not (eGenes vs. non-eGenes).

S20 Fig. Variance explained by eSNPs and heritability. This figure assumes that eSNPs only

account for heritable variation, explaining why some of the eSNPs explain more than 100% of the

heritability. The x-axis has been truncated at 300%.

S21 Fig. Representative photo of the sampled bud flush stage.

S22 Fig. Sampling time and day of bud flush. (A) Box plot distributions of the Julian day of

sampling for the SwAsp sub-populations. (B) The relationship between Julian day of bud flush for

the greenhouse sampled buds and Julian day of bud flush in the field for the same year (2012).

S23 Fig. Motivating example for major genotype filtering. In this case the SNP is heterozygous

in all samples but one, resulting in a minor allele frequency close to 0.5, but a major genotype

frequency close to one. The resulting association turns out very significant, but is only supported by

a single sample.

A

B

C

- Connectivity
- Core vs. non-core
- Expression level
- Expression variance
- eGene vs. Non-eGene

A

Population Latitude

B

rho = 0.766, P < 0.001

Day of year (Field 2012)