Supplementary Protocol

RNA-seq data analysis and identification of tissue-specific genes and transcription factors

Publicly available RNA-seq data from the Jia et al. study (Jia *et al.*, 2023) was used to identify tissue-specific genes and transcription factors (TFs) in five distinct tissues: stem, root, capsule, leaf and petal (**Supplementary Table 1**). The quality of the raw RNA-seq data was assessed using FastQC v0.11.9. (Andrews, 2010) and adapters were trimmed (where applicable) using Trim Galore v0.6.7 (https://github.com/FelixKrueger/TrimGalore). The clean data was aligned against the *Papaver somniferum* reference genome (cv. HN1; accession number GWHAZPJ00000000.1) (Yang *et al.*, 2021) using HISAT2 v2.2.1 (Kim *et al.*, 2019). The mapped reads were sorted using Samtools v1.14 (Danecek *et al.*, 2021) and transcript per million (TPM) counts were generated using StringTie v2.1.3 (Pertea *et al.*, 2015). Principal component analysis (PCA) plots were generated in R v4.3.2 (R Core Team, 2013) using the log2-normalised TPM counts to identify any outliers and ensure that the biological replicates clustered according to tissue type. Tissue-specific genes and TFs were identified using the mean TPM counts of biological replicates for each tissue type and the tau (T) metric (Kryuchkova-Mostacci and Robinson-Rechavi, 2017); a gene was deemed to be tissue-specific if it had the highest mean TPM count in one tissue type relative to the others and had a corresponding $T > 0.8$. Heatmaps showing gene expression of tissue-specific genes were generated using the *Z*-score of log2-normalised mean TPM counts and the pheatmap package in R (Thimm *et al.*, 2004). TFs were annotated using iTAK v1.7a33 (Zheng *et al.*, 2016).

Weighted gene co-expression network analysis (WGCNA)

Since co-expression may involve co-regulation, co-expression modules were generated to identify potential regulators (TFs) of known BIA biosynthesis pathway genes, especially the ones that are involved in the (*S*)-reticuline, noscapine and morphine pathways (**Supplementary Table S3**). First, gene-level quantification was performed on the mapped reads from the Jia et al. (2023) dataset using featureCounts from the Subread v2.0.6 package (Liao *et al.*, 2013, 2014). Genes with low expression variability (*p*-value 0.05) across samples were then filtered out using varianceBasedfilter from the R package DCGL (Liu *et al.*, 2010). A total of 13,037 (out of 55,316) genes remained; these genes were assigned to co-expression modules using the R package WGCNA (soft threshold =18; minClusterSize=50) (Langfelder and Horvath, 2008,

2012). Modules with similar gene expression profiles were merged (cutHeight=0.2) resulting in a total of 13 modules.

Gene regulatory network analysis

WGCNA identified a large number of TFs that are co-expressed with known BIA biosynthesis pathway genes. However, it is unlikely that every TF identified is a potential regulator of BIA biosynthesis. In order to determine which of these TFs share a regulatory link with the genes of interest, gene regulatory networks (GRNs) were constructed using SCION v4.0 (default parameters; clustering=none) (Clark *et al.*, 2021). Since SCION requires a minimum of five samples/replicates to build a network and the Jia et al. (2023) dataset only had three stem samples, we also included the stem samples from the Guo et al. (2018) dataset (Guo *et al.*, 2018).

Given the highly tissue-specific nature of the BIA biosynthesis genes from the three selected pathways ((*S*)-reticuline, noscapine and morphine), two GRNs were generated: a stem-specific GRN and a root-specific GRN. The data used to build the stem-specific network was as follows: 1) row-normalised TPM values of genes expressed in stem tissue (SRA accessions: SRR15146387, SRR15146388, SRR15146389, SRR6782291, SRR6782298); 2) a list of target genes, including BIA biosynthesis genes, that are stem-specific ($T > 0.8$) and found in co-expression modules (**Supplementary Table 2**); 3) a list of TFs that are stem-specific ($T > 0.8$) and found in co-expression modules (**Supplementary Table S4**). BIA biosynthesis genes that did not meet the criteria for tissue-specificity or found in co-expression modules were also included in the list of targets (**Supplementary Table 4**). The above steps were repeated to build the root-specific GRN except that root-specific data (SRA accessions: SRR15146362, SRR15146363, SRR15146367, SRR15146384, SRR15146385, SRR15146386), including root-specific genes and TFs (**Supplementary Table 2**) was used as input instead. Four subnetworks were extracted from the two GRNs: a stem-specific morphine subnetwork, a root-specific morphine subnetwork, a root-specific noscapine subnetwork and a root-specific (*S*)-reticuline subnetwork. The subnetworks were visualised in Cytoscape v3.10.2 (Shannon *et al.*, 2003). For ease of visualisation, the subnetworks were filtered based on the following edge weight cut-offs (0.5 for the stem-specific subnetwork and 0.75 for the root-specific subnetworks).

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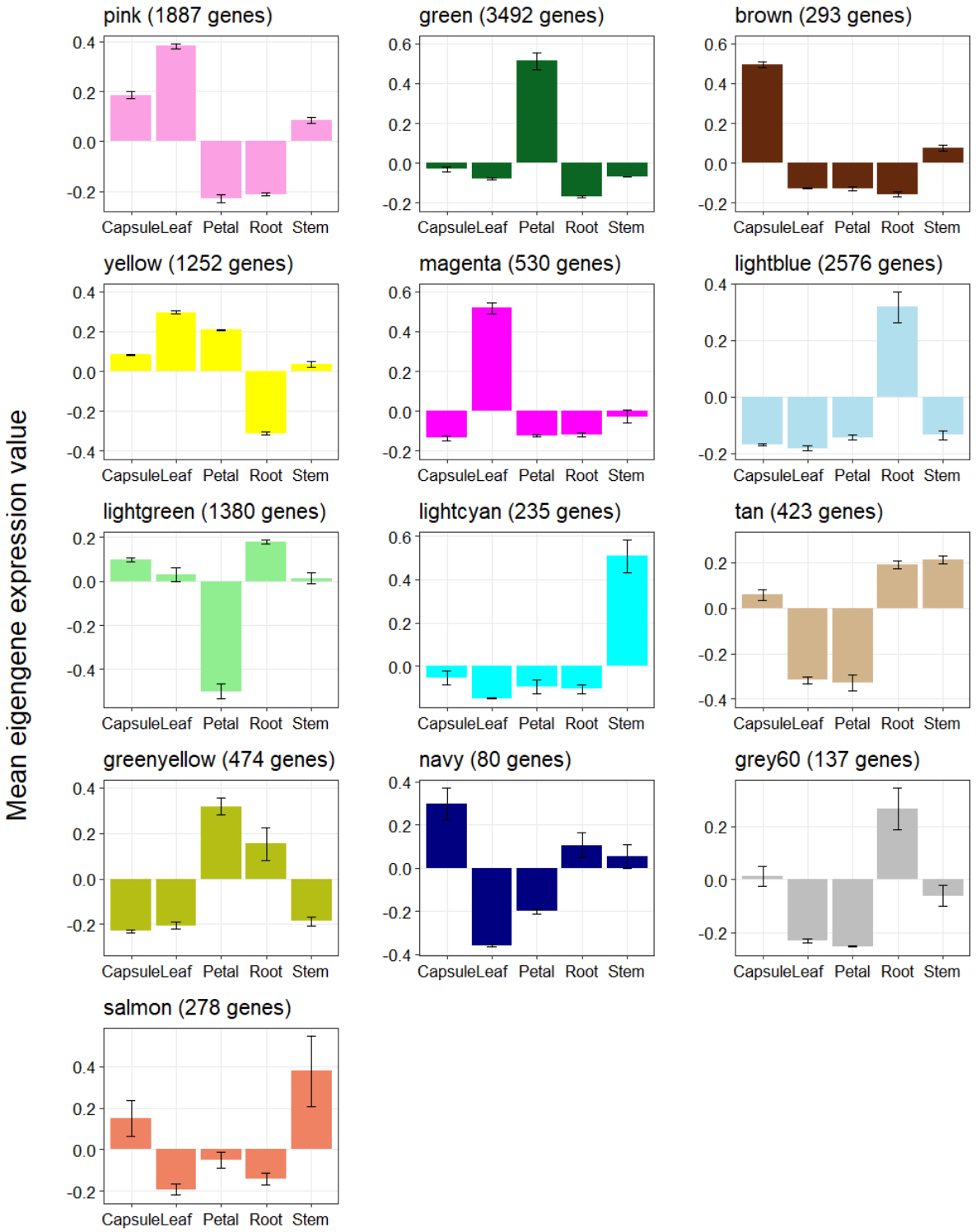
Supplementary Figures



Supplementary Figure 1. Mean eigengene expression value for each tissue across the 13 co-expression modules identified by WGCNA. Numbers in parentheses represent the number of genes and transcription factors in each module. Values are mean ± standard error.