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Evolutionary flexibility in flooding response circuitry in angiosperms

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Abstract

Flooding due to extreme weather threatens crops and ecosystems. To understand variation in gene regulatory networks activated by submergence, we conducted a high-resolution analysis of chromatin accessibility and gene expression at three scales of transcript control in four angiosperms, ranging from a dryland-adapted wild species to a wetland crop. The data define a cohort of conserved submergence-activated genes with signatures of overlapping *cis*-regulation by four transcription factor families. Syntenic genes are more highly expressed than non-syntenic genes, yet both can possess the *cis*-motifs and chromatin accessibility associated with submergence upregulation. While the flexible circuitry spans the eudicot-monocot divide, the frequency of specific *cis*-motifs, extent of chromatin accessibility, and the degree of submergence-activation is more prevalent in the wetland crop and may have adaptive significance.

One Sentence Summary:

Conserved submergence-activated gene families display flexibility in regulatory circuitry.

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Data and material availability: Sequence data deposited in GEO (accession GSE128680), code and resources in http://plant-plasticity.github.io/data-and-code/. All other data are in the main paper or supplement.

Contacts for genetic materials: rice, J.B.-S.; *Medicago*, R.B.D.; tomato, S.B., *S. pennellii*, N.S..

Climate change has increased the frequency and intensity of floods that impact agricultural productivity. Of major crops, only rice (*Oryza sativa*) is resilient to waterlogging of roots and submergence of aerial tissue, due to adaptation to a semi-aquatic habitat. Other angiosperms experience intermittent flooding and are not adapted to these conditions. Submergence triggers signaling in plant cells as a consequence of entrapment of the gaseous hormone ethylene and depletion of available oxygen, (hypoxia) leading to inefficient anaerobic metabolism and energy starvation (1) To understand the variation in response to submergence, we studied rice as a representative monocot and flood resilient species, the legume *Medicago truncatula*, and two *Solanum* species, domesticated tomato (*S. lycopersicum* cv. M82) and its dryland-adapted wild relative *S. pennellii* (Fig. 1A). Roots are the first responders to flooding, and we thus monitored the early response of seedling apical root tips to complete seedling submergence. By monitoring the sentinel response gene family *ALCOHOL DEHYDROGENASE (ADH*), required for anaerobic production of ATP (1) (Fig. 1B), we identified 2 hours, the mid-point of maximal upregulation, as a physiologically relevant time to compare initiation of the submergence response across species.

To conserve energy under hypoxia, stress-induced mRNAs are preferentially translated over transcripts associated with development in the model *Arabidopsis thaliana* (2-4). We therefore considered both transcriptional and post-transcriptional regulation under submergence across the species surveyed. To do so, we deployed Isolation of Nuclei TAgged in specific Cell Types (INTACT) (5) and Translating Ribosome Affinity Purification (TRAP) (6), using constitutive promoters. INTACT was used to profile chromatin accessibility by ATAC-seq (Assay for Transposase-Accessible Chromatin) (7), and measure the abundance of nuclear RNA (nRNA). TRAP was used to monitor ribosome-associated polyadenylated mRNA (TRAP RNA) and evaluate the position of individual ribosomes along transcripts (Ribo-seq) (8) (Fig. 1C, fig. S1-2). We also profiled total polyadenylated mRNA (polyA RNA). Multidimensional scaling analysis confirmed the reproducibility and distinctness of each of the RNA sub-populations and their changes following submergence (fig. S3, data S1-2).

Flood-adapted rice displayed the greatest plasticity in terms of the number of differentially up- and downregulated transcripts (Fig. 1D, fig. S4, data S3). Cultured hairy roots (*Sl*-HR) were used as a contrast to intact roots of tomato (*Sl*) plants, and were more responsive. The clustering of modulated RNAs resolved variation in regulation in all four species (Fig. 1D, figs. S5-9). Rice gene regulation was coordinated across scales (except in clusters 7 and 8 where transcripts were enriched or depleted in the nucleus). In *M. truncatula* and tomato, regulation of gene activity was more evident in the ribosome-associated RNAs, whereas in the dryland-adapted *S. pennellii* regulation was evident as nRNA enrichment or depletion.

Selection likely acts on species-specific traits and adaptation to specific environments that are largely regulated by a common set of gene families. The root meristem is frequently oxygen-deprived due to high metabolic activity and periodic soil inundation; therefore, its capacity to transiently upregulate anaerobic metabolism might be expected in all species. Yet, rice may have evolved a higher proportion of gene family members that are regulated by submergence than flooding-sensitive species. We leveraged gene families (9) to investigate

conservation in submergence-responsive genes of the four species, focusing on the shared families (6685) plus those conserved between the two *Solanums* (3301) (Fig. 1E, data S4). Tabulation of the submergence-responsive gene family members of each species identifyied families with at least one member differentially controlled in any of the RNA populations evaluated (Fig. 1F, fig S10, data S5). This uncovered a set of 68 submergence-upregulated families (SURFs: 249 genes in *Os*, 121 in *Mt*, 137 in *Sl*, 181 in *Sl*-HR and 92 in *Sp*). The 68 SURFs include 17 of the 49 ubiquitously hypoxia-responsive genes of Arabidopsis seedlings (6), demonstrating evolutionarily conservation of gene families activated by submergence and hypoxia (data S5).

The 68 SURFs include one to 13 upregulated genes per family, leading us to investigate whether similar proportions of these families are elevated in each species (fig. S11, data S6). Consistent with overall numbers, rice had the highest and *S. pennellii* the lowest proportion of upregulated genes per family. The restrained response of wild tomato was evident from the 412 *Solanum*-specific gene families that were up-regulated in tomato but not in *S. pennellii*. This motivated exploration of the aerial tissue (shoot apex) response in the *Solanums*, which uncovered more gene families and family members upregulated in shoots of wild than domesticated tomato (fig. S12, data S7). The shoot response of *S. pennellii* showed greater overlap with Arabidopsis shoot-specific hypoxia-responsive genes (10). Distinctions between the two *Solanums* included genes involved in cell elongation and auxin signaling, which predominated in *S. pennellii*.

We reasoned that dynamics in chromatin accessibility and transcriptional activation may be coordinated and conserved for SURF members across species. ATAC-seq exposed open chromatin regions of rice and *M. truncatula* primarily within 1 kb upstream of the transcription start site (TSS) and downstream of the polyadenylation (pA) site of genes (Fig. 2A, data S8). By contrast, *Solanum* roots showed a majority of intergenic ATAC-seq reads (fig. S13). The rice and *M. truncatula*, transposase hypersensitive sites (THS) (11) uncovered a preference for opening of chromatin in response to submergence (Fig. 2B, fig. S13), with increases in 3,497 and 7,501 THSs, respectively. Highly submergence-upregulated genes had elevated accessibility 5’ of their TSS and 3’ of their pA sites (Fig. 2C, figs. S5-6,14), demonstrating nucleosome depletion accompanies activation of transcript production under submergence. Downregulated genes had lower chromatin accessibility overall, particularly in rice (Fig. 2C, figs. S5-6,14).

We exploited the ATAC-seq data to explore conservation in gene regulatory circuitry. A pipeline was developed to identify transcription factor (TF) binding site motif enrichment within promoters and their THS regions of the upregulated SURFs (Fig. 2D). Four significantly enriched TF motifs were identified. These included the Hypoxia Responsive Promoter Element (HRPE), transactivated by low oxygen-stabilized ethylene response group VII (ERFVII) TFs that upregulate genes key to anaerobic metabolism and flooding survival in Arabidopsis (12-14), a basic Helix-Loop-Helix (bHLH), a MYB, and a WRKY-type motif (Fig. 2D, figs. S15-16A, data S9). At least one of the four motifs was present in over 84% of the upregulated SURF genes of rice and *M. truncatula* and over 68% of those of the *Solanums*. HRPE and bHLH motifs predominated near the TSS in all species, with the MYB near the TSS in tomato and WRKY motifs more evenly distributed across the upstream

region (fig S16B). Differential wiring of upregulated SURFs was evident from the HRPE enrichment in rice (55%) versus the MYB or bHLH motif enrichment in these three eudicots (fig. S16A; data S9).

Accessibility of chromatin in response to abiotic stress can be rapid and transient (15, 16). We hypothesized that concordance between a TF binding site and a THS would be representative of a more static regulatory architecture while discordance could reflect the transient propagation of a stress signal. Chromatin accessibility increased during submergence around HRPE and bHLH sites in rice and *M. truncatula* (Fig. S17). A more modest increase was observed for MYB and WRKY sites, potentially representing more rapid and/or transient regulatory interactions (Fig. S17). The co-occurrence of an HRPE and THS corresponded with more pronounced polyA RNA upregulation, with a similar trend observed for bHLH sites in rice and *M. truncatula* (Fig. 2E, fig. S18, data S10). In *M. truncatula*, the presence of a THS alone in the proximal promoter was associated with greater elevation of polyA RNA, and co-occurrence of a MYB and THS corresponded with higher upregulation than the presence of the motif alone (Fig. 2F, fig. S18). Repetitive motifs of the same type within accessible regions coincided with greater up-regulation than with repetitive motifs outside THSs. The incidence of multiple HRPE or WRKY motifs corresponded with higher upregulation in tomato, whereas only an HRPE or multiple bHLH motifs corresponded with upregulation in *S.pennellii*. These results establish a link between the four conserved motifs, chromatin accessibility and transcriptional activation under submergence.

The discovery of the SURFs and four conserved *cis*-regulatory TF binding motifs in submergence-accessible chromatin regions motivated us to evaluate if the conservation prevails in genes maintained at syntenic chromosomal regions (syntelogs). To do so, the gene activity data were re-clustered for the differentially regulated syntelogs across the four species (711) that included 22 of the 68 SURFs (Fig. 3A, fig. S19, data S11). Syntelog clusters 2 and 3 had coordinated upregulation across the scales of gene activity in all species. These comprised seven SURFs with functions in anaerobic metabolism, nutrient transport, abscisic acid (ABA) perception and survival of extreme stress. The upregulated syntelogs included 32 and 53 SURFs in all three eudicots and the two *Solanums*, respectively (figs. S20-22, data S11).

Next, we explored conservation of gene regulation on more recent evolutionary timescales by evaluating the activity of syntelogs of related species (Fig. 3B, fig. S23, data S12-14). Syntenic genes had higher transcript abundance than non-syntenic genes, as reported previously (17). This was evident in all RNA populations under both conditions, with the most pronounced difference between syntenic and non-syntenic genes in the *Solanums*. Rice and *M. truncatula* syntenic gene control regions had slightly higher chromatin accessibility than non-syntenic genes at the global scale (fig. S14), consistent with their higher expression. Transcript elevation was similar for syntenic and non-syntenic SURF genes, especially for the *Solanums* (Fig. 3C, fig. S24, data S14), indicating that upregulated non-syntenic genes have maintained or acquired features enabling their stress activation. Consistent with this, most highly expressed syntenic and non-syntenic SURF genes contained at least one of the four TF motifs recognized (80% rice, 80% *M. truncatula*, >70%

Solanums) (Fig. 3C, fig. S24, data S15). Most TF motifs were coincident with THSs in rice and *M. truncatula*. Although the number of highly expressed but non-syntenic SURF genes was fewer than six in the *Solanums*, all from *S. lycopersicum* contained at least one motif. The four identified TF motifs are therefore a broadly conserved feature of both syntenic and non-syntenic submergence-responsive genes.

To appraise conservation in regulation across eudicots and monocots, we built networks that associate TF motif presence with each upregulated SURF gene for each species (Fig. 4A, figs. S25-S28, data S16). The individual species networks emphasize the presence of species-specific motif biases. The combinatorial nature of target gene regulation was also evident (overlapping outer circles of network) with over 70% of the genes with more than one of the four motifs. Syntenic upregulated SURF genes across the four species (represented with black borders) expose a single conserved putative regulatory network (Fig. 4B, fig. S29, data S16). This network illustrates conservation of TF motifs of syntelogs of responsive genes, in addition to the HRPE regulated by ERFVIIs.

As oxygen levels decline below a threshold, constitutively synthesized ERFVIIs accumulate due to attenuation of their conversion into an N-degron for active turnover (1). The unified SURF network uncovered HRPE conservation across eudicots-monocots in promoters of genes essential to anaerobic metabolism and hypoxia survival including *PLANT CYSTEINE OXIDASE* (*PCO*) genes (Fig. 4C, fig. S30, data S17), which catalyze the oxygen-promoted degradation of ERFVIIs to temper the adaptive response (18). The upregulated SURF genes included *ERFVIIs* in all four species, with at least one with an HRPE motif, suggesting possible autoregulation (fig. S31).

The syntelog network also identified conservation of bHLH motif enrichment in genes not well associated with submergence (i.e., *PYRABACTIN RESISTANCE 1 / PYR1-LIKE [PYL*]) (Fig. 4B, fig. S32) and MYB motif enrichment in genes that contribute to hypoxia tolerance (14) (fig. S33-34). The upregulation of these genes often coincided with a TF motif within a region of submergence-enhanced chromatin accessibility (Fig. 4D-G, fig. S33), supporting functionality of the regulatory sequences. As for the ERFVIIs, the upregulated SURF genes included bHLH, MYB and WRKY family members (fig. S31).

Information from single genes is used in breeding or modifying crops for stress tolerance. The use of multi-scale gene regulatory information of gene families across flowering plant clades to infer regulatory networks demonstrates that conservation of flooding resilience mechanisms is complex and involves diverse regulatory mechanisms. Targeted manipulation of the four submergence-activated modules and seven SURF loci discovered here with the greatest interspecies conservation might be used to enhance flooding tolerance of susceptible crops.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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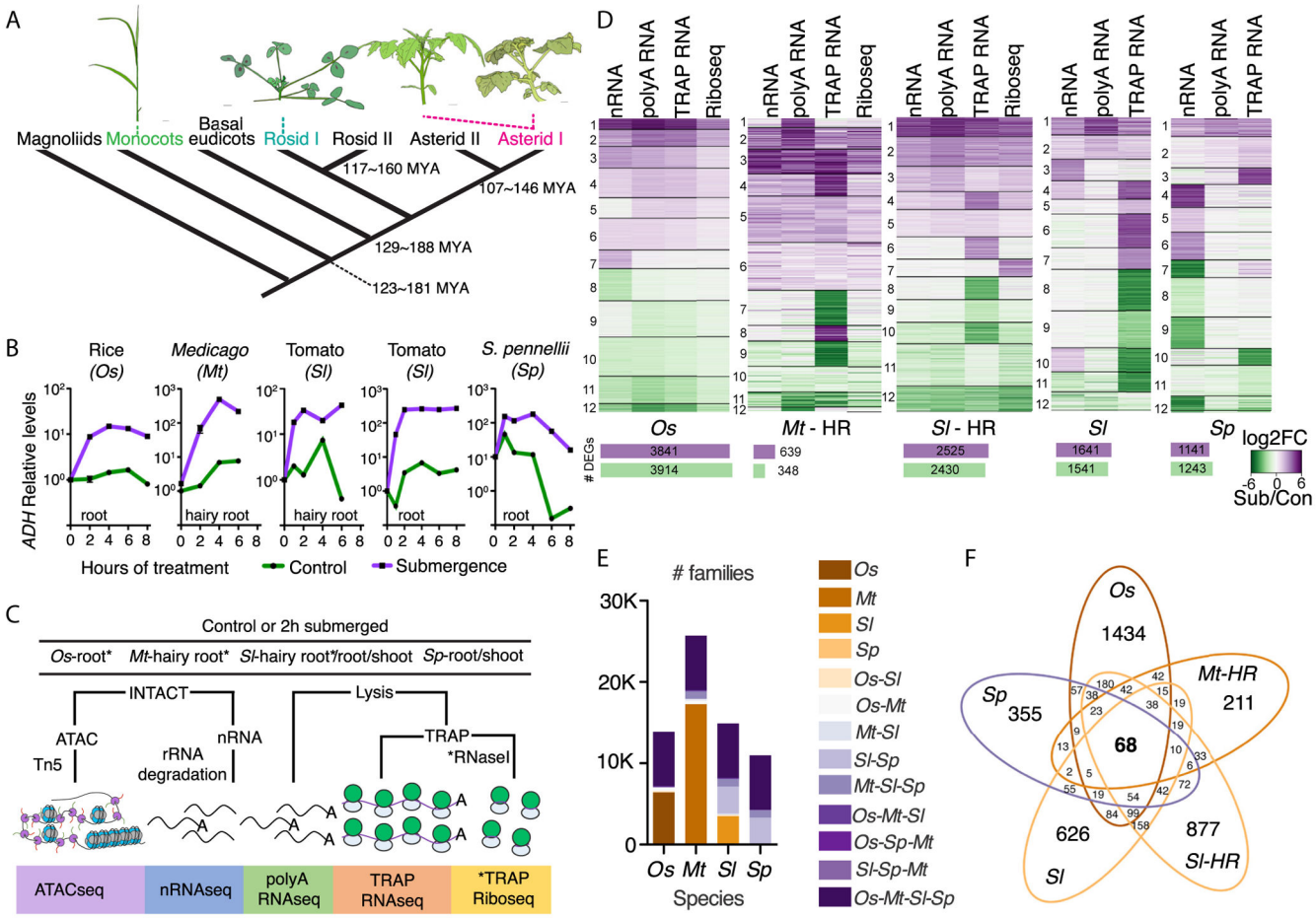

Fig. 1. Multi-tier evaluation of gene activity in four angiosperms identifies highly conserved submergence-upregulated genes.

(**A**) Relatedness of target species (19). (**B**) *ALCOHOL DEHYDROGENASE* (*ADH*) transcript levels of submerged seedlings. (**C**) Overview of experimental strategy. (**D**) Cluster analysis heatmap of $\log_2$ fold change (FC; submergence vs. control RNA) of differentially expressed genes (DEGs; $|\log_2 FC|>1$ and padj<0.01). Below, Bars indicate number of up or down DEGs after submergence $|\log_2 FC|>1$ and padj<0.05. (**E**) Gene families per species and their overlap. (**F**) Conserved submergence upregulated gene families (SURFs).

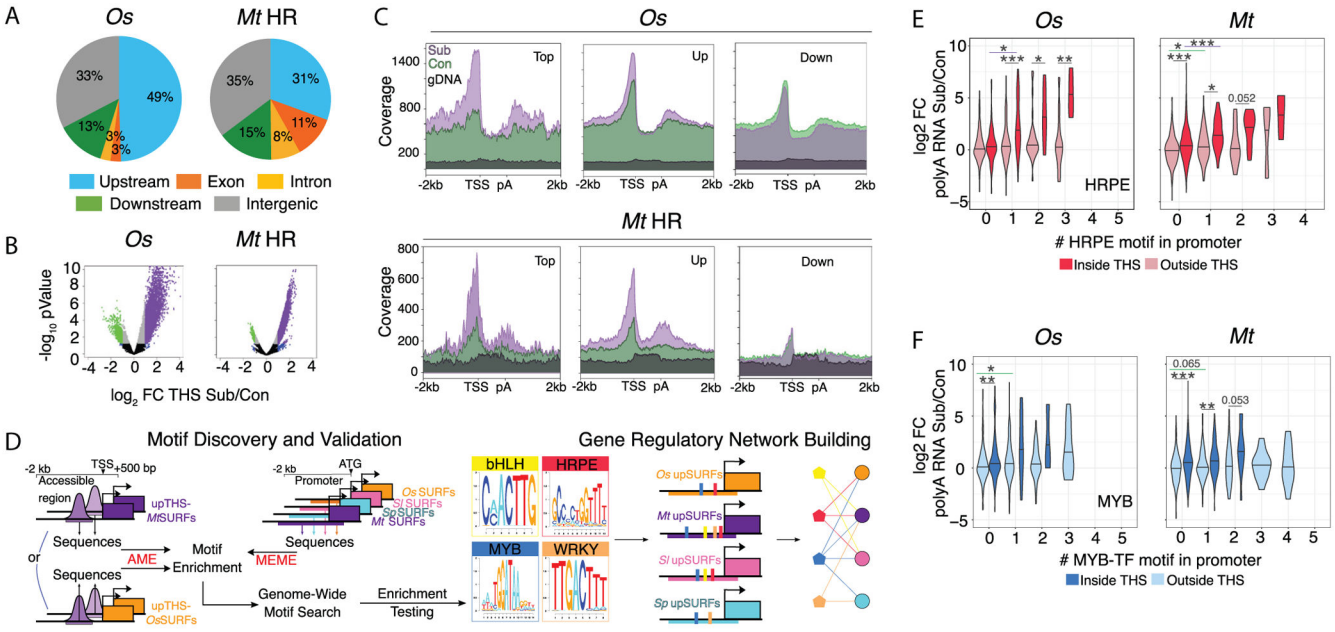

Fig. 2. Enhanced chromatin accessibility and motif enrichment in responsive genes.

(**A**) Accessible chromatin regions (transposase hypersensitive sites [THS]) measured by ATAC-seq. Categories: 2 kb upstream of the transcription start site (TSS), exons, introns, 1 kb downstream of polyadenylation (pA) site, and intergenic. (**B**) THS change in response to submergence. (**C**) Control (Con) and submergence (Sub) ATAC-seq reads on genes of upregulated (Top; cluster 1; Up) and downregulated (Down) clusters from Fig 1D. gDNA is ATAC-seq on naked DNA. (**D**) Discovery pipeline for enriched transcription factor motifs present in upregulated THSs and SURF promoters, using unsupervised (MEME) and supervised (TOMTOM, AME, FIMO) methods. (**E**) **and** (**F**) Distribution of $\log_2$ FC polyA RNA Sub/Con for SURFs arranged by presence and number of HRPE or MYB motif upstream of the ATG, inside or outside THSs. Student's *t* test; * = p<0.05, ** = p<0.01, *** =p<0.001; values ≤0.1.

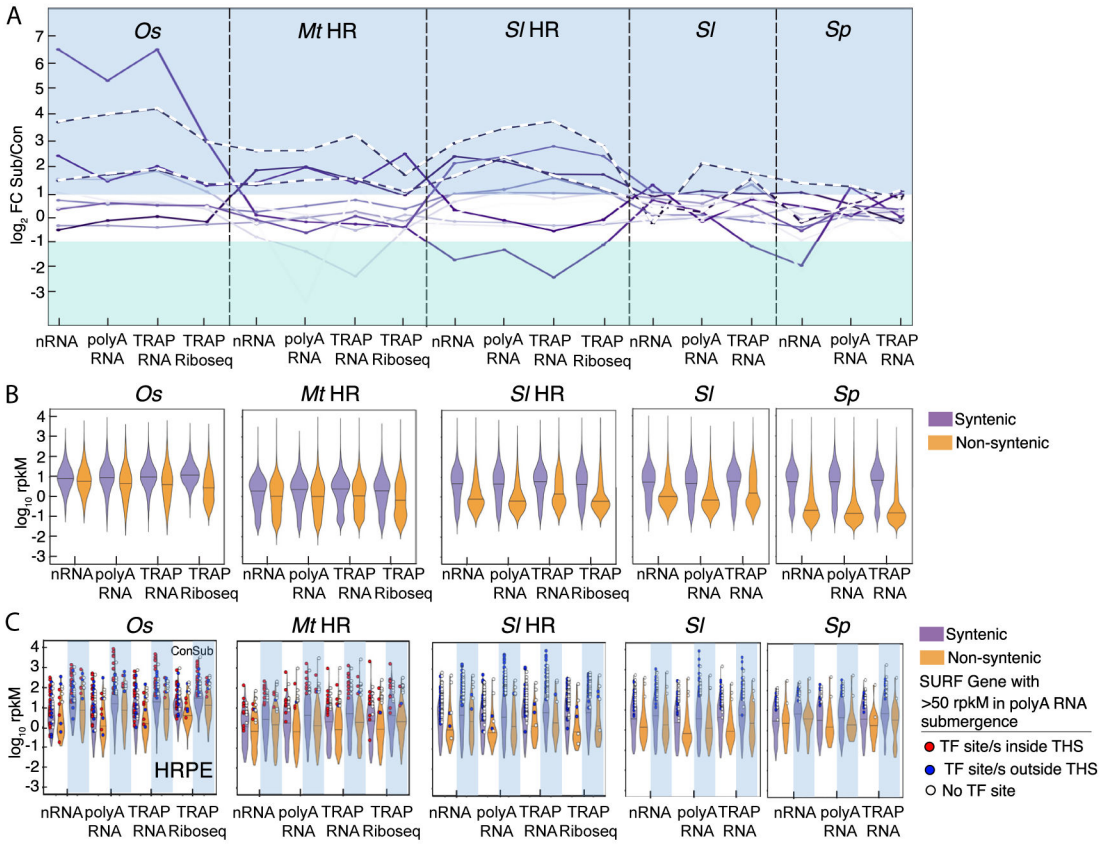

Fig. 3. Syntenic genes are more highly expressed.

(**A**) Median $\log_2$ FC of syntenic genes across four species for eight upregulated clusters. Dashed lines indicate two clusters with conserved interspecies up-regulation. (**B**) Plot of $\log_{10}$ rpkM for all detected syntenic and non-syntenic genes under control condition. Rice synteny was evaluated to *Brachypodium distachyon*, *M. truncatula* to *S. lycopersicum*, and between *Solanums*. Variances between syntenic and non-syntenic genes are significant in every RNA population (F-test). (**C**) Control (white columns) and submergence (blue columns) plots for SURF genes. Highly expressed SURF genes under submergence (>50 rpkM) with a Hypoxia Responsive Promoter Element (HRPE) are depicted as a red or blue dot for those located within or outside a THS, respectively. Central horizontal lines indicate median values.

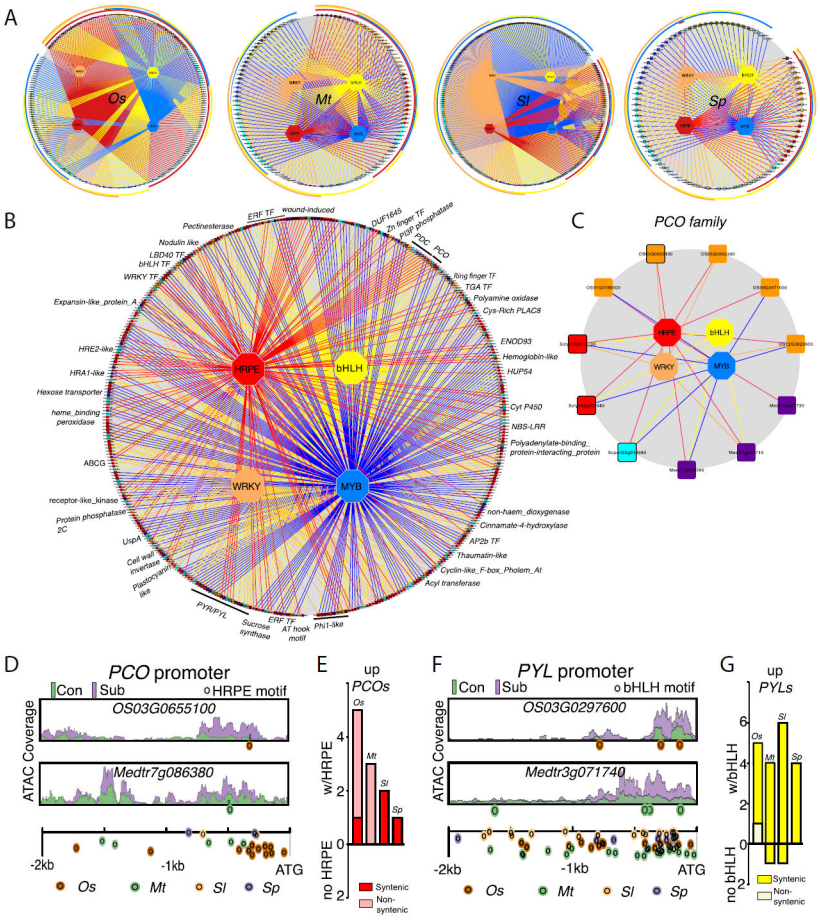

Fig. 4. Conserved transcription factor motifs in SURFs and accompanying chromatin dynamics.

(**A**) Regulatory networks for upregulated SURF genes (expanded in figs. S25-S28). Hexagons, transcription factors (TFs); rectangles, genes; colored lines (edges), interactions of promoter and TF based on motif presence. Outer circles: genes grouped with shared motifs. Genes with black borders have a syntenic ortholog (rice to *M. truncatula*; *M. truncatula* to *S. lycopersicum;* and between *Solanums*). (**B**) Network for syntenic conserved SURF genes across species (expanded in fig. S29). Genes of alternating families have alternating grey or black borders. Families represented in three species are labeled. (**C**) Regulatory network of *PLANT CYSTEINE OXIDASE* (*PCO)* up-regulated genes. Syntenic orthologs have black borders. (**D**) **and** (**F**) Chromatin accessibility in promoters of syntenic *PCO* and *PYL (PYRABACTIN RESISTANCE 1 (PYR1) / PYR1-LIKE (PYL) / REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR*) genes. ATAC coverage scale is the same for genes shown in each panel. Below: locations of HRPE or bHLH motifs for four species. (**E**) **and** (**G**) Number of upregulated genes containing motifs classified by syntenic and non-syntenic.