# Results

## Characterization of shared and species-specific chromatin states

We generated an universal chromatin states (CS) map annotation from ten common epigenomic marks using hiHMM software for the three widely-studied model plant species: *Arabidopsis thaliana*, *Oryza sativa* and *Zea mays*. We focused our analysis on a model with 16 states (**supplemental fig. S1**; see **Methods**). The states were divided into 5 functional groups (bivalent, active, divergent, repressive and quiescent/no-signal), with different levels of genome coverage, TE enrichment and overlap with other genomic features (**fig. 1**).

Co-occurrence of pairs of epigenetic marks exists between these species, but there are clearly specific patterns in both, CS and correlation analyses (**fig. 1**; **supplemental fig. S2**). Despite the diversity of the data, we found some conserved chromatin definitions such as Bivalent TSS/Promoter CS1, strongly linked to all active marks with very low enrichment in H3K27me3 and without clear presence of heavy repressive marks like 5mC and H3K9me2; and Active CS6, established in gene bodies and mainly constituted by H3K36me3, H3K4me2, H3K4me3 and H3K9ac in all the species. On the other hand, most of the states definitions strayed with some species-specific nuances at different levels, which could actually reflect our understanding of species-specific biology and how epigenomic complexity has evolved in plants. From less to more divergent: 1) States which shared genomic distribution and were constituted by marks with same roles, but covered with different marks like Heretochromatin 1 strong CS11 and Heterochromatin 2 weak CS12 (**fig. 1**). Repressive marks, also pinpointed in the correlation analysis with the highest inter-species variance (**supplemental fig. S2**), suggested two distinct types of heterochromatin across species requiring H3K27me3 for strong and H3K9me2 for weak definitions in *A. thaliana*,but not necessary in *O. sativa* and *Z. mays*. 2) Landscapes whose marks and genomic distribution gradually transitioned between species. A good case representing this could be Active weak TSS > TES CS8, mainly dominated by H3K36me3 deposition in gene bodies and TSS in *A. thaliana*,while in the two remaining species H3K4me2 is added and distribution changed towards the TES. 3) Ultimately, divergent region CS10 with a totally different mark and genomic distribution profile. CS10 corresponded to heterochromatic, bivalent and active states in *A. thaliana*, *O. sativa* and *Z. mays*, respectively.

We next performed additional annotation analyses based on non-common chromatin proteins and histone marks tracks to test our states definitions (**fig. 2, bottom panel**). There were evidence supporting our interpretation of the states for each species under study. For example: RNA polymerase II (Pol2) significantly located in all active and several bivalent states, and enrichment of the well-known H3K9-demethylase (IBM1) and transposon-methylase (CMT3) over heterochromatic states in *A. thaliana*. Essentially, all non-common active and repressive histone marks/variants evaluated were enriched in active/bivalent and heterochromatic states, respectively, with only two exceptions: H3K27me1 location in Bivalent Promoter CS2 in *A.thaliana*, which did not impact the state definition because this was already presented as bivalent due to the presence of H3K27me3; and H3K9me1/me3 in Active gradual bivalent flank > intergenic CS7 in *O. sativa*. Although the initial definition included gradual bivalent, this was only alluding to *Z. mays* as *O. sativa* CS7 was absent of any repressive mark, therefore, this would pontentially increase CS7 relation between both Poaceae-family members. We decided to stay conservative and keep our initial interpretation because H3K9me3 data is not available for all the species. It is worth mentioning that most of the transcription factors (TFs) observed in heterochromatin states were related with flowering, organ missed in our collection, and cell-cycle/division functions, previously described as present in chromatin barriers and strictly under control with low levels of expression (Feng & Michaels, 2015; Velay, Méteignier, & Laloi, 2022).

Taking advantage of the inter-species approach, we further evaluated if the states could involve evolutionary information. We observed a remarkable gradient across functional groups, excluding quiescent/no signal from the analysis due to the lack of epigenetic regulation (**fig. 3**; **supplemental fig. S3**). We found a decreasing trend in gene functional convergence (KO and GO), number of protein-coding genes and their corresponding proportion of orthologous relationships following active > bivalent > divergent > heterochromatin order (represented by CS6 > CS1 > CS10 > CS11, respectively). Additionally, most of the PhastCons elements genomic overlaps were located in active and bivalent states (**fig. 2**). Conserved non-coding elements (CNEs) co-localization in the same states for *A. thaliana* and the greater number of CNEs enriched states when comparing both species of monocots, again showed how CS could reflect the closer distance between *O. sativa* and *Z. mays*. Even though the majority of the states enriched in Conserved TF binding-sites (BS) were active and bivalent in *A. thaliana* and *O. sativa*, we did not appreciate a constrained pattern for all the species in TF motifs and genetic variability annotation modules (**fig. 2**). On the opposite side to conservation, these results could indicate that state information is still useful, because significant overlaps were detected, but it would probably reflect species-specific features in genetic variability and TF motifs contexts.

Taking together, these discoveries introduced a plant inter-species chromatin state single annotation as a resource to provide conservation and diversity evolutionary epigenomic information for future research.

## Chromatin states features improve predictions of paralogs functional divergence

In order to exemplify an application of the resource generated, we reproduced two previously published models predicting *A. thaliana* genetic redundancy (Cusack et al., 2021; Ezoe, Shirai, & Hanada, 2021) including CS information. As far as we know, *A. thaliana* was the only organism under study with an experimentally validated set of mutants for paralogous gene pairs which allowed the development of these models. Under the initial hypothesis that two paralogs covered by different state profiles are more likely to have divergent functions, we computed simmilarity and distance metrics between both CS profiles and fed these data to the abovementioned models (**fig. 4, top panel**; see **Methods**).

For the models developed by Ezoe et al.,2021 (**fig. 4a-d**), we first checked if the custom chromatin state metric (CCSM; see **Methods**) proposed could be a determinant of functional divergence using the same paralogous gene pairs as the original article (**fig. 4b**). High and low CCSM values were significantly associated to high and low diversified pairs, respectively (P-value = 3.4e-15, two tailed Wilcoxon rank sum test). In spite of the fact that epigenomic features tested in the reference did not pass this threshold (so the models were computed without including epigenetic information), our CS metric even joined the two best explanatory variables Ka/Ks and Re/Ks (see **Methods**) in terms of relative importance (**fig. 4a**). These results pointed the need to use integrative metrics when predicting genome elements and mechanisms. Logistic regression models (see **Methods**) using different set of features were compared by calculating the area under the curve-receiver operating characteristic (AUC-ROC) and the area under-precision recall curve (AU-PRC) values (**fig. 4c**). Models including CS information had higher AUC-ROC and AU-PRC values and slightly improved the performance of the best final model reported in the original article (Ka/Ks + Re/Ks). This improvement was more obvious in the reduced formula (Ka/Ks + Re/Ks + CCSM) and the small range of improvement between full (Ka/Ks + Re/Ks + CCSM) and reduced formulas also agreed with the information reported by Ezoe et al., 2021. The degree of functional divergence (DFD) can be inferred from the best formula by logistic regression analysis. DFD values close to 0 and 1 reflected low (< 0.5) and high (> 0.5) functional divergence, respectively. To enable potential validation of paralogous pairs DFD in upcoming studies and to minimize the erronous assignment of high and low diversified duplicates, we calculated 5% FDR as a threshold. DFD thresholds were 0.93 and 0.46 for high and low diversified pairs, respectively (**fig. 4d**). A table containing labeled genome-wide predictions with additional filters to assist paralogs redundancy experimental verification (see **Methods**)is available at **supplementary table S3**.

In contrast, for the models developed by Cusack et al., 2021 (**fig. 4e-h**) redundancy was categorized into different definitions and a lot of features with distinct transformations were covered. Therefore, we decided to include all the CS metrics to model redundancy for each of the definitions resulting in four different sets: RD4 (extreme redundancy, single mutants have no abnormal phenotype and the double mutant is lethal; without CS information), RD4C (with CS information), RD9 (inclusive redundancy, general definition which also contained RD4 gene pairs; without CS information) and RD9C (with CS information). The number of variables and the relative importance of the six feature categories in the definitions without CS information mostly confirmed the discoveries in the reference (**fig. 4e**). Very briefly, the ranking from best to worst based on median importance ranks in those categories for RD4/RD9-based models was functional annotation (37/16) > network properties (57.5/64.5) > evolutionary properties (76/110) > gene expression (104/105) > protein properties (145/88) > epigenetic modifications (121/127), while gene expression was the category with the highest number of variables in both cases. These findings validated the reproducibility of the models and guaranteed a rigorous interpretation of the following results. Taking into account RD4C/RD9C-based models, chromatin state category was sixth/second in importance rankings and became the first in terms of number of variables for both cases. Thus, potentially indicating that CS information would be more useful when prediciting general rather than extreme redundancy. This idea was further verified when SVM models (see **Methods**) with different sets were compared using AUC-ROC and AU-PRC values (**fig. 4f-g**). While CS data clearly improved predictions for general redundancy, it also reduced the values for the extreme definition. Finally, we detected that the intersection with the highest number of features was common to all sets suggesting that the core predicting power remained constant for all the models and, again, ensuring accurate comparisons (**fig. 4h**).

Collectively, we revealed that CS information could give clues into duplicates general functional divergence corroborated by the replication of two independent previously published models.

## Defining functional genomics conservation score and database

Evolutionary functional-genomics/epigenomics is an emerging field of study with a growing body of literature reporting massive generation of functional genomics data, yet the determinants underlying these processes are still not well understood for a lack of a holistic point of view. To fill this gap, we adopted an integrative approach and expanded the resource generated with functional genomics conservation scores computed by LECIF algorithm (Kwon & Ernst, 2021). LECIF was applied integrating epigenomic, chromatin states, whole genome alignments and transcriptomic information for all pairwise comparisons. By querying LECIF-scores, we sought to identify regions with similar phenotypic properties (**fig. 5, topleft panel**).

To research elements highlighted by LECIF, we characterized genome distribution of the scores over genetic variability, chromatin states and conservation modules. In all the comparisons, LECIF-scores density decreased in centromeres due to the lower number of alignments in these regions (**fig. 5, middle panel**). As mentioned before, we did not find a constrained pattern in the genetic variability module. Whilst both *Z. mays* contrasts (**fig. 5, topright panel**) and *O. sativa* vs *Z. mays* (**fig. 5 bottomleft panel**) GWAS significant SNPs are enriched in regions with high functional conservation, both *A. thaliana* contrasts (**fig. 5, bottomright panel**) did not reflect any enrichment and *O. sativa* vs *A. thaliana* was even enriched in regions with low LECIF-scores. This could be explained by balanced significant-SNPs distribution through *A. thaliana* genome due to its architecture and higher number of GWA studies, more simmilarity in the traits studied between the monocots and/or *O. sativa* only being able to retain functional conservation information related to the closest species.

In the CS module, genome-wide distributions were shifted to the left because of the higher weights of negative (only aligned) vs positive (aligned and functionally conserved) samples to ensure that only regions with strong functional evidence were underlined (**fig. 5, bottomright-bottomleft-topright panel; histogram**). To validate that LECIF-score displays expected cross-species similarity in functional genomics features, we examined it in relation to CS annotation. In each of the six query vs target comparisons between these three model species, CS linked to strong regulatory or transcription activity tended to have higher mean LECIF-score than the other states (**fig. 5, bottomright-bottomleft-topright panel; violinplots**). We investigated cross-species CS simmilarity for different ranges of the LECIF-score (**fig. 5, bottomright-bottomleft-topright panel; lineplots**). As LECIF-score increased, cross-species CS agreement was gradually higher in active, bivalent and heterochromatin functional groups (i.e aligned regions marked with high scores are expected to present same CS for these functional groups). This pattern was not fulfilled for divergent and quies/no-signal states because simmilarity was not expected by definition and the absence of epigenetic regulation, respectively. To provide further proof, we analyzed CS annotations in regions where functiona genomics (LECIF) and comparative genomics (PhyloP) scores disagreed (**fig. 5, bottomright-bottomleft-topright panel; grouped barplots**). Specifically, for pairs of regions where the LECIF-score was high (percentile rank > 60) and PhyloP-score was low (percentile rank < 40), we computed CS simmilarity. We appreciated that such pairs were more likely to exhibit convergent states for all the groups and vice versa.

We next evaluated more deeply the relationships between functional/comparative-genomics scores and annotations (**fig. 5, bottomright-bottomleft-topright panel; boxplots**). It should be noted that as we are studying distant-related species, the scores of annotations with high coverage % in the aligning regions, like PhastCons/PhyloP (Tian, Yang, Meng, Jin, & Gao, 2020) sequence-based conservation, would be influenced by the high negative:positive weights ratio. We found that regions overlapping PhastCons elements did not have greater average LECIF-score compared to the genome-wide distribution and LECIF-score was not correlated with PhyloP-score (min-max range: 0.04-0.119 and 0.005-0.118 for PCC and SCC, respectively). Interestingly, CNEs followed the same trend as PhastCons elements except for Poaceae-members vs *A. thaliana* pairs, which had higher LECIF-scores. This is reasonable since non-coding elements preserved during longer timescales are more probable to be functionally conserved.

In summary, all these reports suggests that plants LECIF-score can capture functional conservation without being correlated with other comparative genomics and sequence-constraint scores. We expect the LECIF-score and inter-species CS would be useful tools to unify and extrapolate molecular mechanisms discoveries using different model systems, so we developed an integrated hub called PlantFUN(ctional)CO(nservation) to provide interactive user-friendly functionalities for further requests (**fig. 5, topleft panel**; see **Methods**). PlantFUNCO database is available at <https://rocesv.github.io/PlantFUNCO/>.

## Experimental validation of potential divergent duplicates

To illustrate that functional uses of the database could be translated into solutions for complex biological problems, we focused in the experimental validation of mitochondrial alternative oxidases (AOX) redundancy in *A. thaliana*. Despite these pairs do not pass the stringent threshold (**fig. 4d**), they presented high enough DFD values to be considered high divergent paralogs (**fig. 6**). We decided to assess AOX redundancy by monitoring root phenotypes under two different stresses, considering previously described roles of these genes in response and retrograde-signalling (Fuchs et al., 2022); 2/5 paralogs are not root expressed (Papatheodorou et al., 2020), simplifying the system and evaluation in seedling stages. The DFD of duplicates can be inferred based on the phenotypes of knockout plants. When single knockout exhibit abnormal phenotypes related to the wild-type (WT, Col-0) under a specific condition, the duplicates are not compensated by the other gene copies so are assumed to be functional divergent and conversely (Ezoe et al., 2021).

Root phenotypes followed the same pattern for control and mock conditions, there were significant differences for all AOX genotypes in root length (WT > *aox1c* > *aox1a* > *aox1d*), hypocotyl length (*aox1c* > *aox1d* > *aox1a* > WT) and root:hypocotyl ratio (WT > *aox1a/aox1c* > *aox1d*) (**fig. 6**). In PEG x heat stress, significant differences were appreciated too but with two exceptions: *aox1c* root length and *aox1a* hypoctyl length. We decided to establish an additional stress assay using Antimycin A (AA), a complex III inhibitor that can be tolerated in plants due to electon bypass via AOX, but not when the activity of these genes is supressed/dimished (Strodtkotter et al., 2009). Because of the small size of *aox1a* seedlings only root length was monitorized. Again, significant changes were found for all AOX genotypes measured in root length and root:hypoctyl ratio. Hypocotyl length greater p-values in PEG x heat and no significance in AA suggested a general-stress hyopoctyl elogation mechanism in these mutants. In view of AOX genes roles in redox state, DAB staining quantification was performed to have a raw hydrogen peroxide measure. Although both stresses agreed in WT, *aox1d* relevantincrease and *aox1c* no significance, *aox1a* trends were not congruent. Hydrogen peroxide *aox1a* change was nonmeaningul for PEG x heat while a significant increase was detected during AA. Finally, in terms of functional genomics the dominant isoform *AOX1A* seems to be the most crucial because was covered by active CS and was marked with high LECIF-scores when compared to *O. sativa*.

In brief, these findings validated our high divergence predictions and setted a sceneario where *AOX1A* appeared to retain ancestral function allowing the understanding of the remaining AOX genes redundancy relations related to this reference.

# References

Strodtkotter, I., Padmasreea, K., Dinakara, C., Spetha, B., Niazi, P. S., Wojtera, J., … Scheibe, R. (2009). Induction of the AOX1D Isoform of Alternative Oxidase in A . thaliana T-DNA Insertion Lines Lacking Isoform AOX1A Is Insufficient to Optimize Photosynthesis when Treated with Antimycin A. *Molecular Plant*, *2*(2). https://doi.org/10.1093/mp/ssn089

Feng, W., & Michaels, S. D. (2015). Accessing the Inaccessible : The Organization , Transcription , Replication , and Repair of Heterochromatin in Plants. *Annual Review of Genetics*, *49*, 439–459. https://doi.org/10.1146/annurev-genet-112414-055048

Velay, F., Méteignier, L.-V., & Laloi, C. (2022). You shall not pass ! A Chromatin barrier story in plants. *Frontiers in Plant Science*, *13*(September), 1–9. https://doi.org/10.3389/fpls.2022.888102