## Discussion

We introduced PlantFUNCO, a database to allow the community further inspection of the crosstalk between evolution and phenotypic plasticity in terms of epigenomics/functional-genomics. This database is derived from two resources presented and analysed in this work for three well-established plant models. On one hand, we generated inter-species CS using hiHMM (**fig. 1**). While this flexible framework provides a consistent definition of CS across multiple genomes, making easier direct comparison between them, the stack approach allows the understanding of the potential epigenomic regulation over several tissues/conditions such as differentiating constitutively active/repressive regions (Vu & Ernst, 2022). CS link with different types of evolutionary information setted a foundation for the epigenomics inter-species perspective (**fig. 2**; **fig. 3**). It should be noted that all the approaches have trade-offs so this resource should be considered complementary to and not a replacement to other single-species/condition annotations. On the other hand, we obtained functional genomics conservation scores using LECIF. In accordance to the abovementioned framework, LECIF can handle very diverse datasets and take advantage of it to quantify functional conservation. Plants LECIF-score elucidated functional-genomics cross-species agreement without being correlated with other comparative-genomics sources (**fig. 5**). Hence, probably reflecting a complementary side of the evolution. Despite the greater divergence between plants models compared to metazoans (Ho et al., 2014; Kwon & Ernst, 2021), both resources results are coungruent with a higher plant epigenomic/functional complexity probed by more states with species-specific features and lower values of LECIF-scores.

A major focus of this study was to illustrate an application of the generated resources. Due to the holistic approach adopted and exploiting that our inter-species CS could differ between constituvely active/repressive regions, we replicated two previously published models predicting paralogous functional divergence in Arabidopsis (Cusack et al., 2021; Ezoe, Shirai, & Hanada, 2021) including our CS information. We evaluted if CS simmilarity could be a determinant of duplicates degree of functional divergence under the initial hypothesis that two paralogs covered by different state profiles are more likely to present divergent functions. Although models are far from being perfect, useful information about gene features can be extrapolated. These models independently reported CS information as relevant and including this type of data improved general redundancy predictions (**fig. 4**). Thus, showing an example of how PlantFUNCO integrative resources could be effectively employed to genomic elements prediction.

An important goal of a database is to functionally translate applications into solutions for explaining complex biological mechanisms, so we decided to check redundancy predictions of AOX genes. DFD values were high enough to be considered and AOX earlier research made their context of high biological interest. Very briefly, past reports were mainly focused in the dominant isoform *AOX1A* (Giraud et al., 2008) which have a partial redundancy relation described with *AOX1D* (Strodtkotter et al., 2009), but current literature is not congruent with the use of single *aox1a* or double *aox1a-aox1d* mutants to discover retrograde-signalling/metabolism/stress-response causal drivers (Giraud, et al., 2009; Clercq et al., 2013; Oh Khim, et al., 2022; Oh Khim, et al., 2023). Additionally, more AOX isoforms exists but their relationships were still not addressed. To test our redundancy predictions we monitorized seedlings phenotypes in root-expressed AOX single knockout mutants (*aox1a*, *aox1d* and *aox1c*) under drought-heat and oxidative stresses (**fig. 6**). The abnormal seedling growth observed for all the single mutants in control and mock conditions validated our high functional divergent predictions because in case of redundancy other duplicates could rescue these phenotypes (Ezoe, Shirai, & Hanada, 2021). Our findings suggested that the dominant isoform *AOX1A* could retain the ancestral AOX function because it was marked as functionally conserved with the distant-related *O. sativa* and was the only one covered by an active CS, so all the redundancy relations could be pontentially compared to this gene*.* Taking into account that oxidative stress was more severe than drought-heat conditions, we found putative evidence of a probable stress-dependent partial non-mutual redundacy of *AOX1D* to *AOX1A*. While *AOX1D* could partially alleviate *aox1a* raw hydrogen peroxide content in drought-heat (no significant), during more severe oxidative conditions *AOX1D* would not be enough to supply *AOX1A* function (significant) (Strodtkotter et al., 2009). It is defined as a potential non-mutual relation because in all the cases *aox1d* phenotypes remained significant. Finally, nonmeaningful differences in raw hydrogen peroxide content for both stresses and WT-like root length under drought-heat in *aox1c* would probably propose *AOX1C* as a non-stress-responsive gene. This could agree to the already described *AOX1C* AA expression insensitivity (Yoshida & Noguchi, 2009), but we still found root length significant differences in our severe oxidative assay. That said and compared to other genotypes, p-value was close to significance absence so *AOX1C* may only be related to stress under severe conditions and could be probably defined as almost non-stress-responsive. In summary, stress seems to be a crucial evolutionary force driving sub-/neo-functionalization (Panchy, Lehti-shiu, & Shiu, 2016) in AOX genes and we characterized the unknown *AOX1C* asalmost stress-insensitive in seedling stages. Furthermore, extra attention should be taken when using double AOX mutants to interrogate causal determinants of biological processes because all AOX genes evaluated appeared to be functionally divergent during early development.

While we expect PlantFUNCO to be useful, we do note a few limitations. There could be states/regions that are functionally conserved, but have low scores/agreement in the database, since the evidence was not present in our collection. While the interpretation of the resources generated is less ambiguous due to the broad-shallow perspective adopted, we also perceived that PlantFUNCO is limited by the input functional genomics resolution and does not provide the direct information about which particular tracks/conditions supported the evidence. The results promoted the potential application of PlantFUNCO to further test new hypothesis in the context of duplicates evolution and other genomic elements prediction. For example, as CS are determinants of paralog functional divergence and LECIF-scores highlight regions with high phenotypic simmilarity it could be possible to identify genes that are more likely to retain ancestral functions if high scores are found between orthologous in distant-related species (**fig. 5; topleft panel**). Here we focused on *A. thaliana*, *O. sativa* and *Z. mays*, that are widely used models in plant science research with substantial high-quality public data available. Given the increasing availability of epigenomic and functional genomics datasets, the utility of PlantFUNCO will continue to grow and serve as an additional resource to simplify functional conservation annotations for a more diverse set of species like *Chlamydomonas reinhardtii*, *Marchantia polymorhpha* and *Solanum lycopersicum*. All in all, PlantFUNCO aim to leverage data diversity and extrapolate findings from different models to determine the extent of molecular conservation, thus, deepen our understanding of how plants phenotypic plasticity has fascinatingly evolved.

# References

Clercq, I. De, Vermeirssen, V., Aken, O. Van, Vandepoele, K., Murcha, M. W., Law, S. R., … Breusegem, F. Van. (2013). The Membrane-Bound NAC Transcription Factor ANAC013 Functions in Mitochondrial Retrograde Regulation of the Oxidative Stress Response in Arabidopsis. *The Plant Cell*, *25*(September), 3472–3490. https://doi.org/10.1105/tpc.113.117168

Giraud, E., Aken, O. Van, Ho, L. H. M., & Whelan, J. (2009). The Transcription Factor ABI4 Is a Regulator of Mitochondrial Retrograde Expression of. *Plant Physiology*, *150*(July), 1286–1296. https://doi.org/10.1104/pp.109.139782

Giraud, E., Ho, L. H. M., Clifton, R., Carroll, A., Estavillo, G., Tan, Y., … Whelan, J. (2008). The Absence of ALTERNATIVE OXIDASE1a in Arabidopsis Results in Acute Sensitivity to Combined. *Plant Physiology*, *147*(June), 595–610. https://doi.org/10.1104/pp.107.115121

Oh Khim, G. G., Kumari, V., Millar, A. H., & Leary, B. M. O. (2023). Alternative oxidase 1a and 1d enable metabolic flexibility during Ala catabolism in Arabidopsis Research Article. *Plant Physiology*, *192*(4), 2958–2970. https://doi.org/10.1093/plphys/kiad233

Oh Khim, G. G., Leary, B. M. O., Signorelli, S., & Millar, A. H. (2022). Alternative oxidase ( AOX ) 1a and 1d limit proline- induced oxidative stress and aid salinity recovery in Arabidopsis. *Plant Physiology*, *188*, 1521–1536.

Panchy, N., Lehti-shiu, M., & Shiu, S. (2016). Evolution of Gene Duplication in Plants. *Plant Physiology*, *171*(August), 2294–2316. https://doi.org/10.1104/pp.16.00523

Strodtko, I., Padmasree, K., Dinakar, C., Speth, B., S. Niazi, P., 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), 284–297. https://doi.org/10.1093/mp/ssn089

Yoshida, K., & Noguchi, K. (2009). Differential Gene Expression Profiles of the Mitochondrial Respiratory Components in Illuminated Arabidopsis Leaves. *Plant and Cell Physiology*, *50*(8), 1449–1462. https://doi.org/10.1093/pcp/pcp090