DR JAVIER MORENO (Orcid ID : 0000-0001-9763-5325)

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Research review

Molecular mechanisms involved in functional macroevolution of plant transcription factors

Facundo Romani, Javier E. Moreno

Instituto de Agrobiotecnología del Litoral, Universidad Nacional del Litoral - CONICET, Facultad de Bioquímica y Ciencias Biológicas, Centro Científico Tecnológico CONICET Santa Fe, Colectora RN 168 km. 0, Paraje El Pozo, Santa Fe 3000, Argentina.

Authors for correspondence:

Dr. Facundo Romani

Email: romani.facundo@gmail.com

Twitter @facundoromani

ORCID 0000-0003-3954-6740

Dr. Javier E. Moreno

Tel: +54-342-4511370 ext 5019

Email: javier.moreno@santafe-conicet.gov.ar

Twitter @JaviMorenoOK

ORCID 0000-0001-9763-5325

Summary

Transcription factors are key components of the transcriptional regulation machinery. In plants, they accompanied the evolution from unicellular aquatic algae to complex flowering plants that dominate the land environment. The adaptations of the body plan and physiological responses required changes in the biological functions of transcription factors. Some ancestral gene regulatory networks are highly conserved, while others evolved more recently and only exist in particular lineages. The recent emergence of novel model organisms opened the chance for comparative studies, providing new insights to infer these evolutionary trajectories. In this review, we comprehensively revisit the recent literature in transcription factors of non-seed plants and algae focusing on the molecular mechanisms driving their functional evolution. We discuss the particular contribution of changes on DNA binding specificity, protein-protein interactions and *cis*-regulatory elements to gene regulatory networks. Current advances showed that these evolutionary processes were shaped by changes in transcription factors expression pattern but not great innovation in transcription factor protein sequences. We propose that transcription factor role associated with environmental and developmental regulation were unevenly conserved during land plant evolution.

Keywords: transcription factors, gene regulatory networks, bryophytes, Evo-Devo, plant evolution, plant traits.

Introduction

Transcription factors (TFs) are the major contributors to transcriptional complexity, essential for body plan development in multicellular eukaryotic organisms. The colonization of plants to the land environment (~450 Mya) was accompanied by an expansion of transcription factor genes during the Precambrian (Catarino *et al.*, 2016; Bowman *et al.*, 2017; Wilhelmsson *et al.*, 2017). Currently, our knowledge on TFs biological functions is mostly focused on a selected group of angiosperms species.

The study of TFs molecular function and their evolution can be addressed at different levels (Delaux *et al.*, 2019). At sequence level, recently available genomes and transcriptomes helped to elucidate the history of TF-genes evolution during Streptophytes' diversification. It is relatively clear how the birth and death of TF genes paved the road for the adaptation of plants to land (Catarino *et al.*, 2016). However, at the functional or molecular level it is not clear whether these ortholog genes have functioned in the same way or they acquired their current function later in evolution. Recent advances of molecular tools with non-flowering plants are opening the chance to interrogate the evolutionary role of TFs *in planta* and helping to understand the different mechanisms underlying gene regulatory networks (GRN) evolution in a macroevolutionary scale (>100 Mya).

How do transcription factors evolve?

Gene duplications are the main substrate for functional diversification and redundancy in plant TFs (Rensing, 2014; Wu & Lai, 2015). The existence of multiple copies of a TF gene is the first step for subfunctionalization or neofunctionalization (Ohno, 1970; Panchy *et al.*, 2016; Johnson, 2017). Molecular mechanisms involved in these processes can be classified in four major types (Johnson, 2017): changes within the DNA binding domain (DBD), changes outside the DBD, changes in the GRN upstream to the TF, and changes in the GRN downstream to it (see Figure 1).

DBDs are responsible for TF DNA binding specificity and affinity. At the sequence level, DBD are quite conserved across Streptophytes and most of the observed genetic diversity is explained by gene duplication events where a similar DBD is conserved (Catarino *et al.*, 2016; de Mendoza & Sebé-Pedrós, 2019). Studies of several TFs demonstrated that the DNA binding specificity is

highly conserved family- and subfamily-wise and even across a wide evolutionary range of species such as green algae and land plants (Weirauch *et al.*, 2014). This suggests that small changes in DBD protein sequences do not generate major changes in TF sequence specificity. Nevertheless, subtle changes in binding affinity can contribute to the complexity of the GRN. Interestingly, the DBD sequence specificity conservation degree is not the same among families (Lambert *et al.*, 2019). TFs also possess other conserved features outside the DBD that contribute to their interaction with other proteins. These auxiliary domains usually impact oligomerization, activity, subcellular localization, protein stability, among others (Bartlett, 2020).

Changes in upstream GRN impacts on TF expression patterns are common in duplicated genes and are generally associated with mutations in *cis*-regulatory elements (CRE) on TF promoter regions, although other changes in the GRN may alter the final expression pattern. Duplicated genes are usually co-expressed (Panchy *et al.*, 2016) and diverge in accordance with the duplication-event age (Arsovski *et al.*, 2015). Changes in expression pattern in TF functional diversification was well demonstrated in the model flowering plant *Arabidopsis thaliana* and explain several neo and subfunctionalization events in plant species (De Smet *et al.*, 2017). However, their relevance in macroevolution is poorly explored.

In vivo binding of TFs results from a combinatorial effect of binding site selection, competition with other TFs, different affinity of homo- or hetero-mers, and distance of CRE in the promoter region (Galli et al., 2018). These changes are of major significance for TF evolution and affect both upstream and downstream GRN. The conservation of CRE is relatively low in evolutionary times since it encompasses a higher mutation rate compared to coding sequences. Some examples of deeply conserved CRE were described in plants (Lieberman-Lazarovich *et al.*, 2019) but the overall conservation of binding sites is around 8% in Embryophytes (Van de Velde *et al.*, 2016) (see Jones and Vandepoele (2020) for review).

Molecular studies on TF evolution

Comparative studies of specific TFs in non-flowering plants can be used to experimentally test their functional evolution. Within bryophytes, the liverwort *Marchantia polymorpha* and the moss *Physcomitrium patens* stand out as model systems because of their genome sequence availability and well-established genetic engineering tools (Bowman *et al.*, 2017; Rensing *et al.*, 2020). In

addition, heterologous approaches using hornworts, lycophytes, ferns and charophyte algae are also providing insightful findings. An important difference between some of these models is that, with the exception of Physcomitrella and ferns (Li *et al.*, 2018), their genomes did not experience whole-genome duplication events (Bowman *et al.*, 2017; Nishiyama *et al.*, 2018). However, genome evolution is far from a linear process. There are reports of independent expansions of specific gene families even in non-flowering plants, as well as specific secondary losses in several plant genomes. A common experimental pipeline can be followed to approach comparative studies of TF function in divergent groups (Figure 2). In the following sections, we present a comprehensive overview on recent literature highlighting conserved and diverging molecular features.

TFs associated with gametogenesis and sexual reproduction

The role of critical regulators of gametogenesis is well conserved across plants. RWP-RK domain TF, RKD and MID, are conserved in *Marchantia* and green algae (Rövekamp *et al.*, 2016) and the MYB DUO POLEN 1 (DUO1) in *Marchantia* (Higo *et al.*, 2018). A fascinating example showing the power of the evolutionary approach as a tool to find novel genes is the discovery of *bHLH VIIIa BONOBO,* whose role in gametogenesis was firstly described in *Marchantia* and later in *Arabidopsis* (Yamaoka *et al.*, 2018). Moreover, it was also shown that expression of Mp*BONOBO* under the control of the *Arabidopsis BONOBO2* promoter is able to rescue pollen-germination issues of the *Arabidopsis bnb1 bnb2* double mutant and it can partially complement the germ cell specification defects (Yamaoka *et al.*, 2018). Interestingly, specific expression of MpFGMYB in Marchantia gametophytic tissues and its regulation by an antisense locus controls the gametophyte sexual dimorphism (Hisanaga *et al.*, 2019a). These genes, showed striking similarities in their expression patterns and their role during different stages of gametogenesis compared to angiosperms (Hisanaga *et al.*, 2019b).

Higo *et al*. thoroughly studied DUO1 in *Marchantia*, *Arabidopsis* and the charophyte algae *Chara braunii*. DUO1 exemplifies well how each molecular mechanism contributed to their evolution across the Streptophyte lineage (Higo *et al.*, 2018). The authors described that specific expression in the male gametophyte is conserved in land plants but not in algae, likely to mutations found in the promoter region. MpDUO1 also expresses in the male gametophyte of Marchantia controlling

sperm differentiation. Moreover, Mp*DUO1* expressed under the *Arabidopsis* promoter rescued At*duo1* pollen phenotypes but was not able to activate the downstream target At*HTR10*. This was overcome with a chimeric version bearing the *Arabidopsis* C-terminal region, highlighting the relevance of non-DBD protein regions. While, DUO1 DNA binding affinity is conserved in *Marchantia*, *C. braunii* and *Arabidopsis*, it differs in the conjugative algae *Closterium peracerosum* due to changes in the DBD, suggesting its protein activity is only conserved in plants with motile sperm or pollen tube elongation in angiosperms (Higo *et al.*, 2018). Finally, 1 out of 3 downstream targets orthologs are also differentially expressed in Mp*duo1* mutant.

Studies have addressed the co-evolutionary history of the TALE homeodomain KNOX/ BELL TF complex. Their ability to heterodimerize and migrate to the nucleus is conserved in land plants and algae (Bowman *et al.*, 2016; Horst *et al.*, 2016; Dierschke *et al.*, 2020). The KNOX-BELL heterodimerization is required to initiate the diploid program in *Chlamydomonas* zygotes (Lee *et al.*, 2008). In bryophytes, KNOX2 and BELL TFs conserved this function regulating the alternation of generations between sporophyte to gametophyte (Sakakibara *et al.*, 2013; Horst *et al.*, 2016). In sporophyte-dominant plants, TALE TFs are no longer required for reproduction, but they held a role as developmental switches and accompanied the complexity of body plan during embryophytes evolution.

TFs associated with organogenesis

The regulatory module formed by group Ia (SPEECHLESS, MUTE, FAMA-like, or SMF) and IIIb (SCREAM) bHLH TFs in stomata differentiation appear to be conserved in embryophytes. In Physcomitrella, the complete development of stomata requires the interaction of PpSMF1 and PpSCREAM1, similar to angiosperms (Chater *et al.*, 2016). Interestingly, plants from diverse lineages lacking stomata such as *Marchantia* and *Zostera marina* (an aquatic monocotyledonous plant) have lost their orthologs to *SMF* (Chater *et al.*, 2016). This rise an important evolutionary question: what is the ancestral role of conserved TFs associated with traits absent in a diverging lineage? For example, Bryophytes lack vasculature but encode for orthologs to *VASCULAR RELATED NAC-DOMAIN* (*VND*), a group of *NAC* TFs that regulate xylem differentiation in *Arabidopsis* . Surprisingly, in Physcomitrella, mutant plants in *VND* develop fewer hydroid cells (specialized cells for internal water conductance) suggesting that both tissues could share a

conserved regulatory program (Xu *et al.*, 2014). However, hydroids cells are in the gametophyte generation whereas the vasculature of tracheophytes is in the sporophyte generation. This intriguing recurrent behaviour in plant TF evolution may have required changes to adapt gene expression (see below). Another case are LEAFY and MADS TFs, associated with floral identity in angiosperms, although in non-flowering plants regulates cell division (for review see Lai et al. (2020)).

In the same direction, two clades of bHLH TFs TARGET OF MONOPTEROS 5 (TMO5) and LONESOME HIGHWAY (LHW) form a heterodimer to control the vasculature differentiation in Angiosperms. The single ortholog to TMO5 of *Marchantia* phenotypically restored the *Arabidopsis tmo5/t5l1* double mutant defect in vascular bundle patterning, unlike the charophyte algae version from *Klebsormidium nitens* (Lu *et al.*, 2020).=A chimeric version of KnTMO5 with the sole addition of the *Marchantia* ACT-like domain is enough to rescue the *Arabidopsis* phenotype. In contrast, only *LHW* versions from vascular species complemented the *Arabidopsis lhw* mutant (Lu *et al.*, 2020). In this case, neither the ACT-chimeric version restored the phenotype, suggesting the relevance of additional regions to TF-function (Lu *et al.*, 2020). Despite MpTMO5/MpLHW heterodimerization is an ancient feature, *Marchantia* mutants did not present obvious phenotypes at cellular level, suggesting they control development but in independent ways with little overlap in downstream transcriptional responses (Lu *et al.*, 2020).

TF associated with hormone-controlled responses

Most of the core elements of hormonal signaling emerged prior to Embryophytes evolution (Bowman *et al.*, 2017; Blazquez *et al.*, 2020). AUXIN RESPONSE FACTOR (ARF) function in morphogenesis is conserved in land plants (Prigge *et al.*, 2010; Flores-Sandoval *et al.*, 2015; Kato *et al.*, 2015; Lavy *et al.*, 2016; Mutte *et al.*, 2018). At the same time, ARFs constitute a nice example of early diversification. *Marchantia* has a single member of each ARF class, MpARF1 (class A) and MpARF2 (class B) bind similar targets, but MpARF3 (class C) bearing a unique DBD regulates a different set of genes (Flores-Sandoval *et al.*, 2018; Kato *et al.*, 2020). MpARF3 appeared unrelated to auxin signalling with a PB1 domain unable to interact with AUX/IAA as MpARF1 does (Kato *et al.*, 2015). Only PB1 from MpARF1 presents structural properties that connects it to the auxin signalling, whereas MpARF2 interacts with the TOPLESS co-repressor

using a different region (Kato *et al.*, 2020). These differences in protein-protein interactions plus others in their expression patterns in *Marchantia*, builds an antagonistic circuit of ARF regulation that could have been essential for the evolution of the embryophyte meristem and it was maintained following a marked diversification of ARFs in vascular plants.

The two-component system signalling, including ethylene and cytokinin, show strong TF functional conservation even in algae. The canonical ethylene pathway is present and functional in the filamentous alga *Spirogyra pratensis* (Ju *et al.*, 2015). Even more, *Arabidopsis* mutants were partially rescued by an ethylene-signalling TF homologues from *Spirogyra,* the TF ETHYLENE-INSENSITIVE 3 (EIN3) (Ju *et al.*, 2015). It was recently shown that the *Marchantia* EIN3 homolog is involved in dormancy and development in a conserved manner and it contributes to the liverwort ACC-triggered responses in a singular way (Li *et al.*, 2020). The cytokinin pathway of *Marchantia* using loss-of-function alleles of the central TFs controlling the hormone-response known as type-A and B response regulator (RRA/RRB), showed multiple developmental defects including reduced thalli size and hyponastic growth, enhanced rhizoid formation and inhibition of gemma cup formation (Flores-Sandoval *et al.*, 2016; Aki *et al.*, 2019).

The abscisic acid (ABA) dependent TF *ABI3 (ABA-INSENSITIVE 3)*, involved in desiccation tolerance and dormancy in seeds of angiosperms is also necessary for ABA-induced desiccation tolerance in the gametophyte of Physcomitrella (Zhao *et al.*, 2018) and in gemmae dormancy of *Marchantia* (Eklund *et al.*, 2018). Similarly, the function of MYC2 as master TF of the jasmonate pathway, mainly known for its role in plant-inducible defences in land plants, is conserved in *Marchantia* regulating hormone sensitivity and herbivore-induced defences (Peñuelas *et al.*, 2019). In the cases of ABI3A and MYC2, cross-species complementation experiments failed despite their conserved ability to bind similar DNA motifs and induction of similar transcriptional changes, suggesting that specific protein partners are required to conserve TF function (Marella *et al.*, 2006; Yotsui *et al.*, 2013; Peñuelas *et al.*, 2019). PpABI3A presents a weak interaction with its counterpart ABI5 in angiosperms (Marella *et al.*, 2006) and, similarly, MpMYCs interacts with endogenous component of the *Marchantia* mediator complex MED25 but weakly with AtMED25 (Peñuelas *et al.*, 2019), indicating that unique structural features outside the DBD controls their partners.

A fascinating case is the gibberellin (GA) related R2R3-MYB TF, GAMYB. GAsignalling might have evolved in the common ancestor to vascular plants. GAMYB from the lycophyte *Sellaginella*

moeleindorfii can functionally complement the rice *gamyb* mutant, it is induced by GA and it shows localized expression in the microspore, suggesting a strong conservation in vascular plants (Aya *et al.*, 2011). Many components of the GA biosynthesis and signalling pathway are missing in bryophytes (Cannell *et al.*, 2020). However, Aya *et al*. showed that the role of GAMYB in Physcomitrella spore development could be conserved but in a GA-independent fashion (Aya *et al.*, 2011). In both moss and lycophyte, GAMYB would directly regulate the expression of CYP703, involved in the biosynthesis of sporopollenin, a protective component of the surface of spores and microspores (Cannell *et al.*, 2020). This illustrates how TF function, and even targets, could even predate the emergence of hormone signalling evolution that could be the case of other hormone-related TFs.

TF associated with environmental responses

Light mediated regulation by *PHYTOCHROME INTERACTING FACTOR* (*PIF)* and *FAR-RED ELONGATED HYPOCOTYL 1* (*FHY1)* TFs were also found to be functionally conserved in *Marchantia* and Physcomitrella gametophytes. They showed a conserved interaction with the Pfr form of PHYTOCHROME and complemented *Arabidopsis* mutants (Possart *et al.*, 2017; Xu & Hiltbrunner, 2017; Inoue *et al.*, 2019). Similar to angiosperms, the MpHY5-MpCOP1 complex also mediates the ultraviolet-b (UVB) perception controlled by UVR-8 photoreceptor to modulate the accumulation of the phenylpropanoid sunscreen in plant tissues (Clayton *et al.*, 2018). A different evolutionary track followed PpSPA function, that still interacts with COP1 to regulate HY5 stability in darkness, but controls a limited range of photomorphogenesis responses compared to *Arabidopsis* (Artz *et al.*, 2019). The switch from vegetative to reproductive growth of *Marchantia* is also triggered by light signals perceived by MpPHY and MpPIF (Inoue *et al.*, 2019) and core TFs of the circadian clock are also conserved (Linde *et al.*, 2017).

Environmental regulation of anthocyanin biosynthesis in angiosperms involves TF of the R2R3-MYB family, such as AtPAP1. MpMYB02 and MpMYB14 are putative orthologs with interesting singularities. MpMYB14 regulates the accumulation of a unique reddish pigment called auronidins, while flavonols remained unaltered (Albert *et al.*, 2018; Berland *et al.*, 2019). Still, MpMYB14 is induced upon stress and orchestrates defence against pathogens (Albert *et al.*, 2018; Carella *et al.*, 2019) Therefore, it is not clear whether MpMYB14 has a conserved or convergent function compared to PAP1. On the other side, MpMYB02 regulates the biosynthesis of bis(bibenzyl) acids, a characteristic compound of liverworts, but not other phenylpropanoids

(Kubo *et al.*, 2018). The subgroup 9 R2R3 MYB MIXTA involved in cuticle biosynthesis is also functionally conserved in *Marchantia* (Xu *et al.*, 2020).

Also MYC1, another TF associated with JA-ignalling, controls the accumulation of bis(bibenzyl) acids in the liverwort *Plagiochasma appendiculatum* (Wu *et al.*, 2018). Interestingly, Mp*MYB02* is not induced by UVB treatment (Clayton *et al.*, 2018; Kubo *et al.*, 2018). Some parallelism could be traced with *class I TCP* (*TCP1*) of *Marchantia*, whose mutants showed enhanced pigmentation accumulating aminochrome-derivatives, different to those regulated by TCPs in *Arabidopsis* (Busch *et al.*, 2019). These cases highlight to what extent the differences in secondary metabolism of bryophytes and *Arabidopsis* were accompanied by rewiring the associated GRN. The evolution of species-specific metabolic pathway was also observed in vascular plants, and involved the acquisition of CRE in key enzyme genes (Lacchini & Goossens, 2020).

There are other environment-responsive TFs that evolved different functions in different plant lineages, such as the *Marchantia MYC1*-homolog Mp*BHLH12* (Arai *et al.*, 2019) and *class I HD-Zip* (Mp*C1HDZ*) (Romani *et al.*, 2020). In both cases, the *Marchantia* genes are not induced by similar environmental cues to *Arabidopsis* (UVB and osmotic stress, respectively) and plants with altered gene expression developed abnormalities in liverwort synapomorphies, the gemma cup morphology and oil bodies. Also members of the stress-related *AP2/ERF* family (MpERF13 and PpSTEMIN) were reported to play divergent functions in bryophytes (Ishikawa *et al.*, 2019; Kanazawa *et al.*, 2020). In these cases, where there is not an apparent conserved function, it will be relevant to identify the gene functions hindered by redundancy in seed plants to fully understand the ancestral function.

TF involved in meristem regulation

In angiosperms, the main players regulating shoot apical meristem are well known. As we mentioned before, hormone pathways such as auxin and cytokinin are well conserved in bryophytes (Blazquez *et al.*, 2020), but it is still not clear whether these programs work independently in the gametophyte and the sporophyte (see below). Among TFs, a group of *AP2* (*AINTEGUMENTA, BABY BOOM,* and *PLETHORA;* or *APB*) is critical for the sporophytic meristem maintenance in angiosperms. In mosses, the expression of Pp*APBs* is localized in the gametophyte apical cells and the Pp*apb* high-order mutant arrests at the protonema (filamentous-

like structure) stage (Aoyama *et al.*, 2012), suggesting *APBs* are also required for the establishment of the gametophyte meristem. Interestingly, in ferns, the overexpression of the endogenous *AINTEGUMENTA* or a *Brassica napus BABY BOOM* induces the development of a sporophyte without gametes (Bui *et al.*, 2017).

WUSCHEL-related homeobox (WOX) genes control cell proliferation rates in different *Arabidopsis* meristems and are exceptional examples of late functional diversification (Sarkar *et al.*, 2007). However, their role in the gametophyte meristem is not clear. Physcomitrella PpWOX13L regulates cell wall loosening, affects cell regeneration and zygote formation but the mutant did not present any meristem-related defect (Sakakibara *et al.*, 2014). Similarly, in *Marchantia*, the single MpWOX is not required for CLE peptides-mediated meristem regulation (Hirakawa *et al.*, 2020). This suggest that WOX role in sporophyte meristem regulation does not work in the bryophyte gametophytic meristem.

On the other hand, *Class I KNOX* (*KNOX1*) are expressed and participate in sporophyte development but not in the gametophytic meristem in bryophytes and ferns (Sakakibara *et al.*, 2008; Dierschke *et al.*, 2020; Hisanaga, 2020) suggesting it was not co-opted as other regulators (see below). Also, the overexpression of fern or Physcomitrella versions in *Arabidopsis* resembles the AtKNOX1 overexpression leaf serration phenotype (Sakakibara *et al.*, 2008) and Pp*MKN2* can phenotypically complement the *Arabidopsis brevipedicellus* mutant defect in silique-insertion angle, but KNOX1 homologs from vascular plants cannot complement the Physcomitrella *mkn2/4/5* triple mutant defect in sporophyte development (Frangedakis *et al.*, 2017).

Few evolutionary studies approached the *Class III HD-Zip* (*C3HDZ*). In ferns and lycophytes, *C3HDZ* presents a conserved polarized expression pattern in primordia, suggesting their function could be conserved in all vascular plants (Vasco *et al.*, 2016). A possible conserved function could be associated with the coordination of leaf morphology in the moss gametophyte suggesting a possible co-option (Yip *et al.*, 2016). Regardless *C3HDZ* are expressed in both bryophyte sporophyte and gametophyte, but its function in the former has not yet been elucidated.

Sporophyte vs gametophyte

The life cycles of bryophytes and seed plants show contrasting dominant generations. The bryophytes have a dominant gametophytic generation (haploid) with a reduced sporophyte (diploid), whereas seed plants show the opposite. Despite this difference, we mentioned several TFs acting at the same generation (eg. *SMF*, *KNOX1*, gametogenesis-related TFs). An additional example is *TCP2* in Physcomitrella that also plays a conserved function in sporophyte branching (Ortiz-Ramírez *et al.*, 2016).

There are also opposite examples with TFs active in contrasting generations. In Angiosperms, the *miRNA156/529-SPL* module controls the vegetative to reproductive transition, whereas in *Marchantia* controls the transition to sexual reproduction in the gametophyte and is also regulated by light signals (Tsuzuki *et al.*, 2019). Additional TFs examples are *Arabidopsis* LSH1 and rice G1 (ALOG) (Naramoto *et al.*, 2019) and R2R3-MYB TFs ortholog to RAX (Yasui *et al.*, 2019).

Some of the best-studied cases are the *bHLH VIIIc* (*RSL*) and *XI* (*LRL*). *RSL* was found to regulate root hair differentiation in the sporophyte of *Arabidopsis* and rhizoid elongation in gametophyte of bryophytes (Menand *et al.*, 2007; Proust *et al.*, 2016), with homolog functions but in opposite generations. *LRL* genes of *Arabidopsis* and Physcomitrella also regulate root hairs and rhizoids development (Tam *et al.*, 2015). In *Marchantia LRL* knockdown plants present conspicuous epidermal defects in addition to reduced rhizoids (Breuninger *et al.*, 2016). Interestingly, only the *C. braunii* LRL, but not Cb*RSL*, complements *Arabidopsis* mutants (Breuninger *et al.*, 2016). In Chara, the *RSL* expression pattern is not related to rhizoids suggesting that this apparent functional conservation is restricted to land plants (Bonnot *et al.*, 2019).

The model proposed to explain this phenomenon implies a massive co-option of genetic programs to different generations. The current hypothesis suggests the existence of an isomorph generation in the common ancestor and an independent tissue specialization in both groups of embryophytes (Bowman *et al.*, 2019). In general, the co-option model cannot be generalized. The difference in the vegetative meristem regulation in vascular plants and bryophytes highlights the disparity in TFs conservation. Besides, in some cases, the homology among tissues is often controversial and the scenario of convergent evolution should not be discarded.

Neofunctionalization and subfunctionalization

There are several reports of neofunctionalization for duplicated genes in angiosperms acquiring novel functions after divergence. A good example are some of the *Arabidopsis* homologs to Mp*FGMYB* expressed in the sporophytic endosperm controlling fatty acid synthesis, a novel function probably acquired later in angiosperm evolution (Hisanaga *et al.*, 2019a). However, this is not always the case; sometimes the ancestral function is unclear or lost during evolution. Novel functions associated with lineage specific traits are likely acquired after lineage divergence, for example MpC1HDZ and MpERF13 function in liverwort oil bodies (Kanazawa *et al.*, 2020; Romani *et al.*, 2020) or LEAFY in angiosperm floral identity. There are other cases of TFs with a conserved ancestral function that were followed by neofunctionalization in bryophytes without a duplication event. For example, MpTCP1 retains the ability to control growth as their homologs in *Arabidopsis* but controlling the induction of aminochromes (Busch *et al.*, 2019). Likewise, Mp*rkd* mutant display morphological defects in gemmae cups (Rövekamp *et al.*, 2016). It is important to notice, that gene-family expansions followed by neo- or subfunctionalization can also occur in bryophytes (eg. MpBHLH12). It is important to consider multiple scenarios of gain or loss of functions along Streptophytes evolution in order to obtain a full picture of the evolutionary trajectory.

Relative contributions of mechanisms associated with TF evolution

Among possible mechanisms involved in the selection of novel TF functions, changes in expression patterns are probably the most important. In general, the conservation of the expression pattern is tightly associated with functional conservation (e.g. PAP1, HY5, SMF, gametogenesis related TFs), but often in the opposite generation. On the contrary, cases where the function is not deeply conserved showed divergent or lineage specific expression patterns (e.g. DUO1, bHLH VIII, MpMYB02, MpBHLH12, MpC1HDZ).

Protein-protein interactions involving regions outside the DBD are usually conserved, particularly in hormone-related TFs (e.g. ARF, MYC2, ABI3A, PIF, FHY1). This is frequently evidenced by functional complementation of *Arabidopsis* mutants using bryophyte proteins or *vice versa*. Nonetheless, in some cases, co-evolutionary processes have been proposed too (e.g. MYC2, ABI3A, KNOX1). The use of chimeric TFs or domain-swap TFs with their orthologs and cross-

species complementation have helped to understand the contribution of auxiliary domains during evolution (e.g. TMO5, LHW, DUO1, ARFs).

On the contrary, the DBDs and TF sequence specificities are usually conserved across embryophytes, and it was experimentally validated in a plethora of cases (e.g. DUO1, MYC2, MADS, ABI3A, TCP1, LFY). This was also supported by cross-species complementation assays in different land plant lineages. A nice example is DUO1, where the DNA specificity is different in algae but conserved in land plants (Higo *et al.*, 2018).

However, there is a gap of knowledge in the conservation of downstream targets, since most of the works did not compare angiosperms with non-flowering plant transcriptional changes. In general, a single TF can regulate several downstream genes and one-to-one gene regulation conservation is quite unlikely. We discussed here some examples of deep conservation of some downstream targets (DUO1 and GAMYB). In MpTMO5/LHW and MpC1HDZ, downstream transcriptional responses are divergent compared to angiosperms (Lu *et al.*, 2020; Romani *et al.*, 2020). In the case of MpMYCs and MpMYB14 (Albert *et al.*, 2018; Kubo *et al.*, 2018; Peñuelas *et al.*, 2019), target genes are related to similar biological processes but the orthology of these genes was not assessed. GRN are highly dynamic and can gain and lose nodes and edges keeping a similar function (Lacchini & Goossens, 2020).

Concluding remarks

Even after millions of years, several TFs seems to play similar function in distant plant lineages in a conserved fashion. This idea relies on the presumption of homology between different biological processes and tissues, and the unlikeliness of a convergent repurpose of transcriptional regulators for similar function. Evolution and conservation of TFs take different avenues depending on whether they control developmental or environmental responses. Whereas TFs involved in developmental programs are mostly conserved among plant divergent lineages, here we discussed several cases of TFs required for environmental responses with divergent functions or different expression patterns.

It is important to address this uneven conservation in order to understand the process of plant transition to the land environment. A possible reason behind this evolutionary singularity is that

during early land plant evolution both lineages may have developed independent solutions for stress responses using similar components of the genetic toolbox inherited from aquatic algae (Fürst-Jansen *et al.*, 2020). Many of these TFs may have acquired their function after the diversification of tracheophytes.

Recent advances in the functional studies of TFs in non-flowering plants offered novel insights about how genetic innovations took place during plant macroevolution. Some of these works provide a good experimental framework to approach this kind of evolutionary questions (Figure 2) and it will help to think in archetypical trajectories of TF evolution. The deep understanding of GRN will help to differentiate traits that share a common origin from those evolving independently or convergently and to acknowledge the complexity and unique diversity of GRN that exists in divergent lineages.

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Author contributions

FR and JEM conceived and wrote the manuscript. FR and JEM contributed equally to this work.

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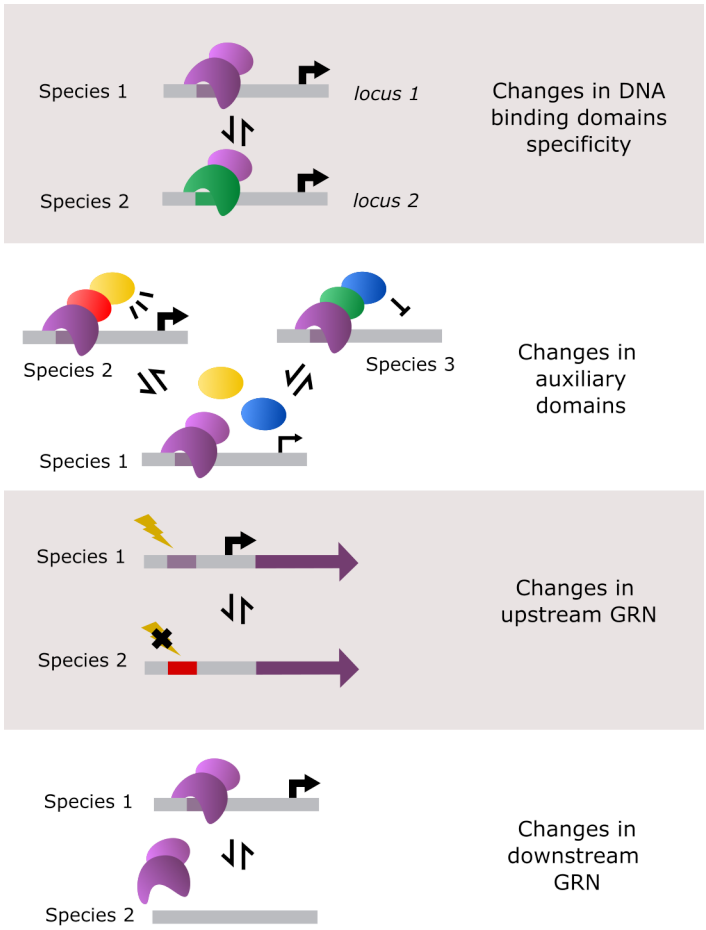
Figure legends

Figure 1. Four major mechanisms of transcription factor (TF) function evolution. The first two mechanisms involve changes in TF-protein sequences, on either the DNA-binding domain (DBD) or the auxiliary motifs. The other two mechanisms affect upstream- or downstream-gene regulatory network (GRN). Changes in upstream-GRN refers to changes in the expression of TFs. Whereas downstream GRN changes are mainly the consequence of altered expression of target genes. Changes of *cis*-regulatory elements are the most frequent cause of the last two mechanisms. Arrow width represent gene expression levels and blunt-ended arrows gene repression.

Figure 2. The reconstruction of transcription factor (TF) functional evolution implies multidisciplinary and comparative efforts that range from sequence analysis, phylogenetics, phenotypic studies on genetically engineered plants, gene expression studies protein-protein interactions, and downstream signalling. We schematically highlight common questions, and techniques used to infer possible evolutionary scenarios. We added some extra questions that should be considered to fully understand the evolutionary processes. Each level, from top to bottom, represents different incremental steps in the reconstruction of TF evolution. See also Delaux *et al*. (2019) for a broader guide for comparative biology evo-devo studies. GO, gene ontology; DBD, DNA-binding domain; DEG, differentially expressed genes; Y2H, yeast two-hybrid; Co-IP, co-immunoprecipitation; FRET, Förster resonance energy transfer; BiFC, bimolecular fluorescence complementation; ChIP-seq, chromatin immunoprecipitation followed by sequencing; PBM, protein binding matrix; SELEX, systematic evolution of ligands by exponential enrichment; qPCR, quantitative polymerase chain reaction.



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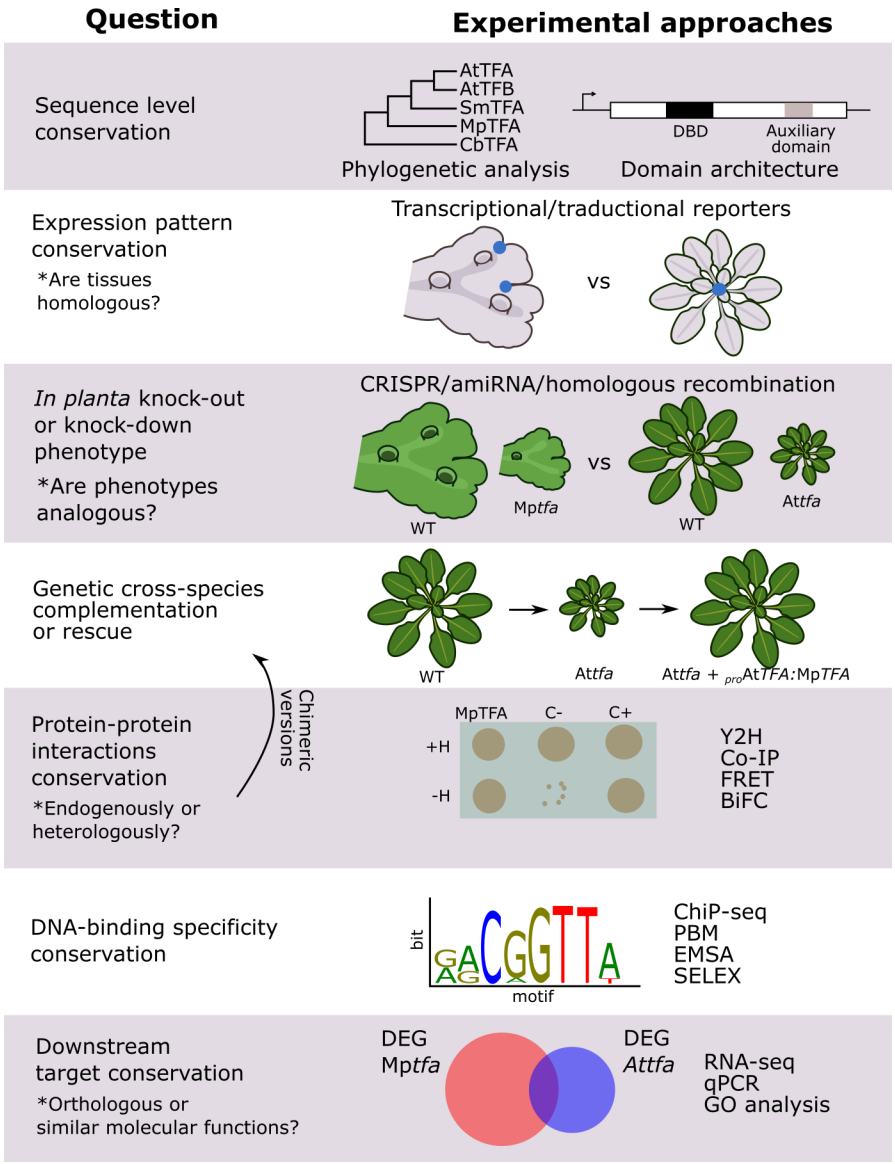

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