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Identification of MeC3HDZ1/MeCNA as a potential regulator of cassava storage root development

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ABSTRACT

The storage root (SR) of cassava is the main staple food in sub-Saharan Africa, where it feeds over 500 million people. However, little is known about the genetic and molecular regulation underlying its development. Unraveling such regulation would pave the way for biotechnology approaches aimed at enhancing cassava productivity. Anatomical studies indicate that SR development relies on the massive accumulation of xylem parenchyma, a cell-type derived from the vascular cambium. The C3HDZ family of transcription factors regulate cambial cells proliferation and xylem differentiation in Arabidopsis and other species. We thus aimed at identifying C3HDZ proteins in cassava and determining whether any of them shows preferential activity in the SR cambium and/or xylem. Using phylogeny and synteny studies, we identified eight C3HDZ proteins in cassava, namely MeCH3DZ1–8. We observed that *MeC3HDZ1* is the *MeC3HDZ* gene displaying the highest expression in SR and that, within that organ, the gene also shows high expression in cambium and xylem. *In-silico* analyses revealed the existence of a number of potential C3HDZ targets displaying significant preferential expression in the SR. Subsequent Y1H analyses proved that MeC3HDZ1 can bind canonical C3HDZ binding sites, present in the promoters of these targets. Transactivation assays demonstrated that MeC3HDZ1 can regulate the expression of genes downstream of promoters harboring such binding sites, thereby demonstrating that MeC3HDZ1 has C3HDZ transcription factor activity. We conclude that MeC3HDZ1 may be a key factor for the regulation of storage root development in cassava, holding thus great promise for future biotechnology applications.

1. Introduction

The world's population has increased three-fold in the last 70 years (www.un.org), reaching over eight billion people. Predicting models indicate that, by the end of the 21st century, it will reach ten billion. Developing countries are experiencing the largest population increase and this growth patterns are predicted to continue over the next decades. Considering the climate change context and the decrease in arable land that population growth entails, quick and targeted improvement of our main staple crops production to ensure food security is a top priority.

Cassava (*Manihot esculenta*) is the fifth staple crop worldwide and the top one in sub-Saharan Africa, where it currently feeds over five hundred million people (www.fao.org). Due to a variety of reasons, cassava is the preferred crop for self-sustained farmers in many sub-Saharan Africa regions. First, cassava displays strong resistance to high temperatures and drought. Second, it presents an outstanding capacity to accumulate carbohydrates in the form of starch in its storage root (from now on SR), the edible part of the plant. Third, the SR remains in good conditions in the soil for a long time after reaching its full development, allowing for gradual harvest depending on needs. Fourth, cassava can be clonally propagated in an extremely easy manner (Lebot, 2009).

In the last couple of decades, strong research efforts have been made to improve cassava in terms of SR nutritional quality, disease resistance and postharvest deterioration (Sayre et al., 2011; Otun et al., 2022; Shakir et al., 2022). Remarkably, although our knowledge about SR development is quite wide at the anatomical and physiological levels, little is known about the molecular mechanisms underlying it (Vanderschuren and Agusti, 2022; Chaweewan and Taylor, 2015; Zierer

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et al., 2021).

The anatomical dynamics behind SR formation in plants were well documented through classic studies in sweet-potato (Wilson and Lowe, 1973; Togari, 1950; McCormick, 1916; Kokubu and Studies, 1973), and several parallelisms exist with cassava. However, specific studies in cassava have helped elucidating specific developmental mechanisms occurring within this species. The mature cassava root system consists of two types of roots that coexist, namely the fibrous root (FR) and the SR (Lebot, 2009). During the first steps of root development, all roots are FR. Later on, through signals that are currently unknown, a subset of these roots experiences several developmental changes to become SRs (Alves, 2002; El-Sharkawy, 2004). Histologically, the differentiation of the two types of roots can be explained by the type of xylem that they develop. Whereas FRs develop wood, consisting of xylem vessels, fibers, xylem rays and axial parenchyma, SRs develop, almost exclusively, xylem parenchyma (Vanderschuren and Agusti, 2022). This does not mean that SRs are unable to produce vessels or fibers. Indeed, they can produce such cell types under certain circumstances (i.e: after flowering or under certain stressful conditions; Lebot, 2009). It is also worth keeping in mind that having held FRs identity in early developmental stages, SRs conserve a central cylinder of lignified xylem.

Xylem vessels are the major water transporters, thus supporting a strong water pressure (Esau, 1961). The fiberś role is mainly complementary to that of vessels, providing mechanical support (Dickison, 2000). For these reasons, vessels and fibers develop thick secondary cell walls that are strongly lignified (Ruzicka et al., 2015). The xylem parenchyma cells do not develop lignified secondary cell walls (Esau, 1961). Instead, these xylem cells possess the ability of synthesizing and storing starch (Esau, 1961; Ruzicka et al., 2015). Considering that xylem cells develop from the cambial meristematic cells, undergoing several maturing steps that lead them to acquire their final identity (Agustí and Blázquez, 2020), understanding how cassava cambial cells proliferate and how developing xylem cells acquire the parenchymatic identity, can tremendously help improving cassava productivity. In this respect, the usage of novel imaging techniques, such as tomography, can help understanding the developmental dynamics at high resolution, which will be key for further genetic and molecular approaches aimed at, among other relevant aspects, disentangle how cambium and xylem proliferate during cassava SR development (Carluccio et al., 2022).

Several studies have helped elucidating the physiology and metabolism events behind SR development. An increase in auxin signaling activity in parallel with a decrease of GA signaling, seems to be a requirement for SR initiation in sweet potato (Singh et al., 2019; Firon et al., 2013; Noh et al., 2010); the former probably canalizing xylem identity acquirement and the latter blocking differentiation from xylem parenchymatous state into fiber or vessel cell types. While these principles in SR development have not been unequivocally proved in cassava, an elegant transcriptomic approach indicated that the role of auxin and GA in SR development observed in sweet potato is probably conserved in cassava (Rüscher et al., 2021). In the same line of argumentation, it is very likely that cytokinin plays a role in cambium cellular proliferation during cassava SR development, as it is the case in sweet potato (Tanaka et al., 2008a; Dong et al., 2019). Interestingly, a recent study (Lamm et al., 2023) also highlighted the relevance of sugar utilization for starch accumulation in the SR, which not only sheds new light on SR physiology but also provides new avenues of research to understand SR development and physiology, as it has been shown that the enhanced starch storage and biosynthesis is, together with delignification, modulation of cell wall metabolism and the above mentioned phytohormone signaling, crucial during SR thickening (Firon et al., 2013; Sojikul et al., 2015; Sun et al., 2015; Utsumi et al., 2022).

Despite the above-described fundamental knowledge generated during the last decades at the anatomical, physiological and transcriptomic levels, little is known, still, about the genetic regulation of cassava SR development (Vanderschuren and Agusti, 2022; Zierer et al., 2021; Rüscher et al., 2021). Forward genetic approaches aiming at identifying root developmental regulators in cassava are scarce. Partially, this is due to the cassava adult plant́s size, the relatively long flowering time, and the fact that the storage root takes several weeks to develop. However, the genetics of root development -including that concerning cambium activity and xylem differentiation- are well documented in the model species *Arabidopsis thaliana*, making it possible to perform gene-targeted approaches aimed at understanding the molecular regulation of root development in cassava based on such knowledge. Thus, here we aimed at identifying genetic regulators of the vascular pattern establishment and xylem identity acquirement in cassava. In Arabidopsis, the C3HDZ family of transcription factors has been shown to play a key role in this respect (Ohashi-Ito and Fukuda, 2003; Ohashi-Ito et al., 2005; Prigge and Clark, 2006; Smetana et al., 2019; Carlsbecker et al., 2010). The C3HDZ family consists of five redundantly acting paralogues in Arabidopsis, namely: PHABULOSA (PHB), REVOLUTA (REV), PHAVOLUTA (PHV), CORONA (CNA) and Arabidopsis thaliana HOMEOBOX 8 (AtHB8) (Carlsbecker et al., 2010; Baima et al., 2001). Consistently with the reported redundancy, most of the genes encoding these proteins show overlapping expression patterns (Carlsbecker et al., 2010).

In general, the *AtC3HDZ* genes are expressed in the cambial zone. Among them, *AtHB8* expresses preferentially in the xylem side of the cambium, supporting the idea that C3HDZ proteins mark cells with xylem identity (Smetana et al., 2019; Carlsbecker et al., 2010; Baima et al., 2001). Genetic and molecular analyses have helped fine-tuning the function of the C3HDZ proteins, demonstrating that, although a general redundancy exists, each member of the family may play dominant roles in specific developmental processes. For example, *rev* loss of function mutants display a lack of interfascicular fibers in the stem (Prigge and Clark, 2006; Zhong and Ye, 1999). Also, plants ectopically expressing *AtHB8* show enhanced secondary growth (Baima et al., 2001), and the *CORONA* gain of function *icu-4* mutant develops more vascular tissue than WT (Ochando et al., 2008). However, single *Atc3hdz* loss of function mutants show no obvious xylem or cambium phenotype; defects in this respect being only found in high order mutants (Carlsbecker et al., 2010). Thus, quadruple mutants show some xylem defects and scattered cambial divisions, and mutants for all five genes display no xylem development (Smetana et al., 2019; Carlsbecker et al., 2010). All in all, these data reinforce the idea that the C3HDZ family is required for cambium activity and xylem establishment and that strong redundancy exists between the members of the family in the regulation of such developmental processes in Arabidopsis. The *C3HDZ* expression levels are negatively regulated by the *miRNA165/166* (Rhoades et al., 2002; Reinhart et al., 2002; Tang et al., 2003; Emery et al., 2003). The differential presence of *miRNA165/166* imposes high or low accumulation of *C3HDZ* transcripts (Carlsbecker et al., 2010; Emery et al., 2003). High *miRNA165/166* accumulation leads to protoxylem differentiation, while low accumulation results in metaxylem differentiation, demonstrating a fundamental role for the family in the determination of xylem cell-type identity (Carlsbecker et al., 2010).

Characterization of C3HDZ TFs in *Zinnia elegans* and rice demonstrated that the essential functions of the family in vascular development are most likely conserved across species (Ohashi-Ito and Fukuda, 2003; Ohashi-Ito et al., 2005; Itoh et al., 2008). Importantly though, in *Populus*, the C3HDZ effect on vascular cambium morphogenesis seems to be mainly capitalized by one single protein, namely PopREV (Robischon et al., 2011), the Populus orthologue of REVOLUTA. This result implies that the observed redundancy in *Arabidopsis* may not equally apply to all species. Thus, in this work we aimed at identifying C3HDZ proteins preferentially operating in the storage root and potentially regulating developmental aspects of SR formation such as cambium activity and xylem parenchyma identity acquisition. To that end, we first used phylogeny and synteny to determine the cassava (*Manihot esculenta*) orthologues of the C3HDZ proteins. Using gene expression analyses, we determined that *MeC3HDZ1*, a member of the *MeC3HDZ* family encoding a CORONA orthologue, not only displays preferential expression in

the storage root in comparison with the rest of vegetative organs but also shows a marked expression in the storage root cambium and developing xylem. Furthermore, we have demonstrated that MeC3HDZ1 can bind to promoters carrying the canonical C3HDZ binding site and regulate the expression of genes downstream of such promoters, indicating that MeC3HDZ1 is able to carry out C3HDZ transcription factor activity. We propose that MeC3HDZ1 may be relevant both for the regulation of xylem parenchyma identity acquisition and for the proliferation of cambium cells during cassava SR development. Results suggest that MeC3HDZ1 may be a good candidate for future biotechnology approaches aimed at improving cassava productivity.

2. Results

2.1. Identification of eight C3HDZ orthologues in the cassava genome

To identify the most likely orthologues in cassava for each of the five C3HDZ transcription factors that exist in Arabidopsis, we first constructed maximum likelihood phylogenetic trees using potential C3HDZ orthologues identified in eleven more species (see Methods and Materials for details). Our BLAST search identified 89 amino acid sequences throughout the twelve species (Supplemental File 1). The tree displayed five different paraphyletic groups, among which we identified eight potential cassava C3HDZ proteins (Fig. 1), from now on MeC3HDZ1 to 8. Paraphyletic groups were named depending on the Arabidopsis C3HDZ protein/s that they contained. While MeC3HDZ1 and 2 clustered in the CORONA (CNA) paraphyletic group, MeC3HDZ3 and 4 fell in the AtHB8 paraphyletic group and the REVOLUTA (REV) paraphyletic group contained MeHDZIP5 and 6. MeC3HDZ7 and 8 were found in the PHABULOSA/PHAVOLUTA (PHB/PHV) paraphyletic group. Identifying two cassava orthologues per Arabidopsis protein is a quite common feature, since cassava experienced a genomic duplication over evolution (Prochnik et al., 2012; Bredeson et al., 2016). All analyzed monocot sequences (from rice or foxtail millet) clustered together within the tree (Fig. 1). Further tests with 1000-bootstrap analyzes confirmed our observation (Supplemental Fig. 1). Synteny analyzes confirmed that cassava genes encoding proteins that fall within the CNA, AtHB8 or REV paraphyletic groups are orthologues of such genes and that MeC3HDZ7 and MeC3HDZ8 could be orthologues either of PHB or PHV (Supplemental Fig. 2).

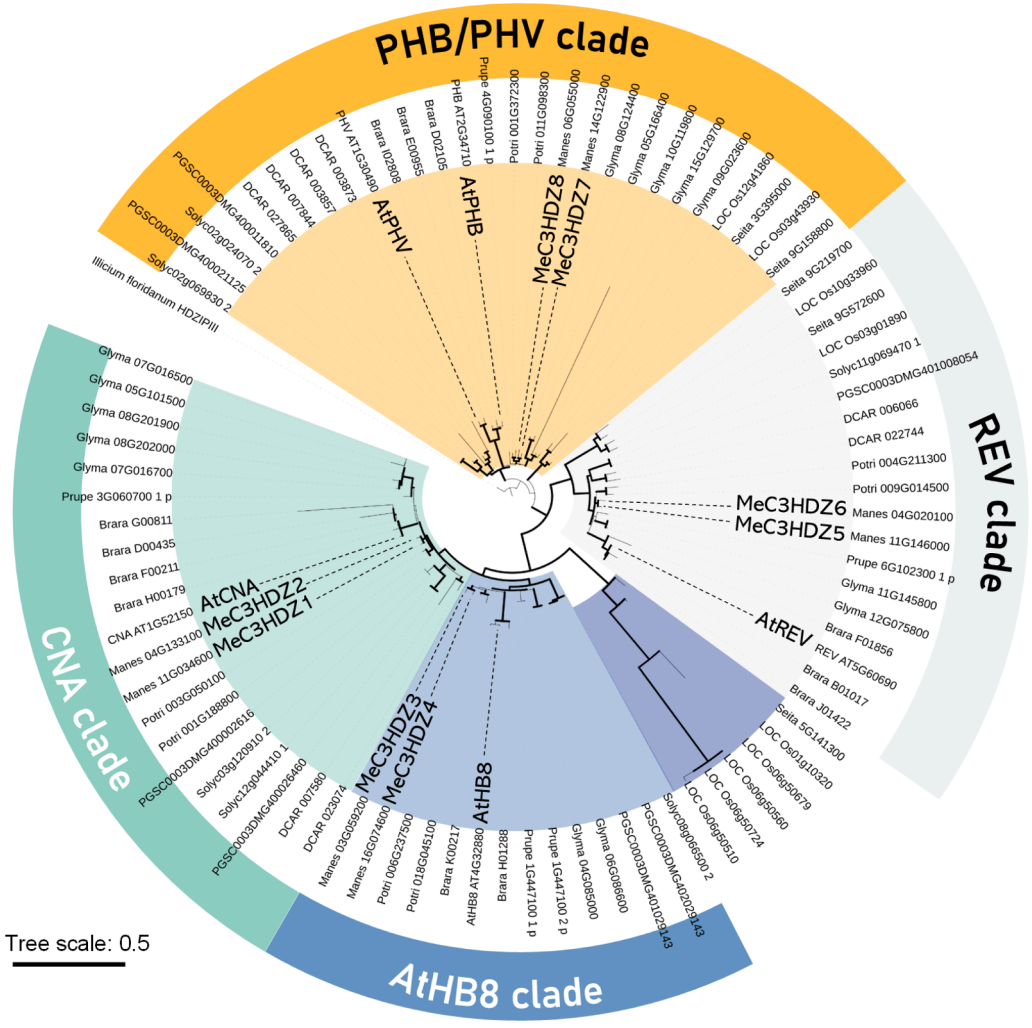

Fig. 1. Identification of putative C3HDZ proteins in cassava. Maximum likelihood tree generated with protein sequences of: cassava (*Manihot esculenta*; proteins code: Manes), Arabidopsis (*Arabidopsis thaliana*; proteins code: AT), field mustard (*Brassica rapa*; proteins code: Brara), tomato (*Solanum lycopersicum*; proteins code: Solyc), potato (*Solanum tuberosum*; proteins code: PGSC), carrot (*Daucus carota*; proteins code: DCAR), soybean (*Glycine max*; proteins code: Glyma), black cottonwood (*Populus trichocarpa*; proteins code: Potri), peach (*Prunus persica*; proteins code: Prupe), rice (*Oryza sativa*; proteins code: LOC Os), foxtail millet (*Setaria italica*; proteins code: Seita) and purple anise (*Illicium floridanum*; ANA clade, outgroup). Clades are defined based on Arabidopsis proteins as follows: yellow PHB/PHV, grey REV, light blue AtHB8, turquoise CNA. Arabidopsis and cassava orthologues are marked. Branch support values are based on Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-like aLRT), and values over 0.8 are marked with thicker black branches.

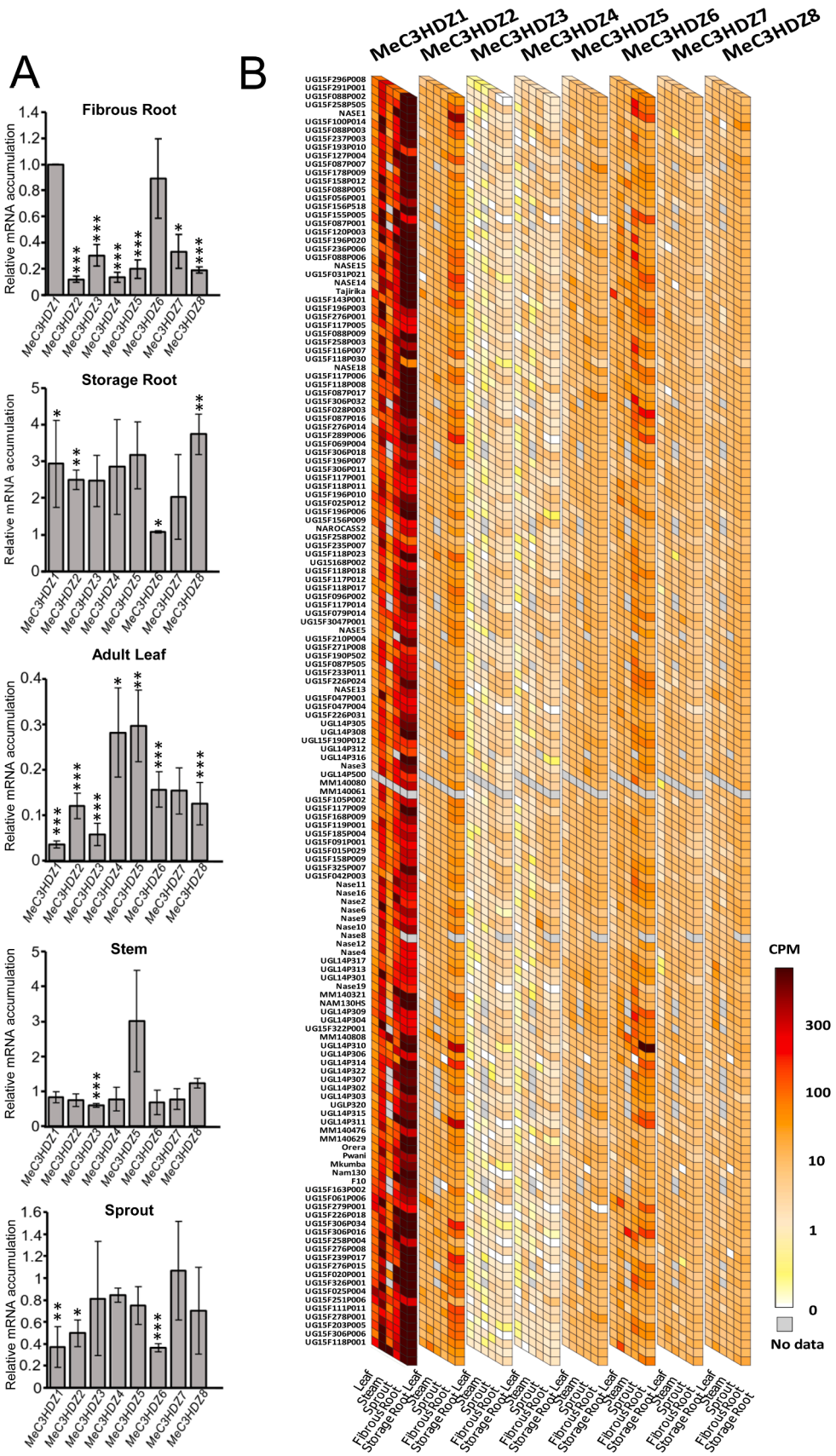

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Fig. 2. Relative mRNA accumulation of the *MeC3HDZ* genes in different plant organs. (A) Quantitative RT-PCR analyses revealed the relative mRNA accumulation of each *MeC3HDZ* gene in fibrous root, storage root, adult leaf, stem or sprout. For fibrous root, mRNA accumulation levels were normalized to that of *MeC3HDZ1*. For the rest of organs, mRNA accumulation of each gene was normalized to that of fibrous root. Bars represent the standard error. Significance was assessed through t-test. *= $p \leq 0.05$; **= $p \leq 0.01$; ***= $p \leq 0.001$ (B) Expression levels of the eight *MeC3HDZ* genes in different plant organs across 150 cassava accessions. Diagram generated from data contained in Ogbonna et al. (2021)

2.2. *MeC3HDZ1 displays preferential expression in the storage root cambium and developing xylem*

Considering the crucial relevance of the C3HDZ proteins in the establishment of the vascular patterns, cambium activity and xylem development (Carlsbecker et al., 2010; Baima et al., 2001), and the critical role that these developmental processes play in SR formation, we searched for *MeC3HDZ* genes preferentially expressed in SR. To that end, we performed qRT-PCR of all eight genes in SR, FR, sprout, stem and leaf. The expression of all genes in each organ was normalized using the expression of *MeUBIQUITIN1* as a reference. After normalization, all values were referred to the (normalized) values in the FR. Our expression analyses revealed that all *MeC3HDZ* genes except *MeC3HDZ6* displayed enhanced expression in SR in comparison to FR (Fig. 2A; Supplemental Table 1). *MeC3HDZ8*, *MeC3HDZ5* and *MeC3HDZ1* displayed the highest differential expression in comparison to FR, with 3.737-fold, 3.167-fold and 2.933-fold, respectively (Fig. 2A, Supplemental table 1). Importantly, when we scored the relative values of each gene within the FR, we observed that, in that organ, *MeC3HDZ1* is the gene displaying highest expression levels and that the expression levels of *MeC3HDZ8* and *MeC3HDZ5* are very weak in comparison to those of *MeC3HDZ1* (Fig. 2A). Thus, in absolute terms, the gene showing highest expression in SR is *MeC3HDZ1*. As for the aerial organs, no gene showed enhanced expression in comparison to FR except for *MeC3HDZ5* in the stem, which displayed 3,019-fold expression in comparison to FR (Fig. 2A, Supplemental table 1).

To test whether the relevance of *MeC3HDZ1* expression in SR is a general feature in cassava or a particular one in the 60444 accession, we made use of a RNAseq-based transcriptomic atlas for 150 cassava accessions (Ogbonna et al., 2021) to check the expression levels of the eight *MeC3HDZ* genes in all vegetative organs in all such accessions. Our results revealed that *MeC3HDZ1* is the highest expressed *MeC3HDZ* gene in the SR in 40% of the accessions and the second highest expressed in 38% (Fig. 2B; Supplemental Fig. 3; Supplemental table 2), implying that *MeC3HDZ1* shows high expression levels in a large number of accessions.

Considering that SR growth and development relies on cambium activity and xylem formation, we aimed at determining whether the observed prevalence of *MeC3HDZ1* expression is reflected in SR cambium and/or SR xylem. Therefore, we checked the expression of the eight *MeC3HDZ* genes in samples enriched in SR cambium or in SR xylem. To complete our analyses, we also included samples enriched in SR phloem. We used the same comparative protocol as described above -i.e: (i) for each gene, data was normalized using the expression of *MeUBIQUITIN1* and (ii) normalized data was compared to that of FR-. Results revealed that *MeC3HDZ1* is the gene displaying the highest expression level in samples enriched in SR cambium (4.369-fold expression in comparison to FR; Fig. 3; Supplemental table 1). In xylem, three genes showed enhanced expression in comparison to FR, namely *MeC3HDZ8* (2,604-fold), *MeC3HDZ4* (2,429-fold) and *MeC3HDZ1* (2,356-fold). Considering that, as it is the case for *MeC3HDZ5* and *MeC3HDZ8*, the expression of *MeC3HDZ4* is very low in comparison to that of *MeC3HDZ1* in FR (Fig. 3), we can conclude that, again, *MeC3HDZ1* is, in absolute terms, the gene undergoing the highest expression level in the SR xylem. In phloem, all genes showed weak expression levels in comparison to FR (Fig. 3; Supplemental table 1).

2.3. *MeC3HDZ-1 possesses C3HDZ activity*

We next aimed at determining whether MeC3HDZ1 possesses C3HDZ activity. To that end, we decided to test whether MeC3HDZ1 can both bind the promoter of theoretical C3HDZ targets and regulate their expression. To put our experimentation in the context of SR, we decided to use as theoretical target a gene showing significant preferential expression in SR in comparison to FR. To that end, we first selected 245 genes displaying the strongest significant preferential expression in SR in comparison to FR making use of previously published transcriptomic data (Wilson et al., 2017) (Supplemental table 3; Supplemental Fig. 4). We then obtained, for each gene, up to the first 500 bp of their promoters (i.e the first 500 bp upstream of the first ATG or the maximum length when the promoter region was less than 500 bp; Supplemental file 2; Supplemental Fig. 4). We used JASPAR (jaspar.genereg.net) (Castro-Mondragon et al., 2022) to look for position frequency matrices for *CORONA*, which binding sites happen to be the C3HDZ canonical one (GTAAT(G/C)AT(T/G)(A/G)C; Fig. 4A; Supplemental Fig. 4). We used the canonical C3HDZ binding sites associated matrices and the 245

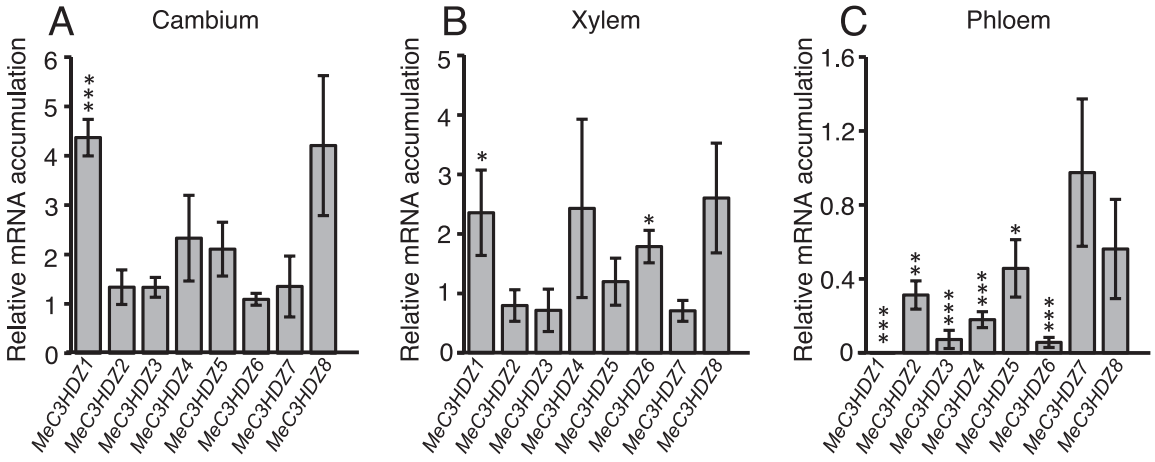

Fig. 3. Relative mRNA accumulation of the *MeC3HDZ* genes in storage root vascular tissues. Quantitative RT-PCR analyses revealed the relative mRNA accumulation of each *MeC3HDZ* gene in storage root cambium (A), storage root xylem (B) or storage root phloem (C). The mRNA accumulation of each gene was normalized to that of fibrous root. Bars represent the standard error. Significance was assessed through t-test. *= $p \leq 0.05$; **= $p \leq 0.01$; ***= $p \leq 0.001$.

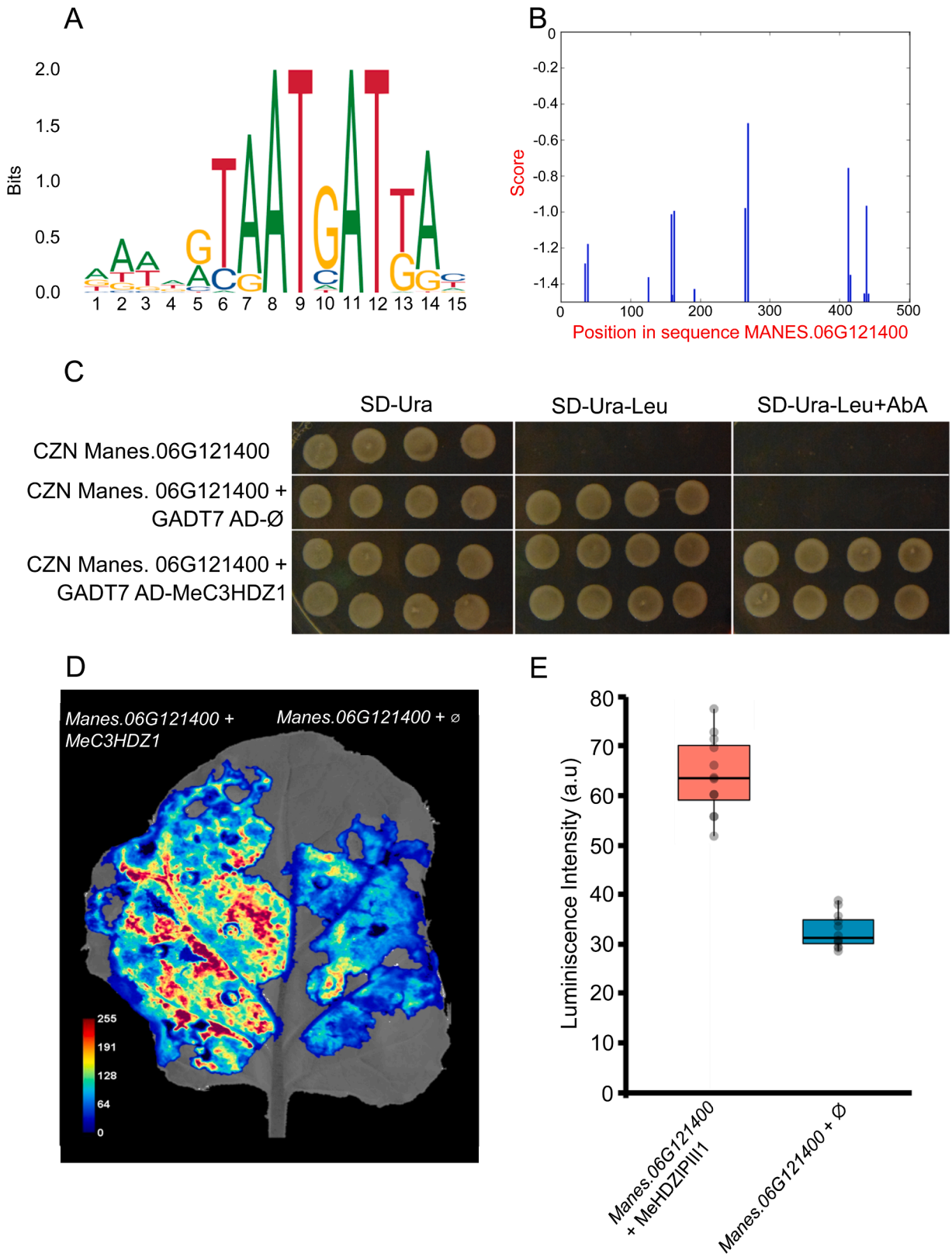

Fig. 4. MeC3HDZ1 possesses C3HDZ transcription factor activity. (A) MEME representing the canonical C3HDZ binding site. (B) Canonical C3HDZ binding sites with high probability for C3HDZ binding are present in the *Manes.06G121400* promoter. (C) Y1H analyses show that C3HDZ1 can bind to the *Manes.06G121400* promoter. (D-E) Transactivation assays demonstrate that C3HDZ1 can regulate the transcription downstream of the *Manes. 06G121400* promoter.

promoter sequences to run the web tool MORPHEUS (Minguet et al., 2015), a software providing scores to promoters according on their likeliness to be bound by a given transcription factor depending on the number of repetitions of the transcription factor binding site and the proximity of such sites to the first ATG. With this search, we identified the promoter of *Manes.06G121400* as our best candidate for further experimentation (Fig. 4B; Supplemental file 3; Supplemental Fig. 4). The workflow for the identification of the *Manes.06G121400* promoter is summarized in Supplemental Fig. 4**.** It is worth mentioning that Manes.06G121400 is highly expressed in the storage root, meristems, and stem (Supplemental Figure 5) tissues. Remarkably, we used the PlantPAN 3.0 database (Chow et al., 2018) providing genome visualization of ChIP-seq data, to analyze the promoter of the *Manes.06121400* Arabidopsis orthologue *AT5G63530*. Results revealed that, as it is the case for the *Manes.06121400* promoter, the *AT5G63530* promoter harbors several C3HDZ binding sites close to the initiation of transcription site (Supplemental Figure 6).

Next, we tested the capacity of MeC3HDZ1 to bind the promoter of *Manes.06G121400*. To that end we performed a yeast one-hybrid assay. Since the first 500 bp of the *Manes.06G121400* promoter contain two C3HDZ binding sites (Fig. 4B; Supplemental file 3), we cloned this sequence in the bait construct. In parallel, the full-length *MeC3HDZ1* coding region was cloned next to the activation domain (AD) in the GADT7 prey vector. We transformed yeast with the bait construct harboring the *Manes.06G121400* promoter. After confirming that autoactivation of the promoter did not take place, the yeast containing the bait was transformed either with the empty prey vector or with the prey vector expressing *MeC3HDZ1*. Our results confirmed that, indeed, MeC3HDZ1 can bind the *Manes.06G121400* promoter (Fig. 4C; Supplemental Figure 7). To test whether MeC3HDZ1 can bind and regulate the transcription of genes downstream of promoters containing C3HDZ binding sites *in planta*, we performed a luciferase transactivation assay in *Nicotiana benthamiana* leaves. For each construct combination, twelve independent transfections were performed and used to take images which were analyzed to calculate the intensity averages produced by each of such construct combination. Leaves infiltrated only with the *pManes.06G121400::LUC* construct displayed residual *LUC* luminescence, probably due to the activity of the *N.benthamiana* native C3HDZ proteins (Fig. 4D and E; Supplemental Figure 8). However, when infiltrated together with the *35S::VP16-MeC3HDZ1,* we observed more than two-fold *LUC* expression activity increase, confirming that MeC3HDZ1 can bind and regulate the expression of genes containing C3HDZ binding sites in their promoter (Fig. 4D and E; Supplemental Figure 8). Our results, thus, demonstrate that MeC3HDZ1 displays C3HDZ transcription factor activity.

3. Discussion

What are the molecular signals that control SR root development in cassava is a question about which little is known to date. Studies in sweet potato have been prolific in determining how SR forms (Wilson and Lowe, 1973; Togari, 1950; McCormick, 1916; Kokubu and Studies, 1973; Singh et al., 2019; Firon et al., 2013; Noh et al., 2010; Dong et al., 2019; Tanaka et al., 2008b) and parallelisms between sweet potato and cassava SR development are likely to exist (Zierer et al., 2021). However, we are only beginning to understand the genetic and molecular control of the process (Zierer et al., 2021; Rüscher et al., 2021; Lamm et al., 2023). Identifying genes potentially operating in upstream positions in regulatory networks controlling key programs for SR development represents a crucial step towards understanding the process.

We used a strategy based on identifying in cassava potential orthologues for genes previously described in model systems such as Arabidopsis or Populus as key regulators for two pivotal programs during cassava SR development: cambium activity and xylem differentiation. In concrete, we focused our attention in the C3HDZ family of transcription factors, known to regulate both processes (Prigge and Clark, 2006; Smetana et al., 2019; Carlsbecker et al., 2010; Baima et al., 2001; Zhong and Ye, 1999; Ochando et al., 2008). We showed that the *MeC3HDZ1* shows higher expression than other members of the *C3HDZ* family in SR. By determining that MeC3HDZ1 has C3HDZ function, we conclude that it is likely that the protein regulates similar (if not equivalent) processes to those regulated by C3HDZ proteins in Arabidopsis and Populus.

Although *MeC3HDZ1* shows very high expression in SR in a very large proportion of accessions, it is worth mentioning that, in some accessions, other genes also show high expression in that organ. This is the case of *MeC3HDZ2* (falling within the same paraphyletic group as *MeC3HDZ1*) and *MeC3HDZ6* (Supplemental Fig. 3). We thus hypothesize that in general, MeC3HDZ1 is the protein contributing the largest proportion of the C3HDZ function that takes place during the SR development, with other proteins of the family also contributing to the function; being the relative contribution of such other members of the family variable depending on the accession. In the same line of argumentation, due to natural variation, there are accessions in which it is likely that other proteins within the family may be of even higher relevance than MeC3HDZ1 in terms of MeC3HDZ function. Future genetic and molecular experimentation will clarify to which extent each member of the family contributes to the C3HDZ function during SR development.

Interestingly, in a high number of accessions, *MeC3HDZ1* expression is also high in stems, implying that MeC3HDZ1 might also regulate cambium activity and/or xylem differentiation in that organ, in turn implying that MeC3HDZ1 is probably not regulating exclusively SR development but also stem secondary development. This is an important piece of information both from a fundamental biology and from a biotechnology point of view. From a fundamental biology perspective, it is remarkable that the anatomy of the xylem tissue of SR is quite different to that of stem and that the anatomical changes taking place during vascular cambium establishment are different in roots and stems (Esau, 1961; Agustí and Blázquez, 2020). Thus, further genetic and molecular experimentation will help elucidating whether (and how) one single TF can contribute significantly to regulate cambium establishment, cambium activity and xylem differentiation in two organs presenting such clear anatomical differences. In this respect, the manipulation of the *MeC3HDZ1* expression might lead to alterations in the developmental processes of both SR and stem, which might enhance the effect of such manipulation. For instance, we hypothesise that generating gain of function lines for MeC3HDZ1 (for example by deleting the miRNA165/166 binding sites within the *MeC3HDZ1* sequence) might result in enhanced sizes of both SR and stems. While the former would have a direct effect on cassava productivity due to its impact on SR size, the latter might enhance the plant́s transport capacity, which might positively impact the general performance of the plant, including also the SR development. Supporting our hypothesis, as mentioned above, a previous work in Arabidopsis revealed that *icu-4*, a gain of function mutant on *CORONA* (the Arabidopsis orthologue for *MeC3HDZ1*) displays enhanced production of vascular tissues (Ochando et al., 2008).

Finally, to date, very little is known about the molecular mechanisms underlying the cell type specification of xylem parenchyma in plants, perhaps because in Arabidopsis this cell type is difficult to access. However, the relevance of this tissue is clear from an agronomical point of view. Thus, research in cassava SR, where xylem parenchyma is very prominent, may provide new concepts on the fundamental aspects of xylem parenchyma biology. In this way, genetic studies aimed at disentangling the specific function for C3HDZ proteins in xylem parenchyma development would be of great importance.

In summary, we have identified a protein possessing C3HDZ activity in cassava which displays high expression in SR and a high prevalence in SR cambium and xylem. Due to its nature and reported regulatory activity of its orthologues in Arabidopsis and other species in terms of cambium activity and xylem development, future studies on C3HDZ1 hold great promise for both, understanding fundamental molecular principles underlying SR development and the establishment of

biotechnology approaches. Considering the widespread high expression of *MeC3HDZ1* in the SR across a large number of cassava cultivars, any biotechnology application with this gene will be highly relevant, as it could be translated into most cassava cultivars.

4. Methods

4.1. Construction of phylogenetic trees and synteny analyses

To construct the C3HDZ phylogenetic tree (Fig. 1; Supplemental File 1) we first searched for potential C3HDZ sequences in the cassava (*Manihot esculenta*), black cottonwood (*Populus trichocarpa*), field mustard (*Brassica rapa*), potato (*Solanum tuberosum*), foxtail millet (*Setaria italica*), tomato (*Solanum lycopersicum*), peach (*Prunus persica*), rice (*Oryza sativa*), soybean (*Glycine max*) and carrot (*Daucus carota*) genomes. To that end we used the amino acid sequences of the *Arabidopsis thaliana* C3HDZ proteins -namely REVOLUTA/AT5G60690, PHABULOSA/AT2G34710, PHAVOLUTA/AT1G30490, ATHB8/AT4G32880 and CORONA/AT1G52150- as bait to carry out BLAST analyzes (Altschup et al., 1990) using the TBLASTN tool of phytozome (https://phytozome-next.jgi.doe.gov/). Rice and foxtail millet were chosen as monocots representatives, while field mustard, potato and carrot were selected due to their common ability with cassava to form underground storage organs. The rest of the species were selected with the view of encompassing as many vascular plant clades as possible. The only reported C3HDZ sequence of purple anise (*Illicium floridanum*) -belonging to the Amborellales-Nymphaeales-Austrobaileyales (ANA) clade, which contained species are assumed as diverged from the angiosperm common ancestor- was used as outgroup. To generate the phylogenetic tree, we followed the PhyML v3.0 maximum likelihood method (Guindon et al., 2010), making use of the NGPhylogeny.fr server (https://ngphylogeny.fr) (Dereeper et al., 2008). Amino acid sequences in FASTA were used as input (Supplemental File 1). Sequences alignment was performed using the MAFFT v7 tool (Katoh and Standley, 2013), using pre-established parameters. Statistical significance was evaluated through aLRT Likelihood statistics (Anisimova and Gascuel, 2006). For the bootstrap analyses we carried out the maximum likelihood method making use of the MEGA 11 software, utilizing the default options (Tamura et al., 2021). Amino acid sequences in FASTA were used as input (Supplemental File 1). Sequences alignment was performed using MUSCLE with pre-established parameters and statistical significance was evaluated with the bootstrap method using 1000 replicates.

Graphic representation of the tree was generated with iTOL v6 (itol.embl.de) (Letunic and Bork, 2021). Synteny analyzes were carried out using the synteny plot tool in Dicots Plaza 5.0 Portal (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v5_dicots/) (van Bel et al., 2022). Default features and options were used, namely window size: 5, strand orientation and clustering: standard.

4.2. Identification of C3HDZ target promoters expressed in storage roots

We used previously published transcriptomic data to identify genes preferentially expressed in the cassava storage root (Wilson et al., 2017). We then obtained the storage root to fibrous root (SR/FR) expression ratio for all such genes and selected the 245 genes that displayed higher values (Supplemental Table 3). Phytozome v12.1 was used to download, for each gene, up to the first 500 bp of the promoter (i.e: upstream of the first ATG) (Supplemental File 2). We searched for promoters containing canonic C3HDZ binding sites using position frequency matrices (PFM) for *CORONA*, obtained from JASPAR (jaspar.genereg.net) (Castro-Mondragon et al., 2022): a database containing curated, experimentally tested transcription factors binding sites stored as PFMs). Next, we used the webtool MORPHEUS (Minguet et al., 2015) on the generated matrices to analyze the C3HDZ binding site of the 245 sequences. Only promoters containing at least two binding sites in the first 500 bp before the first ATG and obtaining a MORPHEUS score above − 15 (which indicates high potential for transcription factor binding) were considered. As an additional validation step for our top candidate promoter, we identified its orthologue Arabidopsis gene and confirmed the presence of C3HDZ binding sites conserved in its promoter using the Promoter Analysais tool from PlantPAN 3.0 http://plantpan.itps.ncku.edu.tw/ (Chow et al., 2018).

4.3. Gene expression and statistical analyses

Cassava plants of 60444 cultivar were grown in the greenhouse for 6 months to ensure proper storage root development, as described (Siebers et al., 2017). Storage root, fibrous root, stem, adult leaf and sprout samples were collected. In addition, storage root samples enriched in cambium, xylem or phloem were manually dissected using a razor blade. Given the size of the storage roots, it is possible to generate such types of samples with a high degree of accuracy (Supplemental figure 9). In all cases, samples were immediately frozen in liquid nitrogen and stored at − 80ºC until needed. RNA was extracted as described (Siebers et al., 2017) and cDNA was generated using the NZY Fist-Strand cDNA Synthesis kit (NZYTech-Genes & Enzymes), following the manufacturers´ instructions. The cDNA was stored at − 80ºC until used. Primers were designed using the Benchling web tool (www.benchling.com). Used primers are listed in Supplemental table 4. Quantitative RT-PCR reactions were carried out in MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems) using Sybr Green Mix 2x, with ROX as reporter (TakaRa). Three biological replicates, from three independent collection events, were used for each of the above-mentioned sample type. In addition, three technical replicates were performed. Statistical analyses were carried out by using the SPSS 22.0 program (SPSS Inc., Chicago, IL, USA). Mean comparisons were calculated using the Duncan test at 5% probability level. In all cases, the expression level was normalized using the *MeUBIQUITIN1* expression as reference. We then used the normalized values in FR as reference to determine the relative value of each gene in each organ or tissue type. In this way, the normalized value for each gene in each organ (other than FR) or tissue type was compared to its normalized value in FR. To determine the relative expression values of each gene in the FR, the normalized value for each gene was compared to that of *MeC3HDZ1*.

4.4. Yeast one-hybrid

Bait strains (CZN Manes.06G121400) were generated by transforming YM4271 with the CZN1018 plasmid harboring the *Manes.06G121400* promoter region and selected on SD-Ura medium. The bait strains were transformed with the empty GADT7 plasmid, which harbors the GAL4 Activation Domain (AD) (GADT7 AD-Ø), and selected on SD-Ura-Leu to obtain our internal negative controls (CZN Manes.06G121400 + GADT7 AD-Ø). Simultaneously, a subset of the same bait strains was transformed with the GADT7 harboring the MeC3HDZ1 coding region to produce our test strains (CZN *Manes.06G*121400 + GADT7 *MeC3HDZ1*). Interaction between MeC3HDZ1 and the *Manes.06G121400* promoter region was examined on SD-Ura-Leu medium supplemented with Aureobasidin A (AbA). The minimal AbA concentration inhibiting growth of the internal negative controls was determined at 150 ng/ml whereas strains showing interaction between the MeC3HDZ1 TF and the promoter region were able to grow on SD-Ura-Leu+AbA medium.

4.5. Luciferase reporter analyses

The first upstream 500 bp before the ATG of the promoter region of Manes.06G121400 were cloned upstream of the LUCIFERASE gene in the pGreenII 800-LUC vector (Hellens et al., 2005). In parallel, the MeC3HDZ1 gene was cloned downstream of 35 S::VP16 into the pAlligator1 vector (Bensmihen et al., 2005). The pManes.06G121400::LUC

construct was co-infiltrated either with the empty pAlligator1 vector 35::VP16 or with the pAlligator1 harboring the 35 S:: VP16 +MeC3HDZ1 construct in *Nicotiana benthamiana* as previously described (Hellens et al., 2005). In brief, transient expression in *Nicotiana benthamiana* leaves was performed as follows, 10 ml cultures of the different strains harboring the constructs of interest were grown at 28 °C overnight. Cultures were collected by centrifugation (10 min at 4000 g) and resuspended in 2 ml of Resuspension Buffer (10 mM MES pH5.6, 10 mM MgCl2, and 200 μM acetosyringone). Cultures of the strains harboring the constructs of interest were combined with the p19 silencing suppressor strain and diluted with additional Resuspension Buffer to an optical density (OD600) of 0.2 for the p19 strain and to an optical density (OD600) of 0.4 for the cultures of the strains harboring the promoter, TF or empty vector. The combined cultures were left at room temperature for at least 3h and then transfections were carried out by infiltrating the abaxial sides of Nicotiana leaves with a needle-less syringe, 200–400 μl of culture per leaf sector (half of the leaf with the control and half of the leaf with the tested constructs combination). After Agro-infiltration, plants were returned to the growth chamber at 26º C in a 16 h light/ 8 h dark cycle. Luciferase luminescence was analyzed at least 48 h after Agro-infiltration in 12 different transfections for each condition (n = 12) using a FujiFilm LAS-3000 Luminescent image analyzer. FIJI (https://imagej.net/software/fiji/) was used to quantify areas and intensities of the luminescence produced by the different leaf sectors and to apply pre-defined LUTs to the gray-scale images obtained with the FujiFilm LAS-3000. The employment of LUT images simplifies the visualization of differences on luminescence intensity.

Author contribution

JB and JA conceptualized the study and designed the experiments. AS-G, AL, DO and CU. performed the experiments. AS-G., AL., DO, CU, JB and JA analyzed, discussed and curated the data. JB and JA wrote the paper. All authors read and approved the final manuscript.

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CRediT authorship contribution statement

Ombrosi Damiano: Formal analysis, Methodology. **Brumos Javier:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Supervision, Writing – original draft, Writing – review & editing. **Agusti Javier:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Software, Supervision, Writing – original draft, Writing – review & editing. **Brumos Javier:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Urbez Cristina:** Data curation, Formal analysis. **Lopez Anselmo:** Data curation, Formal analysis, Investigation, Methodology. **Sole-Gil Anna:** Data curation, Formal analysis, Investigation, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111938.

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