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Vanessa de Almeida Barros

SIGNALING MECHANISMS AND TRANSCRIPTIONAL REGULATION UNDERLYING ABIOTIC STRESS TOLERANCE IN CROPS CULTIVATED ON ACIDIC SOILS

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Vanessa de Almeida Barros

SIGNALING MECHANISMS AND TRANSCRIPTIONAL REGULATION UNDERLYING ABIOTIC STRESS TOLERANCE IN CROPS CULTIVATED ON ACIDIC SOILS

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"Signaling mechanisms and transcriptional regulation underlying abiotic stress tolerance in crops cultivated on acidic soils"

Vanessa de Almeida Barros

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RESUMO

Ocupando metade das terras aráveis do mundo, solos ácidos são comuns em países em desenvolvimento localizados nos trópicos e subtrópicos, onde a população em crescimento exige aumento na produção de alimentos. Em solos tropicais ácidos, as maiores limitações abióticas para a produção agrícola são a toxidez de alumínio (Al), a baixa disponibilidade de fósforo (P) e o estresse hídrico. Inicialmente, neste estudo apresentamos uma revisão buscando por elementos comuns, como fatores de transcrição (FTs) e moléculas de sinalização, que possam mediar o controle pleiotrópico de estresses abióticos coexistentes em solos ácidos. O FT *zinc figer* do tipo C_2H_2, AtSTOP1, é um candidato à esse papel pleiotrópico, pois está envolvido na regulação da tolerância ao Al, deficiência de P e respostas à seca em Arabidopsis. Além disso, AtWRKY46 atua na tolerância ao Al e na resposta à seca, com uma possível função na melhoria da aquisição de P, e AtMYB2 regula tanto a deficiência de P quanto a tolerância à seca. O possível papel unificador desses e de outros FTs na tolerância a estresses abióticos os torna potencialmente úteis em programas de melhoramento visando a adaptação do sorgo à solos tropicais. Em sorgo, o transportador localizado na membrana plasmática, SbMATE, confere tolerância ao Al via liberação de citrato ativado por Al na rizosfera. Foi demonstrado que os FTs, SbWRKY1 e SbZNF1, se ligam a *cis*-elementos em regiões repetidas que flanqueiam um elemento transponível no promotor do gene *SbMATE*, cooperativamente ativando sua expressão. Assim, nós objetivamos investigar os mecanismos moleculares pelos quais SbWRKY1 e SbZNF1 regulam transcricionalmente o gene *SbMATE*. Identificamos que SbWRKY1, pertencente ao grupo III dos FTs WRKY, contém um raro heptapeptídeo, WRKYGEK, e reconhece um novo *cis*-elemento de interação ao DNA de FTs WRKY. Ensaios de localização subcelular mostraram que SbZNF1 e SbWRKY1 estão localizados na membrana plasmática e no núcleo, respectivamente. Ambos os FTs interagem fisicamente na membrana plasmática e, sob exposição a Al^{+3}, SbZNF1 é mobilizado para o núcleo de uma maneira dependente de SbWRKY1. Este estudo mostra que SbZNF1 detecta o Al^{+3} e transmite o sinal para o núcleo, o que pode contribuir para a ação sinérgica previamente observada de SbZNF1 e SbWRKY1 na expressão de *SbMATE*.

Palavras-chave: Solos ácidos. Estresses Abióticos, Toxidez de Alumínio. Seca. Deficiência de Fósforo. Fatores de Transcrição. SbWRKY1. SbZNF1. DHHC. Tolerância ao Alumínio.

ABSTRACT

Occupying half the world's arable lands, acidic soils are common in developing countries in the tropics and subtropics, where growing populations continuously demand increases in food production. On tropical acidic soils, the most significant abiotic limitations to crop production are aluminum (Al) toxicity, low phosphorus (P) availability and drought stress. In this study, we present first a review aiming at the identification of common elements such as transcription factors (TFs) and signaling molecules that could mediate pleiotropic control of co-existing abiotic stresses on acidic soils. We found that the C_2H_2-type zinc finger TF, AtSTOP1, is a candidate for such a pleiotropic role, as it is involved in the regulation of Al tolerance, P deficiency and drought responses in Arabidopsis. Also, AtWRKY46 acts on Al tolerance and drought response, with a possible function in improved P acquisition. Additionally, a MYB TF, AtMYB2, regulates both P efficiency and drought tolerance. The possible unifying role of these and other TFs on abiotic stress tolerance make them potentially useful in breeding programs targeting sorghum adaptation to tropical soils. In sorghum, the plasma membrane-localized transporter, SbMATE, confers Al tolerance via Al-activated citrate release to the rhizosphere, where citrate detoxifies rhyzotoxic Al^{3+}. The TFs, SbWRKY1 and SbZNF1, were previously found to bind to *cis*-elements in repeats flanking a transposable element in the *SbMATE* promoter, cooperatively regulating *SbMATE.* Hence, we set out to investigate the molecular mechanisms whereby SbWRKY1 and SbZNF1 transcriptionally regulate *SbMATE*. We found that SbWRKY1, which belongs to WRKY group III, contains a rare heptapeptide variant, WRKYGEK, and recognizes a novel *cis*-element for WRKY DNA-interaction. Subcellular localization assays showed that SbZNF1 is located in the plasma membrane, whereas SbWRKY1 is found in the nucleus. Both TFs physically interact in the plasma membrane and, under Al^{3+} exposure, SbZNF1 is mobilized to the nucleus in a SbWRKY1-dependent manner, where they positively regulate *SbMATE*. This study shows that the plasma membrane-localized TF, SbZNF1, senses Al^{3+} and transmits its signal to the nucleus, which may contribute to the previously observed synergistic action of SbZNF1 and SbWRKY1 on *SbMATE* expression.

Keywords: Acidic Soils. Abiotic Stresses. Aluminum Toxicity. Drought. Phosphorous Deficiency. Transcription Factors. SbWRKY1. SbZNF1. DHHC. Aluminum Tolerance.

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INTRODUCTION

Acidic soils are widespread globally and predominantly found in the tropics and subtropics (von Uexkull and Mutert, 1995). The major constraints for crop production on these soils are aluminum (Al) toxicity, low phosphorus (P) availability and drought stress. At low pH (pH < 5.5), rhyzotoxic Al^{+3} ions are solubilized in the soil solution, damaging the root system and inhibiting root growth, which results in nutrient deficiency (Kochian et al., 2015), particularly phosphorus (P) deficiency. On tropical soils, P is strongly bound to Al and iron oxides in the soil clay fraction, becoming highly unavailable for uptake by the plants. Moreover, plants undergoing Al toxicity are more susceptible to drought stress, because the rapid inhibition of root growth leads to insufficient water uptake (Foy et al., 1993). Thus, crops cultivated on acidic soils may simultaneously be exposed to Al stress, low-P availability and drought stress. Chapter I presents a review in which we search for signaling/regulatory proteins, such as transcription factors (TFs), that could play a pleiotropic control of these co-existing stresses, which may be useful for breeding programs aiming at sorghum adaptation to the typical multiple stress condition on acidic soils.

Over the past years, several Al-tolerance genes were isolated broadening our knowledge of the molecular and physiological basis of crop Al tolerance (Kochian et al., 2015). A well-documented Al-tolerance mechanism is Al exclusion from sensitive sites in the root apices, a process driven by plasma membrane transporters mediating Al-activated release of organic acids (most commonly malate or citrate) from the root apex into the rhizosphere, where the organic acids chelate and detoxify Al^{+3} (Kochian et al., 2015). Two important transporters that provide crop Al tolerance are the wheat Al-activated malate transporter, TaALMT1 (Sasaki et al., 2004) and the sorghum Al-activated citrate transporter, SbMATE, which is a member of the multidrug and toxic compound extrusion family of membrane transporters (Magalhaes et al., 2007). In addition, some species can detoxify Al internally, for example, via Al uptake by plasma membrane transporters and subsequent sequestration into vacuoles (Negishi et al., 2012). In general, Al tolerance transporter genes are transcriptionally induced by Al and, in the last years, important advances have been made in the understanding of their transcriptional regulation (Zhang et al., 2019). The C_2H_2-type zinc finger TF in Arabidopsis, STOP1 (Sensitive to proton rhizotoxicity 1), and its homologous in rice, ART1 (Aluminum resistance transcription factor 1), are the best-characterized TFs regulating Al tolerance genes. AtSTOP1 transcriptionally regulates *AtALMT1* (Sawaki et al., 2009), the major Arabidopsis Al tolerance gene, which encodes an Al-activated malate transporter (Hoekenga et al., 2006). Additionally,

AtSTOP1 regulates the expression of other transporters required to Al tolerance in Arabidopsis, such as the Al-activated citrate transporter, *AtMATE1* (a homolog of sorghum SbMATE), and the ABC (ATP binding cassette) transporter-like protein, *AtALS3* (Liu et al., 2009; Sawaki et al., 2009). In rice, ART1 is implicated in the regulation of 31 genes in response to Al (Yamaji et al., 2009), including transporter genes involved with both external and internal detoxification of Al. Some of the ART1-regulated transporters are MATE, Al-activated citrate transporter, FRDL4, OsSTAR1 and OsSTAR2 (encoding the nucleotide-binding domain and the transmembrane domain, respectively, of a functional ABC transporter), OsNrat1 (Nramp family Al transporter 1) and OsALS1 (a half-type ABC transporter) (Yamaji et al., 2009; Yokosho et al., 2011; Huang et al., 2009; 2012). Moreover, *ALMT1* expression in Arabidopsis is regulated by the WRKY transcription factor, AtWRKY46, which represses *AtALMT1* expression and reduces malate exudation in the absence of Al, possibly controlling carbon losses (Ding et al., 2013).

The Alt_{SB} locus, which harbors the plasma membrane-localized SbMATE, controls approximately 80% of the phenotypic variation for Al tolerance in sorghum. SbMATE belongs to the MATE family and confers Al tolerance via Al-activated citrate released from root cortical cells into the rhizosphere (Magalhaes et al., 2007). It was demonstrated that *SbMATE* expression is regulated by a WRKY (SbWRKY1) and a DHHC-type zinc finger (SbZNF1) transcription factor (Melo et al., 2019). Al transcriptionally induces *SbWRKY1* and *SbZNF1* in the root apex from Al-tolerant lines, similarly to *SbMATE*, while in Al-sensitive lines, the expression of both TFs decrease over time. SbWRKY1 and SbZNF1 synergistically induce *SbMATE* expression by binding to a tandemly repeated sequence flanking a transposable element (MITE, Tourist-like miniature inverted-repeat transposable element, Wessler et al., 1995) found in the *SbMATE* promoter (Melo et al., 2019). In chapter II, we focused on the characterization of SbWRKY1 and SbZNF1 in the transcriptional regulation of the *SbMATE*. We performed a phylogenetic analysis to understand their relationship with proteins from other plant species and aimed to determine their binding site in the *SbMATE* promoter. Also, we investigated both the subcellular localization of SbWRKY1 and SbZNF1 and the physical interaction between both TFs. Finally, we used subcellular localization approaches to obtain insights about the signaling mechanisms by which SbZNF1 and SbWRKY1 respond to Al stress leading, inducing *SbMATE* expression.

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CAPÍTULO 1

Root Adaptation via Common Genetic Factors Conditioning Tolerance to Multiple Stresses for Crops Cultivated on Acidic Tropical Soils

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REVIEW
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Root Adaptation via Common Genetic Factors Conditioning Tolerance to Multiple Stresses for Crops Cultivated on Acidic Tropical Soils

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Crop tolerance to multiple abiotic stresses has long been pursued as a Holy Grail in plant breeding efforts that target crop adaptation to tropical soils. On tropical, acidic soils, aluminum (Al) toxicity, low phosphorus (P) availability and drought stress are the major limitations to yield stability. Molecular breeding based on a small suite of pleiotropic genes, particularly those with moderate to major phenotypic effects, could help circumvent the need for complex breeding designs and large population sizes aimed at selecting transgressive progeny accumulating favorable alleles controlling polygenic traits. The underlying question is twofold: do common tolerance mechanisms to Al toxicity, P deficiency and drought exist? And if they do, will they be useful in a plant breeding program that targets stress-prone environments. The selective environments in tropical regions are such that multiple, co-existing regulatory networks may drive the fixation of either distinctly different or a smaller number of pleiotropic abiotic stress tolerance genes. Recent studies suggest that genes contributing to crop adaptation to acidic soils, such as the major Arabidopsis Al tolerance protein, AtALMT1, which encodes an aluminum-activated root malate transporter, may influence both Al tolerance and P acquisition via changes in root system morphology and architecture. However, *trans*-acting elements such as transcription factors (TFs) may be the best option for pleiotropic control of multiple abiotic stress genes, due to their small and often multiple binding sequences in the genome. One such example is the C2H2-type zinc finger, AtSTOP1, which is a transcriptional regulator of a number of Arabidopsis Al tolerance genes, including *AtMATE* and *AtALMT1*, and has been shown to activate *AtALMT1*, not only in response to Al but also low soil P. The large WRKY family of transcription factors are also known to affect a broad spectrum of phenotypes, some of which are related to acidic soil abiotic stress responses. Hence, we focus here on signaling proteins such as TFs and protein kinases to identify, from the literature, evidence for

unifying regulatory networks controlling Al tolerance, P efficiency and, also possibly drought tolerance. Particular emphasis will be given to modification of root system morphology and architecture, which could be an important physiological "hub" leading to crop adaptation to multiple soil-based abiotic stress factors.

Keywords: acid soils, aluminum toxicity, aluminum tolerance, phosphorus deficiency, phosphorus efficiency, drought resistance, transcription factor, signaling

INTRODUCTION

Acidic soils (soils pH < 5.5) are quite extensive worldwide, comprising up to 50% of the world's potentially arable lands (Von Uexküll and Mutert, 1995). As the acidic weathered soils are particularly prominent in the humid tropics and subtropics where many developing countries in sub-Saharan Africa and Asia are located, and food production must keep pace with population growth (Godfray et al., 2010), acidic soils are a major constraint for developing world agriculture The two most significant limitations to crop production on acid soils from the plant nutrition perspective are aluminum (Al) toxicity and phosphorus (P) deficiency (Kochian et al., 2015). Both arise from the unique chemical properties of highly weathered acid soils. Aluminum is the most abundant metal in the earth's crust as it is a major component of clays, as aluminosilicates. At soil pH values of pH 5.5 and below, Al^{3+} ions are solubilized into the soil solution. Al^{3+} is quite toxic to roots, inhibiting both root elongation and root meristem cell division (see, for example, Kochian, 1995; Ma et al., 2001; Kobayashi et al., 2013b, and references therein). This results in major reductions in yields due to insufficient water and mineral nutrient uptake by the root systems. Low-P soil levels and availability also arise from the chemical properties of acidic soils as soil weathering exposes Al and Fe oxides/hydroxides on the surface of clay minerals, which bind soil P (as the phosphate anion) tightly, reducing its bioavailability (Marschner, 1995). Soils with low P availability will be designated henceforth as low-P soils for brevity. The third related stress we address in this review is drought stress, which is found on all soil types, including acidic soils. The unique aspect to acidic soils is that crop adaptation to drought on those soils requires that the plants be both Al tolerant to maintain a healthy root system to facilitate water absorption, along with specific adaptations to drought which are ubiquitously found in crop species on all soil types. Because crops acidic soils have had to adapt to all three stresses simultaneously, it is not surprising that especially in recent literature common features in adaptation to these three abiotic stresses are being uncovered. This is the theme we address in this review.

In searching for classes of genes involved in mediating resistance concurrently to these three stresses, it is more likely that "upstream" genes that control regulatory and signaling networks such as transcription factors (TFs), kinases and phosphatases would be more likely candidates than structural genes such as root plasma membrane transporters that mediate efflux of Al-binding organic acid anions that have been shown to be involved in crop Al tolerance (Ma et al., 2001; Ryan et al., 2001; Kochian et al., 2004, 2015). Regulatory genes, such as transcription factors, bind to very small *cis* elements in the promoter region. Depending also on more complex aspects such as chromatin structure, this gives TFs potential for promiscuous binding to many targets, giving rise to complex regulatory circuits. A good example of how the promiscuity of TF binding sites can impact evolutionary adaptation is presented in Pougach et al. (2014), where they show that duplication of a transcription factor gene allowed the emergence of two independent regulatory circuits in yeast. Since TFs are often regulators of response to multiple stresses, they are excellent candidates for breeding programs searching for pleiotropic control of co-existing stresses in acidic soils such as Al toxicity, low P availability, and drought (Baillo et al., 2019).

In this review, we have focused on signaling/regulatory proteins such as TFs and protein kinases to identify, from the literature, evidence for unifying regulatory networks controlling Al tolerance, P efficiency and also possibly drought tolerance. Particular emphasis will be given to modification of root system morphology and architecture, which could be an important physiological "hub" leading to crop adaptation to multiple soil-based abiotic stress factors.

ALUMINUM TOXICITY AND TOLERANCE

Transcriptional Regulation Involved in Al Tolerance

Aluminum (Al) on acidic soils intoxicates root regions involved in root growth (meristem and elongation zone). Cells in these regions are subject to rapid alterations in Al-induced transcription, resulting in the induction of expression of several Al tolerance genes associated with root tip Al exclusion and detoxification mechanisms (Kochian et al., 2015).

Several TFs (TFs) have been reported to be involved in crop Al tolerance, and the majority of these TFs belong to zinc finger and WRKY transcription factor families. Among them, *STOP1* in Arabidopsis and *ART1* in rice are the best characterized TFs regulating Al tolerance. STOP1, a C2H2-type zinc finger transcription factor, was identified via positional cloning of a low-pH-sensitive Arabidopsis mutant. Although *AtSTOP1* expression is not induced by Al, the *stop1*-mutant is also Al hypersensitive (Iuchi et al., 2007). AtSTOP1 has four functional zinc finger domains that bind to a 15-bp long sequence in the *AtALMT1* promoter. *AtALMT1* is the major Arabidopsis Al tolerance gene (Hoekenga et al., 2006), closely related to the primary wheat Al tolerance gene, *TaALMT1* (Sasaki et al., 2004). These two ALMT genes and similar ones in other plant species encode root

cell plasma membrane Al-activated malate efflux transporters that are one of the key genes involved in root Al exclusion via release of Al-binding organic acid anions. Mutations in the STOP1 binding sites and in AtSTOP1 zinc finger domains critically suppress *AtALMT1* expression, indicating that STOP1 binding is essential for *AtALMT1* expression and Al tolerance in Arabidopsis (Tokizawa et al., 2015). Furthermore, AtSTOP1 also regulates the expression of other transporters required for Al tolerance in Arabidopsis, including *AtMATE* (Al-activated citrate transporter) and *AtALS3* (ABC transporter-like protein) (Liu et al., 2009; Sawaki et al., 2009).

AtSTOP1 is ubiquitously expressed in the root with higher expression in the root tip, and its expression is not affected by Al stress. In turn, AtSTOP1 downstream genes (*AtALMT1*, *AtMATE*, and *AtALS3*) are induced by Al (Liu et al., 2009; Sawaki et al., 2009). These findings suggest that Al might induce *AtSTOP1* regulation at the post-transcriptional/-translational level. Recently, an F-box protein that regulates the level of AtSTOP1 protein, RAE1, was identified in Arabidopsis (Zhang Y. et al., 2019). The authors showed that RAE1 interacts with SKP1, a protein involved in ubiquitination and subsequent proteasomal degradation of target proteins. These two proteins interact to form a functional SCF-type E3 ligase complex, physically binding to STOP1 and driving its degradation via the ubiquitin 26S proteasome. As such, *AtALMT1* and other STOP1 regulated genes, including *AtMATE* and *AtALS3*, are overexpressed in the *rae1* mutant. Interestingly, AtSTOP1 binds to the *RAE1* promoter and positively regulates its expression, indicating that there is a negative feedback loop between *AtSTOP1* and *RAE1*. Finally, the authors suggest that the feedback loop might be important in controlling AtSTOP1 homeostasis, enabling the degradation of accumulated AtSTOP1 after Al stress.

The transcriptional regulation of *AtALMT1* expression by STOP1 is fairly well characterized. However, as stated in Tokizawa et al. (2015), the structure of the *AtALMT1* promoter indicates that other factors may be acting on its expression. The authors identified several *cis*-elements in the *ALMT1* promoter related to: (1) Al-induced early and late expression; (2) root tip-specific expression; and (3) repression of *ALMT1* expression. In addition, it was reported that the transcription factor, *CAMTA2* (Calmodulin binding *trans*-activator 2), binds to the *AtALMT1* promoter at a *cis*-element in a different binding region than STOP1, and appears to be involved in induction of *AtALMT1* expression only in late Al stress. Previously, it was also demonstrated that AtWRKY46 binds to W-box sequences in the *AtALMT1* promoter, repressing its expression in the absence of Al (Ding et al., 2013), indicating that regulation of *AtALMT1* is not restricted to STOP1.

Sharing 41.2% sequence identity with AtSTOP1, the rice homolog, OsART1, was identified by map-based cloning of an Al-sensitive rice mutant. ART1 is also a C2H2-type zinc finger transcription factor involved in the regulation of a number of rice Al responsive genes, but, unlike *AtSTOP1*, it is not responsive to low pH. Microarray analyses showed that OsART1 regulates at least 31 downstream genes in response to Al (Yamaji et al., 2009). This transcription factor directly binds to a GGNVS core sequence in the *OsSTAR1* promoter, which is present in 29 of the 31 ART1-regulated genes (Tsutsui et al., 2011). Some of the ART1-regulated genes have been functionally characterized as being involved in Al tolerance, including a number of transporters mediating Al uptake into the root and the vacuole, a Mg uptake transporter, and an ABC transporter that helps mediate the release of UDP-glucose into the cell wall to possibly minimize Al binding (Huang et al., 2009, 2012; Xia et al., 2010, 2013; Yokosho et al., 2011; Chen et al., 2012). Interestingly, the GGNVS core promoter sequence is also found in the promoter of genes regulated by AtSTOP1, suggesting that STOP1 and ART1 recognize similar DNA sequences (Tokizawa et al., 2015).

STOP1/ART1-like proteins, and their function in the regulation of Al tolerance genes, are broadly conserved among land plant species, including dicots, monocots, woody plants, and even bryophytes (Chen et al., 2013; Sawaki et al., 2014; Fan et al., 2015; Daspute et al., 2018; Huang et al., 2018; Wu et al., 2018; Ito et al., 2019; Kundu et al., 2019). The genome of the moss, *Physcomitrella patens* has a functional STOP1-like protein, and knock out of *PpSTOP1* results in an Al sensitive phenotype, suggesting that plants acquired *STOP1* at a very early time during land adaptation of plants, protecting roots from toxic environments including Al and low pH (Ohyama et al., 2013).

In addition to OsART1, OsWRKY22 also regulates the Al-induced expression of *OsFRDL4*, which encodes the rice root plasma membrane citrate efflux transporter. *OsWRKY22* is rapidly induced by Al and works as a transcriptional activator of the *OsFRDL4* expression via binding to W-box *cis*-elements in the *FRDL4* promoter. OsWRKY22 has not been shown to regulate other *ART1*-regulated genes, however, OsWRKY22 and OsART1 are essential for the full activation of Al-induced *FRDL4* expression and root citrate secretion in rice (Li et al., 2018).

Recently, through QTL mapping, GWAS, and functional analyses, two novel TFs in sorghum were identified, SbWRKY1 and SbZNF1, which positively regulate *SbMATE* expression (Melo et al., 2019). Previously, it was reported that miniature inverted-repeat transposable elements (MITE) in the *SbMATE* promoter play a critical role in its expression, and the number of MITE repeats is strongly correlated with *SbMATE* expression level and Al tolerance in sorghum (Magalhaes et al., 2007), which is consistent with the findings of (Salvi et al., 2007) showing that allelic polymorphisms due to MITE insertions can affect the transcription of regulated genes. These two TFs directly bind to sequences flanking the transposable element and, according to the proposed model, the expanded number of MITE repeats found in Al-tolerant genotypes provides an increased number of binding sites for SbWRKY1 and SbZNF1, resulting in higher sorghum *SbMATE* expression and Al tolerance (Melo et al., 2019). Similar to *SbMATE*, other studies have shown that the diversity of the promoter structures contributes to differences in Al tolerance between tolerant and sensitive genotypes in several crops. Al responsive genes of tolerant accessions of wheat (*TaALMT1*), *Holcus lanatus* (*HlALMT1*) and rice (*OsFRDL4*) carry more STOP1/ART1 binding sites in their promoters and exhibit higher expression levels than the same genes in the respective sensitive accessions (Chen et al., 2013; Tokizawa et al., 2015; Yokosho et al., 2016). These findings indicate that the enrichment of transcription factor binding sites in Al-tolerance gene promoters

leads to enhanced transcription factor recruitment, which might explain, at least partially, Al tolerance in several crop species.

In addition to zinc-finger and WRKY TFs, *ASR1* and *ASR5* (Abscisic acid, Stress and Ripening protein 1 and 5) are involved in Al tolerance in rice (Arenhart et al., 2013, 2014, 2016). *ASR5* is induced by Al and binds to the *OsSTAR1* promoter and functions together with OsART1 as transcriptional activators of the *OsSTAR1* expression. This study also demonstrated that *ASR5*-silenced plants impair the expression of two other rice Al tolerance genes, *OsNrat1* and *OsFRDL4*, suggesting that ASR5 is also involved in their transcriptional regulation (Arenhart et al., 2014). Subsequently, it was reported that *ASR5*-silenced plants exhibited a similar Al tolerance phenotype as wild type plants. This was attributed to the transcription factor *ASR1*, which, under the silencing of *ASR5*, is highly induced and regulates ASR5-target genes, including *STAR1*, in a non-preferential manner.

Recently, studies searching for novel regulators of Al resistance have identified TFs related to the modification of the cell wall properties (Li C.X. et al., 2019; Lou et al., 2020). In Arabidopsis, it was found that the *wrky47* mutant has reduced Al tolerance and altered subcellular Al distribution, i.e., increased Al accumulation in symplast, and decreased Al content in the root apoplast. According to the authors (Li C.X. et al., 2019), these effects occur due to the reduction of cell wall Al-binding capacity, conferred by decreased hemicellulose-I cell wall content in the mutant. It was demonstrated that AtWRKY47 directly binds to and activates the expression of genes encoding *EXTENSIN-LIKE PROTEIN (ELP)* and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE-HYDROLASES17 (XTH17)*, that are involved in cell wall modification. Within those, XTH17 works in modifying hemicellulosic polymers during cell expansion (Zhu et al., 2014), and ELP is involved in cell wall extension (Li C.X. et al., 2019). These findings indicate that *WRKY47* is involved in Al resistance by increasing cell wall bind of the rhizotoxic Al^{3+} ion, minimizing its effect on the cell wall and also reducing uptake into the root cytoplasm (Li C.X. et al., 2019). Another study showed that *VuNAR1* (*Vigna umbellata NAC-type Al Responsive1*), a rice bean *NAC* transcription factor, is up-regulated by Al in the root apex (Lou et al., 2020). In this paper, it was demonstrated that VuNAR1 binds to the *AtWAK1* (Arabidopsis wall-associated receptor kinase 1) and *VuWAKL1* (*Vigna umbellata WAK1-like*) promoters, positively regulating their expression (Lou et al., 2020). In Arabidopsis, *WAK1* is rapidly induced by Al, and the *AtWAK1* overexpression increases Al tolerance (Sivaguru et al., 2003). Lou et al. (2020) showed that the phenotype of the *Atwak1* mutant is higher root cell wall pectin content under Al stress, and it is believed that the binding of Al ions to the negatively charged carboxylic acid residues in pectin is involved in one of the components of Al rhizotoxicity, with methylation of the pectin carboxyl groups correlating with reduced Al toxicity (Yang et al., 2008).

Other Signaling Molecules

We still don't know how plants sense Al ions to trigger Al-dependent gene regulation. However, several signaling molecules have been identified that appear to be involved in initiating Al-induced transcriptional regulation. For example, Al-induced changes in cytosolic Ca^{2+} and pH (H^+), have been implicated as sensing/signaling molecules in Al signaling [see review by Kochian et al. (2015) and references therein]. In addition to these ions, several other endogenous species, reactive oxygen species (ROS), phytohormones, and the phosphatidylinositol pathway, appear to be involved in Al signal transduction.

Reactive Oxygen Species

Reactive oxygen species including peroxides, superoxide, hydroxyl radical, singlet oxygen are produced in response to a range of stress responses (Banti et al., 2010; Miller et al., 2010; Shahid et al., 2014; Hieno et al., 2019). Biomolecules including lipids, proteins, and DNA/RNA are oxidized by ROS, and this oxidative damage leads to organelle disfunction and programmed cell death (PCD) (Van Breusegem and Dat, 2006; Mittler, 2017). To protect the oxidative stress, plants activate antioxidant systems (i.e., ROS scavenging pathways) and also induce/activate a series of heat shock proteins (HSPs, e.g., molecular chaperon) (Sharma et al., 2012; Driedonks et al., 2015; Mishra et al., 2018; Waszczak et al., 2018). Al toxicity has been shown to trigger ROS, including hydrogen peroxides (H_2O_2), and Al/H_2O_2-mediated PCD was reported in various plant species (Yamamoto et al., 2002; Sivaguru et al., 2013; Huang et al., 2014). To protect against this, Al induces multiple genes associated with antioxidant production, such as peroxidase and superoxide dismutase, and they play a likely secondary role in Al tolerance (Ezaki et al., 2000; Basu et al., 2001). On the other hand, ROS also can act as signal molecules with the best characterized of these involved in plant defenses against pathogens and pests (see review by Bhattacharjee, 2012, and references therein). With regards to plant Al toxicity and tolerance, Sivaguru et al. (2013) showed there is a strong correlation between Al-induced ROS production and *SbMATE* expression, both temporally and spatially in the sorghum root tip. Subsequently, Kobayashi et al. (2013a) showed that *AtALMT1* and *AtMATE* expression are induced by H_2O_2 without Al. However, H_2O_2 cannot activate malate release from the roots, suggesting that protein activation of ALMT1 is regulated by a H_2O_2-independent pathway. In addition, several proteome analyses of Al stress revealed that several heat shock proteins (HSPs) are induced by Al stress (Zhen et al., 2007; Jiang et al., 2015), and the ER resident chaperon, *AtBIP3*, was identified as a possible Al-tolerance gene which is highly expressed in Al tolerant Arabidopsis accessions (Kusunoki et al., 2017). Interestingly, Enomoto et al. (2019) recently reported that AtSTOP1 directly regulates *AtHSF2A*, which is a master regulator of a series of HSPs, under hypoxic conditions. It is known that hypoxia stress involves ROS-mediated oxidative stress (Blokhina et al., 2003; Schmidt et al., 2018). These results suggest that activation of chaperon proteins including HSPs might be involved in signaling leading to tolerance of Al-induced oxidative stress in plants.

Nitric oxide (NO) is also induced by Al and has been suggested to be involved as a signaling molecule in Al signal transduction. There are several reports describing that Al-induced root growth inhibition is alleviated by application of the NO donor, sodium

nitroprusside (Wang and Yang, 2005; Zhang et al., 2011; He et al., 2012). More detailed research into the mechanistic basis for NO-regulated Al stress alleviation is still needed, but it may involve Al tolerance based on the following findings: (1) NO enhancement of antioxidant systems to prevent Al-induced oxidative stress (Wang and Yang, 2005; He et al., 2019), (2) NO modulation of OA metabolism and secretion under Al stress (Yang et al., 2012a,b), and (3) Al induction of endogenous ABA that may be a positive regulator of Al resistance (see phytohormone section below) (He et al., 2012).

Phytohormones

The root apex is the primary site of Al toxicity, and one of most active sites in the plant for phytohormone signaling (Ryan et al., 1993; Jung and McCouch, 2013). Auxin (i.e., Indole-3-acetic acid [IAA]) is a key regulator for plant root growth and development (Overvoorde et al., 2010). An appropriate auxin gradient with a maximal auxin gradient in the root apex are essential for continuous root growth (Petersson et al., 2009). Several membrane-localized PIN-FORMED (PIN) proteins, which are auxin-efflux transport proteins, play a major role in the regulation of the formation and maintenance of this gradient (Wiśniewska et al., 2006; Grieneisen et al., 2007). Al toxicity disturbs this auxin gradient in the root apex (Kollmeier et al., 2000; Shen et al., 2008); moreover, Al interferes with the appropriate membrane localization of PIN2 in Arabidopsis root tip cells (Shen et al., 2008). In addition, Al sensitivity was altered by knock-out or over-expression *PIN* genes in rice and Arabidopsis (Sun et al., 2010; Wu et al., 2014, 2015). These results suggest that abnormal PIN-mediated auxin flux in the root apex under Al stress is one of reasons for Al-induced root growth inhibition. Additionally, several Al-inducible IAA synthesizing genes, *AtTAA1* and *AtYUCCA*, encode proteins that regulate IAA accumulation in the root transition zone (TZ) which is located between the root meristem and zone of elongation (Yang et al., 2014; Liu et al., 2016). These genes are specifically induced in the TZ in response to Al, and activate IAA biosynthesis, resulting in root growth inhibition. Lastly, a recent study showed that the multidrug and toxic compound extrusion transporter, DETOXIFICATION 30 (DEX30), regulates auxin homeostasis in the TZ under Al stress, and contributes to Arabidopsis Al tolerance (Upadhyay et al., 2020).

Abscisic acid (ABA) also appears to be involved in Al signaling. Similar to several other phytohormones, endogenous ABA levels are upregulated under Al stress (see, for example, Kasai et al., 1995). However, unlike auxin and ethylene, ABA positively regulates Al tolerance. Al-induced root growth inhibition is alleviated by exogenous ABA application in barley, soybean, and buckwheat (Kasai et al., 1993; Shen et al., 2004; Hou et al., 2010; Reyna-Llorens et al., 2015). Additionally, co-treatment of ABA and Al induce greater root tip organic acid release than Al alone in soybean (Shen et al., 2004). In addition, ABA induces *AtALMT1* and *AtMATE* expression and malate release without Al in Arabidopsis (Kobayashi et al., 2013a). Therefore, Al-induced ABA production may contribute to the activation of OA transporter expression and increased OA release, which leads to Al resistance. Interestingly, IAA also induces *AtALMT1* expression, but it cannot activate malate release from roots without Al. This result is consistent with the finding that IAA treatment does not enhance Arabidopsis Al resistance.

Phosphatidylinositol

Recently, Wu et al. (2019) reported that blockade of phosphatidylinositol (PI) signaling, especially the Phosphatidylinositol 4-kinase (PI4K) and phospholipase C (PLC) pathways, leads to down-regulation of a number of Al-inducible genes, including *ALMT1*. PI and its derivatives are membrane lipids and conserved as signaling molecules among eukaryotes, and are involved in various important biological process such as membrane trafficking, root hair and pollen tube tip growth, and stress responses in plants (Meijer and Munnik, 2003; Thole and Nielsen, 2008; Ischebeck et al., 2010; Hou et al., 2016). In the screening, PIK-75 (Inhibitor for phosphatidylinositol 3-kinase [PI3K] in human) was identified that inhibits Al-induced malate secretion due to reduction of *ALMT1* expression. *In silico* docking analysis suggested that PIK-75 can interact with PI3K and PI4K in Arabidopsis. They confirmed that the PI4K and the subsequent PLC pathways play critical roles in Al-inducible *ALMT1* expression. Additionally, the blocking of the PI4K/PLC pathways significantly suppresses several Al-inducible genes, including STOP1-dependent target genes. The PI3K inhibitor does not affect Al-induced gene expression, suggesting that the PI4K/PLC pathways uniquely regulate signaling pathways associated with Al-inducible gene expression. However, PI3K is involved in plant Al signal transduction, because the PI3K inhibitor reduces root malate exudation via activation of the ALMT1 protein. More than 20 years ago, (Jones and Kochian, 1995) already speculated that the relationship between Al toxicity and membrane lipids included phosphatidylinositol. They found that Al directly and strongly binds to several plasma membrane lipids. PI(4,5)P2, the intermediate product between the PI4K and PLC pathways, has highest binding affinity with Al^{3+}. In addition, inositol trisphosphate, which is one of final products in the PI4K/PLC pathways, is transiently accumulated in culture coffee cells under Al stress (Poot-Poot and Teresa Hernandez-Sotomayor, 2011). These findings suggest that Al alters PI signaling/metabolism, and this could be a possible sensing mechanism for Al stress in plants.

P DEFICIENCY STRESS AND RESPONSES

Root system architecture (RSA) alterations leading to longer and thinner ageotropic lateral roots in the topsoil (where P levels are highest) is essential for the plants to more effectively forage for P in the soil, increasing P acquisition under low soil P availability (Lynch, 2011). The main processes that affect RSA and increase root exploration capacity stem from cell division in the root pericycle ahead of generation of lateral root meristems, which allows for indeterminate growth, and the formation of seminal and lateral roots arising from lateral meristem initials in the pericycle of the root stele (López-Bucio et al., 2003).

Root remodeling in soils with low-P availability is related to two types of signaling pathways. Local signaling is associated with RSA modifications regulated by changes in the rhizosphere P concentration in the soil, with the root apical meristem (RAM) being the site sensing the P changes in the soil (Chien et al., 2018). Under low-P conditions, the differentiation of meristematic and stem cells especially in the pericycle, where lateral roots arise, are triggered (Sánchez-Calderón et al., 2005; López-Bucio et al., 2019; Wang et al., 2019). The second P signaling pathway is systemic signaling, where low soil P availability results in lower shoot P availability, triggering systemic responses transmitted to the root to reprogram root processes enhancing P acquisition. The primary conduit for these systemic responses is the phloem, which in addition to sugars produced by photosynthesis in mature leaves, also contains hundreds or thousands of different RNA species and proteins that can serve as signaling molecules for plant responses.

The best example of this is plant response to P deficiency, which triggers massive changes in the phloem transcriptome and proteome (Zhang et al., 2016; Zhang Z. et al., 2019). The first example of P deficiency systemic signaling involves the *microRNA 399* (*mirR399*), which is induced early in the low-P response in leaves and moves to the root in the phloem to interact with its target, the *PHO2* gene (Fujii et al., 2005; Chiou et al., 2006; Hu et al., 2015). The transcription factor AtMYB2 acts as a direct transcriptional activator of *miR399* (Baek et al., 2013), and *miR399* then can directly cleave *PHOSPHATE 2* (*PHO2*) mRNA in some species (Bari et al., 2006; Ramírez et al., 2013; Ouyang et al., 2016). PHO2 is a ubiquitin-conjugating E2 enzyme (UBC24) that negatively regulates P transporters, inhibiting P uptake and root-to-shoot translocation under sufficient P conditions (Aung et al., 2006; Bari et al., 2006). Subsequent studies showed that PHO2 targets proteins that are involved in expression of the root high affinity uptake transport genes, *Pht1;8* and *Pht1;9*. *miR399* is strongly induced by P deficiency in source leaves and then loaded into the phloem, where it is translocated to the root and silences *PHO2*, which in turn allows high expression of *Pht1;8/Pht1;9* and increased root P uptake (Fujii et al., 2005; Bari et al., 2006; Chiou et al., 2006; Hsieh et al., 2009). In the Zhang et al. (2016) paper cited above, the authors directly identified and quantified mRNAs that move from the shoot toward the root in the phloem, and whose abundance are altered by P deficiency. They used the appearance of *miR399* in the phloem as a bioassay for the plant perceiving P deficiency in the shoot and found it appeared in the lower source leaf phloem rapidly, within 12 h after withholding P from the roots. In this study they found that imposition of Pi stress induced large and rapid changes in the mRNA population in the phloem, and grafting studies demonstrated that many hundreds of phloem-mobile mRNAs are delivered to specific sink tissues, including the root. From these findings the authors proposed that the shoot vascular system acts as the site of perception for root-derived Pi stress signals, and the phloem delivers a cascade of signals to the different plant sinks, in order to coordinate P status throughout the plant. The molecular mechanisms for both local and systemic signaling that orchestrate P sensing and activation of pathways induced by low-P availability are not fully understood. The cross-talk between regulatory networks certainly occurs, but the information available is still fragmented, so this topic will focus on the transcriptional networks and molecules involved in P response and root remodeling.

MicroRNA 399 plays this key role in Pi-starvation signaling network in many plant species other than Arabidopsis. Its rice homolog, *LEAF TIP NECROSIS1* (*LTN1*), is associated with root morphology changes under low-P, and the lack of function *ltn1* mutant exhibits elongation of primary and adventitious roots under P starvation. In rice, *LTN1* is a key component downstream of *miR399* in the P starvation response (Hu et al., 2011). In maize, *miR399* transcripts are strongly induced in maize by P deficiency. Moreover, lines overexpressing *MIR399b* accumulated more P in their shoots, showing P-toxicity phenotypes and presented significantly lower abundance of the long-noncoding RNA1 (*PILNCR1*) in P-efficient lines, indicating that the interaction between *PILNCR1* and *miR399* is important for tolerance to low-P (Du et al., 2018). Finally, the overexpression of the transcription factor, *WRKY74*, in rice led to a larger root system phenotype, enhanced P acquisition and grain yield (Dai et al., 2016). These authors also showed that OsWRKY74 likely is a positive regulator of *miR399*.

PHOSPHORUS-STARVATION TOLERANCE 1 (PSTOL1) genes

To date, there are not many genes that directly link root morphology and P acquisition, particularly in crop species cultivated in soils with low-P availability. A receptor-like cytoplasmic kinase gene named *PHOSPHORUS-STARVATION TOLERANCE 1* (*PSTOL1*) described by Gamuyao et al. (2012), is the first candidate P efficiency (tolerance to low soil P) gene identified. This gene encodes a receptor-like kinase and is responsible for a major quantitative trait locus for rice root P uptake (Wissuwa et al., 2002). Rice lines overexpressing *PSTOL1* showed greater root total length and root surface area (Gamuyao et al., 2012), and enhanced phosphorus uptake and grain yield under low-P conditions compared to the control. PSTOL1 is expressed in the crown root primordial and parenchymatic cells located outside of the peripheral vascular cylinder, where crown roots are formed in rice (Gamuyao et al., 2012). Although P-starvation induced (*PSI*) genes were not differentially regulated by PSTOL1, constitutive genes with regards to P supply, such as HOX1 (Scarpella et al., 2005), a transcription factor that is a positive regulator of root cell differentiation, was up regulated in lines overexpressing *PSTOL1* in the Gamuyao et al. (2012) study, which is consistent with the proposed role of PSTOL1 in regulating early crown root development and root growth in rice.

In sorghum, multiple homologs of *OsPSTOL1* were shown by candidate gene association mapping to be associated with P efficiency in the field (grain yield and P uptake on low-P soil) and/or in the lab (changes in root topology and growth, and P uptake; Hufnagel et al., 2014). In this study, these sorghum *SbPSTOL1* genes appear to modify root system morphology and architecture, leading to increases in grain yield in field studies on a low-P Brazilian soil, and also exhibited enhanced

biomass accumulation and P content in sorghum landraces from West Africa using native soils. These data suggest a stable effect of the target alleles across environments and sorghum genetic backgrounds (Hufnagel et al., 2014; Bernardino et al., 2019). In maize, homologs of *OsPSTOL1* that were preferentially expressed in roots and co-localized with QTLs associated with root morphology and P acquisition traits (Azevedo et al., 2015), mapped in the same region as QTLs for grain yield on a low-P soil (Mendes et al., 2014).

TFs Involved in Plant Low-P Response/P Efficiency

The maize transcription factor (TF) ROOTLESS CONCERNING CROWN AND SEMINAL ROOTS (RTCS) has been shown to be involved in altering root development and architecture (Hetz et al., 1996; Taramino et al., 2007). More recently, Salvi et al. (2016) also reported co-mapping of a quantitative trait loci controlling the number of seminal roots in maize, with the *RTCS* gene. RTCS contains a Lateral Organ Boundaries (LOB) domain, LBD, that is induced by auxin. RTCS acts downstream of ARF34 (and is responsible for the initiation of embryonic seminal and postembryonic shoot-borne roots (Xu et al., 2015). Other RTCS LBD proteins are involved in several developmental processes; for example, Arabidopsis LBD16/ASYMMETRIC LEAVES18 (ASL18) is involved in the regulation of lateral root formation, downstream of ARF7 and ARF19 TF's (Lee et al., 2009). RTCS was highly expressed in a P efficient maize genotype under low-P conditions when compared to a P inefficient genotype (De Sousa et al., 2012), indicating that is modulated by maize P status.

The TF *PTF1* (*phosphorus starvation transcription factor*) is a member of the *BASIC HELIX-LOOP-HELIX* (*bHLH*) family of TF's and plays a role in low-Pi tolerance response in rice, maize and soybean (Yi et al., 2005; Li et al., 2011; Li Z. et al., 2019). In maize, ZmPTF1 is involved in the promotion of lateral root development and also binds to the promoter and positively regulates the transcription of a number of other TF's including *9-cis-epoxycarotenoid dioxygenase (NCED)*, *C-repeat-binding factor (CBF4)*, *ATAF2/NAC081*, and *NAC30*. RNA-seq data showed that genes related to the auxin signaling pathway are also up-regulated in *ZmPTF1* overexpression lines (Li Z. et al., 2019). These authors suggested that ZmPTF1 acts upstream of signaling pathways related to biosynthesis and activation of phytohormones such as ABA and auxin, which are associated with root system development and the Pi starvation and drought tolerance responses.

There are a number of other WRKY TFs involved in P deficiency stress. One of these is WRKY6, which negatively regulates *PHO1* expression under normal, sufficient P conditions. PHO1 is the phosphate efflux transporter that mediates xylem loading of Pi in the roots. When the plant experiences P deficiency, WRKY6 is degraded via 26S proteasome-mediated proteolysis (Chen et al., 2009). Its homolog in Arabidopsis, WRKY42, also negatively regulates *PHO1* transcription under P sufficiency, but under the same plant P status, it positively regulates expression of the gene encoding the root Pi uptake transporter, PHT1;1 (Chen et al., 2009; Su et al., 2015). Under P deficiency, like WRKY6, WRKY42 is also degraded by the 26S proteasome. Another related TF, *WRKY45*, whose expression is root-specific, binds to two W box elements in the promoter of *PHT1* and regulates its transcription (Wang et al., 2014). WRKY75 appears to play dual roles in P deficiency responses. It is an activator of expression of a number of P deficiency induced genes, including phosphatases and P transporters (Devaiah et al., 2007). But it also is a negative regulator of root development associated with P deficiency. That is, when it is knocked out, lateral root length and number, and root hair density, were significantly increased. Hence, *WRKY75* is the first WRKY transcription factor to be shown to regulate both a nutrient deficiency response and root development and architecture.

Major Al Tolerance Genes That Are Also Involved in P Deficiency Stress Pathways

As plants that have adapted to highly acidic soils have had to deal with the dual stresses of Al toxicity and low soil P availability/high P fixation (Kochian et al., 2015), it is not surprising researchers have recently begun to discover that what were believed to solely be Al tolerance genes also can be involved in P deficiency responses and possibly P efficiency. These findings come from research on Arabidopsis, and the three key players in this scenario are STOP1, the TF that regulates Al-induced expression of a number of Al tolerance genes (Iuchi et al., 2007), and two of the genes regulated by STOP. These are *ALMT1*, the root tip PM malate anion channel that is activated by Al and releases Al chelating malate into the acid soil rhizosphere (Hoekenga et al., 2006), and *ALS3*, whose function is more varied and puzzling. ALS3 was first shown by Larsen et al. (2005) to be an Al tolerance gene that encodes an ABC transporter that in the shoots, is localized to the vasculature and hydathodes. The authors showed in the shoot it was PM-localized and speculated it could confer Al tolerance by loading Al into the phloem, thus moving it away from the site of toxicity in the root tip.

More recently, Dong et al. (2017) found that in Arabidopsis roots, knockout of *ALS3* results in hypersensitivity to low-P. In this study, ALS3 was found to be part of a root tonoplast ABC transporter complex with AtSTAR1, which is the counterpart of rice OsSTAR1, which in rice pairs with OsSTAR2 (the rice counterpart of ALS3) to form a cytoplasmic vesicle ABC transporter involved in rice Al tolerance (Huang et al., 2009). The Arabidopsis ALS3/AtSTAR1 transporter complex was shown to mediate electrogenic transport in oocytes (transports net charge across the membrane), but it is not clear what solute AtSTAR1/ALS3 transports across the root-cell tonoplast. This study is one of several (the others being; Müller et al., 2015; Balzergue et al., 2017; Mora-Macías et al., 2017) that together explain the primary Arabidopsis P deficiency response, which is inhibition of primary root growth and continued lateral root growth under low-P growth conditions. This response involves the genes initially shown to be involved in Al tolerance, *ALS3*, *ALMT1* and *STOP1*. The low-P inhibition of primary root growth requires Fe to occur, and under low-P conditions, Fe

accumulation both into the root symplasm and the cell wall is increased. The path of events that start with P deficiency under sufficient/high Fe growth conditions and end with inhibition of Arabidopsis primary root growth are both elegant and relatively complex. These events are summarized here:

(1) P deficiency inhibition of Arabidopsis primary root growth requires available Fe in order to occur.
(2) Under P deficiency, STOP1 induces *ALMT1* gene expression; subsequently the ALMT1 protein releases malate into the root tip apoplast and rhizosphere where it increases Fe availability in the apoplast via chelation of Fe^{3+} from the rhizosphere.
(3) At the same time, P deficiency induces the release of the multicopper ferroxidase, LPR1, from the ER to the cell wall of RAM cells surrounding stem cells in the RAM. LPR1-mediated reduction/oxidation of ferric/ferrous ions in this cell wall region generates peroxide, which catalyzes lignification and cell wall stiffening, accounting for the initial rapid inhibition of root growth.
(4) Concurrently, the ROS generation from LPR1-mediated ferroxidase activity triggers callose formation in this region of the RAM, which blocks plasmodesmata between the stem cells and cells surrounding the stem cell niche.
(5) This prevents for cell-to-cell transport of the TF, SHORT-ROOT, which is essential for stem cell division. This inhibition of stem cell division exhausts the meristem, resulting in the slower inhibition and termination of primary root growth.

The way that cells in the RAM perceive P deficiency is not understood, however, it is known that the accumulation of AtSTOP1 in the nucleus is the on-off switch for the regulatory mechanisms involved in the inhibition of primary root growth associated with P deficiency and Fe accumulation. Wang et al. (2019), building upon the research presented in Dong et al. (2017), showed that STOP1, ALMT1, and LPR1 act downstream of ALS3/STAR1 in controlling Arabidopsis primary root growth in response to P deficiency. Furthermore, they found that the tonoplast ABC transporter, ALS3/STAR1, represses STOP1 protein accumulation in the nucleus, thus inhibiting *ALMT1* transcriptional activation. They suggested that an unknown metabolite or ion is sequestered in the vacuole by ALS3/AtSTAR1, and this metabolite or ion is necessary for STOP1 accumulation in the nucleus. Subsequently, Godon et al. (2019) found that the stability of AtSTOP1 in the nucleus is triggered by Fe^{3+} accumulated in root cells under P deficiency, and not the decrease in P itself. They also found that Al^{3+} had the same effect as Fe on stimulating STOP1 accumulation in the nucleus, which is consistent with the abundance of toxic Al^{3+} ions in acidic soils. The authors suggested that the AtALS3/AtSTAR1 transporter may be mediating the accumulation of either ionic Fe or Al, or Fe/Al chelates in the vacuole, and in the case of P deficiency, this transporter controls cytoplasmic Fe homeostasis via the stability of AtSTOP1 in the nucleus under low-P conditions.

Involvement of Posttranslational Modification in P Deficiency Responses

SUMO E3 LIGASE (SIZ1) is responsible for post-translational modifications based on the addition of small Ubiquitin-like Modifier (or SUMO) proteins, which can affect protein function (Gareau and Lima, 2010). The MYB-like transcription factor, PHOSPHATE STARVATION RESPONSE1 (PHR1), which modulates RSA under P starvation, is one example of a protein modified by sumoylation (Miura et al., 2011). In rice, OsMYB2P-1 positively regulates P starvation signaling and lines overexpressing this gene have a longer primary root and more lateral roots compared to the wild type under low-P conditions (Dai et al., 2012). PHR1 and its homolog PHL1 (PHR1-Like1) directly bind to the *cis*-element, P1BS (Rubio et al., 2001), which is prevalent in the promoters of many P starvation induced genes, including *PHO1*, *miR399*, *IPS1* (*INDUCED BY PHOSPHATE STARVATION1*), and *RNS1* (*RIBONUCLEASE1*) (Poirier et al., 1991; Bariola et al., 1994; Martín et al., 2000; Bari et al., 2006). PHR1 has also been found to be sequestered from the nucleus in a P-dependent manner by SPX1, a nucleus-localized SYG/PHO81/XPR1 domain protein, inhibiting PHR1 activity (Puga et al., 2014). In rice, SPX4 negatively regulates *PHR2*; under low-P, *SPX4* degradation is accelerated through the 26S proteasome pathway, releasing PHR2 into the nucleus and activating the expression of *PSI* genes (Lv et al., 2014). Getting back to sumoylation, a loss-of-function *siz1* mutant exhibited reduced primary root growth and increased lateral root and root hair length and density, which is apparently independent from the PHR1/SIZ1 signaling pathway (Miura et al., 2011). SIZ1 is also involved in the negative regulation of auxin patterning to modulate RSA in response to low-P (Miura et al., 2011).

This *siz1* mutation also revealed a dual role of the SIZ1 E3 ligase in the regulation of P homeostasis in rice. In *siz1* rice plants grown under P deficiency, two root high-affinity P transporter genes, *OsPT1* and *OsPT8*, were more highly expressed compared to the WT, whereas *OsPT2* and *OsPT6* (which are expressed in both roots and shoots) were down-regulated (Wang et al., 2015). OsPT2 and OsPT8 are phosphorylated by CASEIN KINASE2 (CK2), which inhibits their interaction with PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (OsPHF1) under normal conditions. OsPHF1 is a SEC protein that facilitates the trafficking of Pi transporters from the ER to the PM (González et al., 2005). The retained phosphorylated phosphate transporters in the endoplasmic reticulum lead to a reduced P absorption from the rhizosphere (Chen et al., 2015). Also, rice PROTEIN PHOSPHATASE95 (OsPP95), a PP2C protein phosphatase negatively regulated by OsPHO2, positively regulates P homeostasis and remobilization, through the interaction with OsPT2 and OsPT8. OsPP95 acts antagonistically with CK2 to regulate the reversible phosphorylation of phosphate transporters (Yang et al., 2020b).

Another transcriptional factor with a role in P homeostasis is WRKY6, which was shown to negatively regulate the expression of *PHO1* (Chen et al., 2009), which is a phosphate efflux transporter localized to the Arabidopsis root vasculature and

is key in loading Pi absorbed by roots from the soil into the xylem for translocation to the shoot (Hamburger et al., 2002). Its closest Arabidopsis homolog, WRKY42, also negatively regulates *PHO1* transcription under P starvation, (Chen et al., 2009; Su et al., 2015). Interestingly, under Pi-sufficient conditions, WRKY42 positively regulates *PHT1;1* expression, which is a root high and low affinity Pi uptake transporter in Arabidopsis (Shin et al., 2004). WRKY42 accomplishes this by binding directly to the *PHT1;1* promoter, and this binding is abolished by low-Pi stress. During Pi starvation, the WRKY42 protein is degraded through the 26S proteasome pathway. These results show that AtWRKY42 modulates Pi homeostasis by regulating the expression of *PHO1* and *PHT1;1* to adapt to environmental changes in Pi availability.

Members of the Proteaceae Family Have Evolved Unique Adaptations to Acquire P From Low-P Soils

Some plant species of the Proteaceae family develop cluster or proteoid roots in response to growth on low-P soils. Cluster roots are specialized primary lateral roots that develop one or more clusters of rootlets along their axes. Cluster roots synthesize large amounts of organic acid, such as citrate and malate, which are subsequently released into the rhizosphere to increase P availability by chelating metals such as Fe, Al, and Ca that are fixing the phosphate anions in the soil (Keerthisinghe et al., 1998; Neumann et al., 2000; Peñaloza et al., 2002). A number of genes are involved in the developmental and biochemical responses in cluster roots. These include upregulation of the root high-affinity phosphate transporters, LaPT1, and phosphoenolpyruvate carboxylase 3 (LaPEPC3) under P deficiency. Also, it was found that white lupine homologs of the Arabidopsis SCARECROW (AtSCR), LaSCR and LaSCR1 are localized to the root endodermis and presumably help drive the developmental processes that result in these impressive clusters of laterals, which play such an important role in lupine adaptation to low-P soils (Peñaloza et al., 2005; Sbabou et al., 2010).

Recently, a cultivated accession of white lupin was sequenced and de novo assemblies of a landrace and a wild relative were also performed (Hufnagel et al., 2020). The modern accession displays an increased soil exploration capacity through early establishment of lateral and cluster roots (Hufnagel et al., 2020). The authors identified the presence of AP2/EREBP, a large multigene family that is key to control of lateral root development. They also identified several mature microRNAs expressed in cluster root sections and related to P deficiency responses, such as *miRNA156*, *miRNA166*, *miRNA211139*, and members of *miRNA399* family, that were not detected previously in white lupin. Moreover, Hufnagel et al. (2020) identified five genes that are targets of the detected miRNAs, including the TFs *LaWRKY* (*Lalb_Chr07g0182001*) and *LaPUCHI*-3 (*Lalb_Chr18g0055601*). Activation of key regulatory genes may trigger the early establishment of the root system, and consequently P-uptake and P efficiency (increased grain yield on low-P soils).

DROUGHT STRESS AND TOLERANCE

Drought stress is the most widespread abiotic stress affecting crop yield and quality. Due to the sessile nature of plants, evolutionary adaptations have enabled plants to develop sophisticated mechanisms to tolerate or avoid drought. When plants sense water deficit in the surrounding environment, it leads to the generation of drought stress signals (Blackman and Davies, 1985; Kuromori et al., 2014; Batool et al., 2019). These primary and secondary drought response signals are perceived by receptor molecules which leads to direct changes in the expression of genes or expression of TFs that regulate expression drought-responsive genes, which ultimately leads to drought adaptation (Kuromori et al., 2014). Drought signaling networks are presumably complex and to date poorly understood, but it is clear they involve both intercellular and intracellular signaling (Kuromori et al., 2014). Because this review focuses on root adaption to multiple stresses, here we will focus on the role of drought-related communication between the roots and shoots involving intercellular signaling networks and TFs responsive to drought.

Drought Signaling Molecules

Hormones

Several studies have shown that phytohormones act as signaling molecules in response to drought. ABA is one of the most widely studied phytohormones in part because of its role in regulating stomatal conductance in response to different related abiotic stresses that impact plant water status including drought, salinity, high and low temperatures. Jones and Mansfield (1970) showed that external application of ABA to roots led to a reduction in stomatal aperture suggesting that ABA was involved in regulation of stomatal conductance. This led a number of researchers to conduct plant water stress studies investigating the hypothesis that root-derived ABA is a primary candidate for root to shoot drought signaling. It had been generally accepted that stomatal closure in response to drought was triggered by reductions in leaf water potential due to the drought conditions. A key finding in changing thinking about drought signaling came from the work of Blackman and Davies (1985), which showed that reduced water content in roots in response to drought led to stomatal closing or reduction in stomatal aperture without changes in leaf water potential. This indicated that a signal was likely traveling from the root to leaves to help induce stomatal closure. As described above regarding the earlier work of Jones and Mansfield (1970), ABA was already known to decrease stomatal opening and thus it became a logical candidate for a drought-induced root signal transmitted to leaves. Subsequently, it was shown that upon soil drying, the ABA concentration was increased in maize roots and xylem sap (Zhang and Davies, 1989, 1990a), and these findings further strengthened the idea of ABA as a key drought induced signal in root to shoot signaling. Subsequent work with a number of plant species, including maize, sycamore, lupin, wheat, castor bean and grapevine (Loveys and Kriedemann, 1974; Zhang et al., 1987; Henson et al., 1989; Zhang and Davies, 1990b) all showed that soil water deficit induced ABA synthesis in roots, and the newly synthesized ABA was then translocated to leaves via the xylem to induce stomatal closure.

Drought stress can cause arrested shoot growth; however, it has been shown that under those conditions root elongation can continue due to ABA-mediated plant adaptations (Sharp and Davies, 1989; Saab et al., 1990; Sharp et al., 1994) observed that primary root elongation was maintained under water limited conditions, and in subsequent work it was suggested that increased ABA accumulation in the root under drought conditions (water potential [ψ_w] of −1.6 MPa) might play a key role in the prevention of root growth elongation inhibition under drought stress (Saab et al., 1990). In the Saab et al. (1990) study, two treatments were employed. These involved both the root application of fluridone, an inhibitor of carotenoid biosynthesis that provides the precursors for ABA biosynthesis, and the use of the *vp5* mutant that is deficient in carotenoids that leads to reduced ABA synthesis. They showed that in both of these treatments, the roots did not maintain continued root elongation at a lower water potential compared to wild type maize plants. They also conducted these experiments in a dark environment with saturated humidity to rule out the indirect effects of ABA deficiency on photosynthesis and/or alterations in stomatal control. Subsequently, Sharp et al. (1994) confirmed the role of ABA in maintenance of tap root growth under water limited conditions by applying exogenous ABA, which recovered the wild type root growth phenotype in both the *vp5* mutant and fluridone-treated plants under drought. Based on an earlier report by Wright (1980) on the interaction between ABA and ethylene, researchers from the Sharp lab also investigated whether ABA-dependent inhibition of ethylene synthesis was involved in the maintenance of root elongation under water limited conditions (Spollen et al., 2000). In this study, wild type root growth elongation was recovered by applying ethylene synthesis and action inhibitors in the vp5 mutant and in fluridone-treated maize plants, suggesting that the ability of root growth to better tolerate drought compared to shoot growth does involve interactions between ABA and ethylene.

Despite this body of work supporting the hypothesis of root to shoot translocation of ABA in response to drought, other studies suggesting the importance of leaf ABA synthesis have been carried out in a number of labs using reciprocal grafting between wild type and ABA deficient mutants in tomato, Arabidopsis, and sunflower. In these studies, drought stress was imposed on the wild type shoot/mutant roots and mutant shoots/wild type roots grafting combinations. When these different grafted "genotypes" were water stressed, the shoot genotype was shown to control stomatal behavior, suggesting that shoot-derived ABA was also important drought response (Jones et al., 1987; Fambrini et al., 1995; Holbrook et al., 2002; Christmann et al., 2007; Dodd et al., 2009). In summary, despite the general acceptance in the plant water relations that the primary mode of ABA signaling occurs via root ABA synthesis, followed by translocation via xylem to the leaves, it is clear that the field of plant ABA signaling is not unified behind this hypothesis. As supported by the findings reported in the publications summarized in this section of the review, ABA signaling may involve both roots and leaves, with a systemic response involving ABA that is made in roots under drought and transported to leaves, and a more local response within the drought-stressed leaf.

The ABA signaling pathway has been studied extensively in the model plant, *Arabidopsis thaliana*. ABA receptors have been identified in Arabidopsis, and during ABA signaling, ABA has been shown to bind to intracellular ABA receptors from the PYR/PYL/RCAR family, triggering a signal cascade that results in ABA-mediated stomatal closure (Fujii et al., 2009; Nishimura et al., 2009; Santiago et al., 2009; Cutler et al., 2010; Gonzalez-Guzman et al., 2012). The binding of ABA to its receptor leads to interactions with a Type 2C protein phosphatase (PP2C), which inhibits PP2C-mediated activation of an OST (Open stomata) 1 kinase. This kinase is responsible for stomatal opening by controlling anion channels in the guard cell plasma membrane, and blocking its activation results in stomatal closure (Ma et al., 2009; Park et al., 2009; Hauser et al., 2017). Hence, PP2C is a negative regulator of the ABA signaling pathway, resulting in stomatal closure. Interesting recent findings from Belda-Palazon et al. (2018) showed that the PYL8 ABA receptor is responsible for root perception of ABA though a non-cell-autonomous mechanism. In this study the PYL8 transcript was localized to the root meristem epidermis and stele, while the PYL8 protein was also found in adjacent tissues. The authors go on to show that both inter- and intracellular trafficking of PYL8 appears to occur in the RAM. This study shows that ABA receptors can interact with ABA in the root. It doesn't appear that this interaction plays a role in drought signaling to the shoot. Instead the authors hypothesize that the binding of ABA to the PYL8 receptor in the root may be involved in well documented roles of ABA in root function including root growth associated with hydrotropism and salt stress, and root plasticity in response to variation in nutrient availability (Barberon et al., 2016; Feng et al., 2016; Dietrich et al., 2017).

There are a number of published papers indicating that ABA biosynthesis occurs in both shoots and roots, and this occurs first via biosynthetic processes in plastids, and then the ABA precursors made in the plastid are transported to the cytosol, where they are converted to ABA (Thompson et al., 2007; Fujii et al., 2009; Nishimura et al., 2009; Santiago et al., 2009; Cutler et al., 2010). With regards to drought signaling regulation of stomatal function, cytosolic ABA has been found to bind to PYR1-type ABA receptors also located in the cytoplasm (Fujii et al., 2009; Nishimura et al., 2009; Santiago et al., 2009; Cutler et al., 2010). Based on the findings presented above for (Belda-Palazon et al., 2018), it is clear that ABA receptors are both functioning in the root and the leaf. In the studies showing that ABA binds to PYR1-type ABA receptors in leaf tissue, although not directly stated, the clear implication is that this ABA interaction with its receptor occurs in the guard cell cytoplasm, although that has not yet been shown.

Components of the ABA signaling pathway that are involved in moving ABA either into guard cells or from roots to leaves have been found. Kuromori et al. (2010) identified an ABC (ATP binding cassette) efflux transporter gene *AtABCG25* that encodes an ABC transport protein localized in the root vascular parenchyma plasma membrane. This transporter exports ABA accumulated in root xylem parenchyma cells into xylem vessels

in response to drought stress. Studies also showed that transgenic Arabidopsis plants overexpressing *AtABCG25* had higher leaf temperatures and lower transpirational loss of water from detached leaves, compared to wild type plants. This is consistent with more ABA being provided to guard cells from the root, leading to stomatal closure. Furthermore, Kang et al. (2010) has identified an ABA uptake transporter, AtABCG40 (also known as Pleiotropic drug resistance transporter PDR12). This ABC transporter is localized to the plasma membrane and predominantly expressed in leaf guard cells, where it acts to transport ABA that is delivered via the xylem, into guard cells. In summary, many of the structural components of the root to shoot ABA signaling network are being identified, including root-localized ABA biosynthetic pathways, transporters involved in xylem translocation of ABA to the shoot, and transporters in the leaf moving ABA into guard cells.

Another hormone that appears to be involved in drought signaling is cytokinin. Reduced maize stomatal aperture due to exposure to dry soil was reversed by the application of cytokinin to the roots (Blackman and Davies, 1985). Whereas xylem levels of ABA are increased in response to drought treatment of rice seedlings, cytokinin levels are decreased under the same drought conditions (Bano et al., 1993). These findings suggest that both hormones are involved in drought signaling, acting antagonistically to more finely regulate stomatal aperture related to plant water status (Blackman and Davies, 1985; Bano et al., 1993).

Peptide Hormones

The CLE (CLAVATA3/EMBRYO-SURROUNDING REGION) family of peptides are small peptides that function as plant hormones via release from cells into the extracellular space, where they function as intercellular signaling molecules. They have been shown to bind to receptor-like proteins at the outer surface of the plant cell plasma membrane to help mediate signal transmission. CLE peptides have been shown to regulate a range of physiological and developmental processes, including drought responses. CLE proteins have recently been shown to be a mobile signal transmitted from roots to shoots and involved in increased ABA biosynthesis after dehydration stress (Takahashi et al., 2018). In this study, synthetic isotope-labeled CLE25 was externally applied to roots and its accumulation was detected in leaves of treated plants using nanoscale nLC-MS/MS. The CLE25 peptide has been shown to be involved in regulation of ABA biosynthesis in leaves after the roots sense drought conditions in the soil (Takahashi et al., 2018). CLE25 is expressed in vascular tissues and its expression is induced in response to drought. Subsequently, the CLE25 peptide moves from the roots to leaves, where it enhances ABA synthesis and accumulation, helping trigger stomatal closure. It does this by binding to BARELY ANY MERISTEM (BAM) receptors in leaves. It is possible that CLE25 plays a role in the leaf-mediated regulation of stomatal function described above from the earlier publications reporting on the physiology of drought induced stomatal closure. If this is the case, then CLE2 could be a second root-to-shoot drought signal (the other being ABA itself) that triggers leaf-localized ABA regulation of stomatal responses to drought.

Other Signals

Plant cellular and apoplastic pH has been proposed to be another signaling factor that could play a role in stomatal aperture regulation (Hartung et al., 1998; Wilkinson, 1999). Drought stress has been shown to trigger an increase in xylem sap pH (Gollan et al., 1992; Wilkinson and Davies, 1997; Hartung et al., 1998). Under these conditions, Wilkinson and Davies (1997) found that this led to an increase in apoplastic ABA in the leaves. They hypothesized that as drought increases ABA concentrations in the xylem sap, and are then transported to the leaves, the higher apoplastic (xylem) pH will deprotonate acid groups in the ABA molecules and the increased charge of the ABA anion will reduce passive ABA flux through the lipid bilayer of the leaf cell plasma membrane. Hence, they speculated that extracellular ABA may be important in triggering stomatal closure. This is a topic that will require more research to more clearly define both the role of xylem pH in drought signaling and the role of apoplastic ABA in directly regulating stomatal response to drought.

Recent studies have identified specific microRNAs that are responsive to drought stress (Bakshi and Oelmüller, 2014; Bakhshi et al., 2016; Aravind et al., 2017) and this could be part of another drought signaling mechanism, as microRNAs can regulate genes post-transcriptionally (Aukerman and Sakai, 2003). Bakhshi et al. (2016) identified 61 known and 11 novel microRNAs involved in drought signaling in rice by performing experiments with a divided root system where half of the root system was water stressed and the other half kept well-watered. They identified miRNAs that exhibited differential expression when the entire root system is exposed to drought stress, along with miRNAs whose expression was altered in response to divided root system drought versus well-watered signaling. The results for differential expression of many of the miRNAs were validated via qRT-PCR. Furthermore, *in silico* target analysis led to the identification of two to three hundred novel target genes for the drought stress response of the entire root system, along with responses of the divided root system to drought and well-watered conditions. From the target analysis, the authors proposed these miRNAs could be involved in a number of drought response pathways, including ABA and calcium signaling, detoxification of free radicals induced by drought, and stimulation of lateral root initiation and growth, which could lead to bigger and deeper root systems that could more effectively acquire water located deeper in the soil profile under drought.

TFs Responsive to Drought

It is well known that transcription factor (TF) proteins can play major roles in regulatory and signaling networks, and plant drought response is no exception. A number of recent studies have been conducted to identify TFs responsive to drought and in some these studies, the function of the identified TFs has been elucidated (He et al., 2016; Lee et al., 2016, 2017; Chen Y. et al., 2018; Kumar et al., 2019). TFs responsive to drought are members of several different TF families including: (1) the AREB/ABF (ABA responsive binding or ABRE binding factor) family; (2) the AP2/ERF (ethylene response element binding factor) family; (3) the bZIP (the basic leucine zipper) family; (4) the NAC (NAM,

ATAF1,2, CUC2) family; and (5) the WRKY transcription factor family (Joshi et al., 2016).

With regards to a TF in the AREB/ABF family involved in drought signaling, Marinho et al. (2016) showed that soybean transgenic lines overexpressing *AtAREB1* exhibited enhanced performance under drought without any penalty on yield. From changes in expression profiles for phosphatases (PP2C) and kinases (SnRK2) in the *AtAREB1*-overexpressing transgenic plants, the authors noted that the observed lower expression of phosphatases and higher expression of kinases are known to be linked to ABA-dependent stomatal closure, and the resulting reduced stomatal conductance to water in the OE lines could explain the observed drought resistance. This overexpression line also had a higher leaf area index and elevated intrinsic water use in subsequent research by Fuganti-Pagliarini et al. (2017). In rice, overexpression of *OsERF71* altered expression of genes that regulate lignin biosynthesis and cell wall loosening enzymes, leading to increased root radial growth, more cell layers in the vasculature, and increased root aerenchyma, and these root structural changes were associated with reduced water transpiration and increased drought tolerance (Lee et al., 2016). OsERF71 belongs to the AP2/ERF TF family and is mainly expressed in the root endodermis, meristem and pericycle tissues. It was not clear how these root structural changes confer drought resistance, but the authors noted that increased radial growth has been observed in other studies in response to drought. The authors pointed out that in these previous studies, it has been suggested that observed increases in aerenchyma could reduce the carbon cost required to produce bigger root systems (Zhu et al., 2010). In the *OsERF71* overexpressing lines, the putative lignin biosynthesis genes, cinnamoyl-coenzyme was expressed ten-fold higher than in wild type plants. This was associated with quantification of higher lignin accumulation in roots tissues by phloroglucinol staining in the transgenic plants. Increased lignin biosynthesis might be required for additional root layer formation for wider radial root growth to accommodate larger aerenchyma.

NAC TFs have been characterized in transgenic wheat and it was reported that *TaRNAC1*-overexpressing lines exhibited changes in root growth and structure, which resulted in larger and deeper root systems and increased performance under drought, presumably due to enhanced water acquisition (Chen D. et al., 2018). Finally, He et al. (2016) evaluated Arabidopsis transgenic lines overexpressing the wheat *TaWRKY33* transcription factor for drought tolerance. They observed that *TaWRKY33* overexpression was associated with increased expression of *ABI5*, which encodes a basic leucine zipper transcription factor that in involved in the regulation of seed germination and early seedling growth under abiotic stress conditions that involve ABA. It has been shown that ABI5 is involved in the receptor-mediated ABA signaling described above (PYR/PYL/RCAR ABA receptors, PP2C phosphatases and SnRK2 kinases), through its interaction with ABSCISIC ACID RESPONSE ELEMENT (ABRE) motifs in target gene promoters. Hence, ABI5 has been shown or proposed to be involved in many ABA-related activities, including seed germination, seedling stress tolerance, integration of hormone interactions, and ABA biosynthesis (for a review, Skubacz et al., 2016).

In the He et al. (2016) publication, the authors suggested that the TaWRKY-mediated increase in *ABI5* expression was likely central to the observed improved performance under drought, possibly due to increased ABA synthesis under drought conditions. In the OE lines they also observed reduced transpirational loss of water from excised leaves, which would correlate with increased ABA accumulation resulting in greater stomatal closure.

ERECTA- A Leucine Rich Repeat Receptor-Like Kinase

The *ERECTA* gene has been shown to be involved in the regulation of leaf transpirational water loss through stomata by altering leaf anatomy (Masle et al., 2005). Leaf carbon isotope discrimination, which is due to the discrimination against the naturally occurring carbon isotope, ^{13}C, in favor of the more abundant ^{12}C isotope during photosynthetic CO_2 fixation by the rate-limiting enzyme, Rubisco, is negatively related to leaf transpiration efficiency (ratio between transpirational water loss and photosynthetic CO_2 assimilation). Hence, leaf isotope C discrimination can be used as a proxy phenotype for transpirational efficiency. Using this approach, leaf isotope C discrimination was used to phenotype an Arabidopsis Col-4 x Ler RIL population. Genetic analysis of the data yielded a significant QTL for transpirational efficiency for leaf isotope C discrimination that was then fine mapped on chromosome 2 (48.96–51.02 cM) and explained up to 64% of the phenotypic variation for this trait in the RIL population. The population parents, Col and Ler, contain *ERECTA (ER)* and *er1* alleles, respectively. Upon screening of candidate genes residing in that region, they found that the *ERECTA* gene was located in the center of the QTL interval. They also observed contrasting values of leaf isotope C discrimination for individuals with the *ER* or *er1* alleles. For functional validation of the candidate gene for transpirational efficiency, multiple ERECTA mutants were compared with near-isogenic lines containing *ERECTA* allele homozygotes. They observed that all of the *er* mutants exhibited higher leaf isotope C discrimination and therefore lower transpirational efficiency than lines homozygous for *ERECTA* allele. Further, in transgenic lines which complemented the mutation with the *ERECTA* allele, they confirmed the identity of *ERECTA* as a transpirational efficiency gene. By dissecting leaf anatomical features, lower stomatal conductance because of lower stomatal density caused by epidermal cell expansion, was observed as the anatomical effect of the *ERECTA* gene. Loosely packed fewer and smaller mesophyll cells were also observed, and it was concluded that all of these phenotypes collectively are affecting transpirational efficiency. These anatomical phenotypic traits were maintained under drought stress which suggests that the *ERECTA* gene could be an important genetic tool to increase transpirational efficiency in crops in drought stress environments.

Zheng et al. (2015) also showed that the expression of two wheat *TaER* genes were positively correlated with

transpiration efficiency and yield traits. Gene sequences for ERECTA orthologs in wheat were identified by Linzhou et al. (2013) using a homology-based cloning approach. Zheng et al. (2015) subsequently found the physical chromosomal location of these genes on chromosomes 6 and 7 by using *in silico* approaches to compare *TaER* cDNA sequences to a wheat genome sequence database. The authors also observed significant variation in expression of these genes among 48 wheat varieties in the flag leaves at grain filling and at the heading growth stages. There was a significant positive correlation in TaER expression with water use efficiency, flag leaf area and yield traits (biomass and gain yield), whereas the rate of transpiration, stomatal density and rate of photosynthesis were negatively correlated. These results were consistent with Masle et al. (2005) and further strengthened the role of ER genes in regulation of transpiration efficiency. In addition to the above studies, Li H. et al. (2019) also showed increased drought resistance in Arabidopsis and maize plants by overexpressing the sorghum *ER* (*SbER2-1*) gene and the transgenic overexpression lines exhibited increased rates of photosynthesis and water use efficiency.

POSSIBLE COMMON DETERMINANTS OF AL TOLERANCE, P EFFICIENCY AND DROUGHT TOLERANCE

C2H2 TFs

Water deficit may disrupt the lipid bilayer in cell membranes, triggering protein denaturation and accumulation of cellular electrolytes, which may lead to osmotic imbalance in plant cells (Fernando and Schroeder, 2016). Hence, osmotic adjustments play a role in plant adaptation to dehydration via turgor maintenance and by the production of osmoprotectants that maintain proper cellular function (Blum, 2017). Cys2/His2-type (C2H2), zinc fingers are known to play a role in plant abiotic stresses tolerance and emerge as a possible hub controlling tolerance to Al toxicity, low-P and also drought stress. Possible mechanisms whereby C2H2 zinc fingers influence drought tolerance have been recently reviewed by Han et al. (2020). Those mechanisms involve the biosynthesis of solutes in the cell leading to osmotic adjustments, reactive oxygen species scavenging via enhanced antioxidant enzyme activity and ABA-dependent signaling pathways.

As previously described, there is evidence linking the C2H2 transcription factor, STOP1, to both Al tolerance and P deficiency tolerance (see section "Major Al Tolerance Genes That Are Also Involved in P Deficiency Stress Pathways"). This emerging pleiotropic role of STOP1 in abiotic stress tolerance has been further supported by the recent finding that *stop1* knockout lines showed enhanced drought tolerance in Arabidopsis (Sadhukhan et al., 2019). Among the genes suppressed in the *stop1* mutant was the *CBL-interacting protein kinase 23* (*CIPK23*), which may be involved in K^+/Na^+ homeostasis via regulation of K^+ transporters. In agreement with K^+ involvement in stomatal opening (Munemasa et al., 2015), further complementation experiments suggested that the STOP1 function in drought tolerance occurs via ABA-mediated stomatal closure elicited by CIPK23. A protein phosphatase 2C-family protein, PP2C61, was also repressed in the *stop1* mutant, which provides further indication of ABA-dependency for STOP1. This scenario points toward a highly pleiotropic nature of the transcription factor *STOP1*. In this context, STOP1 enhances *AtMATE*- and *AtALMT1*-mediated Al tolerance (see section "Transcriptional Regulation Involved in Al Tolerance"), inhibits primary root growth and enhances lateral root proliferation under P deficiency, possibly favoring P acquisition via Fe-mediated RAM exhaustion (see section "Major Al Tolerance Genes That Are Also Involved in P Deficiency Stress Pathways"). In addition, STOP1 may also influence both salt and drought tolerance (Sadhukhan et al., 2019). However, STOP1 was suggested to negatively impact drought tolerance in Arabidopsis (Sadhukhan et al., 2019), which may conflict with a possible general role of STOP1 in crop adaptation to acidic, tropical soils, where Al toxicity, P deficiency and drought stress usually co-exist.

NAC and bHLH Transcription Factors

There are many reports linking NAC TFs including NAM, ATAF, and CUC TFs with drought tolerance (Nakashima et al., 2012), which largely involves ABA-dependent pathways. However, some NAC TFs show very early responses to ABA treatment, probably before endogenous ABA accumulates (Tran et al., 2004). Hence, some NACs are also thought to function through ABA-independent pathways (Singh and Laxmi, 2015), at least to some extent. Mutant analysis targeting class III SnRK2 protein kinase genes resulted in repression of the NAC gene, *RD26*, indicating that the expression of stress-inducible NACs is under control of the central ABA perception and signaling module (Nakashima et al., 2012; Fernando and Schroeder, 2016). Overexpression of the stress responsive, NAC gene, *SNAC1*, has been reported to lead to salt and drought tolerance in rice without a penalty in yield (Hu et al., 2006). SNAC1 was shown to bind to the promoter of the stress-induced gene, early responsive to drought 1 (*OsERD1*), and many stress-related genes were up-regulated in the *SNAC1*-overexpressing rice plants. The transgenic lines were also more sensitive to ABA and showed reduced water loss due to enhanced stomatal closing (with apparent no effect in photosynthesis), possibly by drought induction of *SNAC1* in guard cells (Hu et al., 2006). Also, OsNAC5 has been found to improve drought tolerance in rice via up-regulation of stress-inducible genes, and both OsNAC6 and OsNAC10 may also improve rice drought tolerance (reviewed by Nakashima et al., 2014; Singh and Laxmi, 2015).

Basic helix-loop-helix (bHLH) TFs have been implicated in drought regulation of stress-related genes via a wide-range of possible tolerance mechanisms, including stomatal development, ABA signaling, trichome and root hair development, osmoregulation, photosynthesis and growth regulation, in addition to ROS scavenging (reviewed by Castilhos et al., 2015; Sun et al., 2018). For example, AtMYC2 (bHLH) and AtMYB2 (MYB) TFs interact with *cis* elements in the promoter of the dehydration-responsive gene, *rd22*, to function

as transcriptional activators in ABA-inducible gene expression under drought stress in Arabidopsis (Abe et al., 2003). However, strong alterations of stomatal development elicited by some bHLH TFs may hinder their practical application in cultivar development (Castilhos et al., 2015).

The bHLH family member, PTF1, has been found to play a role in low-Pi tolerance in rice, maize and soybean (see section "TFs Involved in Plant Low-P response/P Efficiency"). In maize, ZmPTF1 was also shown to enhance lateral root development and confer drought tolerance (Li Z. et al., 2019). Enhanced drought tolerance in the *ZmPTF1*-overexpression lines might be a result of activation of ABA and auxin signaling pathways and enhanced lateral root growth, which may be at least in part caused by up-regulation of NAC TFs. In fact, ZmPTF1 was shown to bind to the promoter of *NAC30* and other TFs, acting as a positive regulator of those genes (Li Z. et al., 2019). Thus, a possible connection between NAC-mediated tolerance to both drought and low-P conditions may be mediated at some extent via bHLH-dependent regulation of NAC TFs.

Interestingly, NAC TFs may also connect with Al tolerance based on up-regulation of *VuNAR1* in the *Vigna umbellata* root apex (Lou et al., 2020) and Al inducibility of other NACs (Escobar-Sepúlveda et al., 2017; Jin et al., 2020). Since VuNAR1 was shown to bind to the promoters of wall-associated receptor kinase genes, this NAC gene may confer Al tolerance through regulation of cell wall pectin metabolism (Lou et al., 2020). Interestingly, this mechanism could possibly feedback on P acquisition, since wall-associated kinases have been shown to affect root growth (Kanneganti and Gupta, 2008, 2011; Kaur et al., 2013).

MYB TFs

Transcription factors possessing a conserved MYB domain involved with DNA binding are important players in abiotic stress tolerance and are intimately involved in cross-talk between different types of abiotic stresses. MYB TFs may influence drought tolerance via regulation of root growth and development, leaf development, stomatal movement in response to drought, cell wall biosynthesis, cuticle and suberin biosynthesis, and antioxidant activity via accumulation of flavonoids (reviewed by Baldoni et al., 2015). MYB TFs are also closely associated with changes in root morphology, which involves rather complex responses to different hormones. MYB77 has been implicated in auxin signaling via interaction with auxin response factors (ARFs), changing lateral root growth (Shin et al., 2007). MYB involvement with ABA signaling stems from the interaction between the ABA sensing gene, PLY8, and MYB77 (Zhao et al., 2014). By increasing auxin signaling, this interaction leads to a recovery of lateral root growth following inhibition by ABA. An important role for MYB TFs in abiotic stress cross-talk is also suggested by the joint role of AtMYB60 (Oh et al., 2011) and AtMYB96 in both stomatal movement and lateral root growth, with an integrative role in ABA and auxin signaling being proposed for AtMYB96 (Baldoni et al., 2015). Another MYB transcription factor working in a similar way is SiMYB75, which function in an ABA-dependent manner to promote root growth and drought tolerance, which results from enhanced stomatal closure to reduce water loss (Dossa et al., 2020).

By far the most compelling case of a MYB transcription factor jointly modulating drought stress and P deficiency tolerance arises from AtMYB2 regulation of *miR399* (Baek et al., 2016, 2017). As previously described (Section 3), *miR399* is a key component in P homeostasis (Fujii et al., 2005; Baek et al., 2013). Baek et al. (2016) have shown that AtMYB2 regulation of miR399 is involved in drought responses, with transgenic Arabidopsis plants overexpressing *miR399f* exhibiting ABA resistance and drought hypersensitivity. This response is thought to be a consequence of ABA-signaling, with *miR399* targeting CSP41B and ABF3 (Baek et al., 2016).

WRKY TFs

WRKY TFs can act both as activators or repressors of gene expression and are involved both with abiotic and biotic stress responses (Bakshi and Oelmüller, 2014). The function of WRKY genes in drought tolerance is closely related to ABA signaling, which gives rise to a multi-pronged mode of action on plant performance under drought including stomatal closure and changes in RSA. Studies with a promoter::reporter gene construct for the sorghum member of the WRKY family, SbWRKY30, indicated that the *SbWRKY30* promoter responds to different phytohormones such as ABA, GA and Me-JA (Yang et al., 2020a). Expressed both in the tap root and leaf, *SbWKRY30* was induced by drought stress and conferred drought tolerance both in Arabidopsis and rice by affecting RSA. This drought tolerance response may also be a result of enhanced ROS scavenging elicited by SbWRKY30. This transcription factor was also found to influence the transcription of a number of stress-responsive genes, including *SbRD29* (Yang et al., 2020a).

Among other WRKY proteins, AtWRKY40 has been shown to interact with the C-terminal of the ABA receptor ABAR, with ABA acting to remobilize AtWRKY40 from the nucleus to the cytoplasm (reviewed by Rushton et al., 2012). Since AtWRKY40, AtWRKY18 and AtWRKY60 are negative regulators of ABA signaling, this mechanism leads to de-repression of ABA-dependent pathways and, hence, induction of ABA-responsive genes (Shang et al., 2010). With ABA sensing by its receptors, ABI5 is de-repressed and activates AtWRKY63, which activates stress-inducible genes such as *RD29* and *COR47* (Ren et al., 2010; Rushton et al., 2012).

Although there are many reports of WRKY TFs influencing drought responses (Rushton et al., 2012; Rabara et al., 2014; Singh and Laxmi, 2015), examples of common WRKY proteins also acting on Al tolerance and P deficiency tolerance are rather scarce, which might merely reflect less research focus on the involvement of WRKY TFs in abiotic stresses other than drought. Transgenic Arabidopsis with constitutive expression of the stress-response transcriptional coactivator, *multiprotein bridging factor 1c* (*MBF1c*), were more tolerant to bacterial infection, heat, and osmotic stress (Suzuki et al., 2005). *AtWRKY46* expression was found to be elevated in the transgenic lines, albeit only slightly, suggesting that AtWRKY46 could possibly be involved with stress tolerance in the *MBF1c* lines. Somewhat more compelling is the observation that *AtWRKY46* was induced by drought,

salt and oxidative stress (Ding et al., 2014). AtWRKY46 was expressed in guard cells and may regulate stomatal opening and drought stress. Additionally, AtWRKY46 was found to regulate lateral root development in osmotic and salt stress conditions, possibly via ABA-signaling (in part via ABI4 regulation) and auxin homeostasis (Ding et al., 2015).

This suggests that AtWRKY46 could act on stress tolerance beyond its repressor role on the expression of the Al tolerance

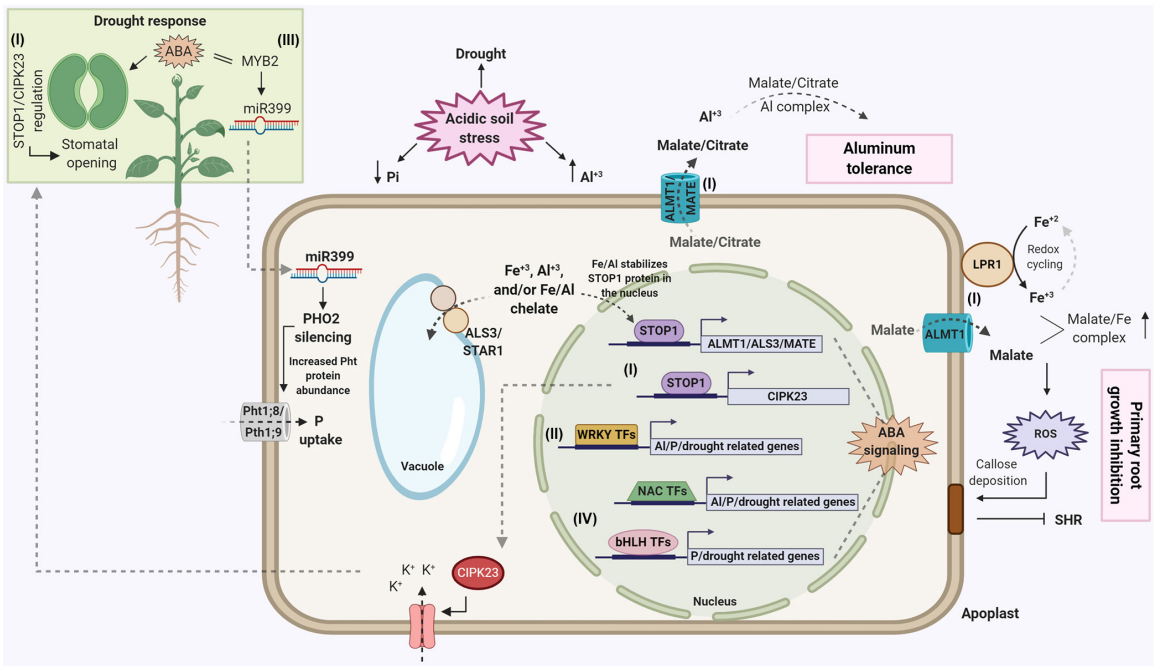

FIGURE 1 | Model for cross-talk between Al toxicity, low-P availability and drought stress. Four transcriptional regulatory networks are highlighted that we identified in this review that may be involved with plant responses to these three abiotic stresses. **(I)** The *SENSITIVE TO PROTON RHIZOTOXICITY 1* (*STOP1*) transcription factor is involved in Al tolerance, P deficiency and drought stress. In addition to *AtMATE*, *STOP1* transcriptionally activates the *ALUMINUM ACTIVATED MALATE TRANSPORTER1* (*ALMT1)* which encodes the root PM anion channel that mediates Al activated malate release from roots, detoxifying Al in the rhizosphere. P deficiency induces the release of the multicopper ferroxidase (LPR1) that reduces/oxidizes Fe. P deficiency also induces expression of *ALMT1*, and the malate released via the ALMT1 protein chelates and facilitates accumulation of Fe in the cell wall where its oxidoreduction is catalyzed by LPR1, generating peroxides, which triggers lignification and cell wall stiffening, and rapid inhibition of root growth. At the same time, ROS generated from oxidoreduction of Fe triggers callose formation, which blocks plasmodesmata between stem cell initials and cells of the RAM outside the stem cell niche. This blockage of the plasmodesmata prevents cell-to-cell transport of the transcription factor, *SHORT-ROOT*, which is needed to drive stem cell division. This exhausts the RAM and terminates primary root growth. The Fe accumulated in the cell wall also drives increased Fe influx into the cytoplasm of RAM and the increased Fe helps stabilize and enhance STOP1 accumulation in the nucleus. Under P sufficient conditions, the tonoplast ABC transporter, ALS3/STAR1, is hypothesized to transport the Fe or Al (depending on soil conditions) or possibly Fe/Al-chelates into the vacuole and the reduction of Fe/Al levels in the cytoplasm and nucleus represses STOP1 protein accumulation in the nucleus, inhibiting *ALMT1,* transcriptional activation. With regards to STOP1's involvement in drought response, *STOP1* also transcriptionally activates *CBL-INTERACTING PROTEIN KINASE 23* (*CIPK23*) expression, and the CIPK23 protein is an activator of high affinity K^+ transporters in the root and possibly the shoot, which could result in enhanced stomatal opening and a reduction in drought tolerance due to increased transpirational water loss. **(II)** A second family of candidate TFs are the large WRKY family. There are several WRKY transcription factor family members involved in responses to drought and Al toxicity. For example, *AtWRKY46* represses *ALMT1* expression in the absence of Al and also is expressed in guard cells in response to drought stress; although its role in drought response remains to be elucidated **(III)** The AtMYB2 transcription factor co-regulates P efficiency and is involved in drought response via regulation of *miR399*. AtMYB2 induces *miR399* in response to ABA and salt stress, and overexpression of *miR399* results in salt and ABA tolerance but interestingly, is associated with drought sensitivity. *miR399* plays a well-documented role in long distance P deficiency signaling in the phloem as it is synthesized in response to P deficiency in mature source leaves and is translocated in the phloem to the root, where it silences *PHOSPHATE 2* (*PHO2)*, which encodes a ubiquitin-conjugating E2 enzyme that negatively regulates P transporters under P sufficiency. **(IV)** NAC and Basic helix-loop-helix (bHLH) transcriptional factors have been implicated in drought regulation of stress-related genes via a wide-range of possible tolerance mechanisms, which involves ABA-dependent and independent pathways. The bHLH family member, PTF1, plays a role in low P tolerance, enhancing lateral root development. ZmPTF1 bind to the promoter of NAC30 and other TFs, acting as a positive regulator of those genes. Thus, a possible connection between NAC-mediated tolerance to both drought and low-P conditions may be mediated at some extent via bHLH-dependent regulation of NAC TFs. NAC TFs may also connect with Al tolerance based on up-regulation of VuNAR1 root apex and Al inducibility of other NACs. VuNAR1 also binds to the promoters of wall-associated receptor kinase genes, conferring Al tolerance through regulation of cell wall pectin metabolism. Although there is no evidence for direct interaction of STOP1, *AtMATE1* and *AtALS3* promoters, it is clear that STOP1 is crucial for induction of these genes. Not shown here is a possible link between Al tolerance and drought tolerance via ERF transcription factors, whose supporting evidence is more limited and preliminary. The figure was created with BioRender.com.

gene, *AtALMT1* (Ding et al., 2013), possibly influencing P acquisition and tolerance to drought stress. While enhanced lateral roots by AtWRKY46 might be expected to lead to enhanced P acquisition on low-P conditions, the nature of the impact of AtWRKY46 on drought tolerance, whether negative or positive, is yet to be unraveled in detail. Another possible case of cross-talk between drought stress tolerance and tolerance to low-P conditions involves WRKY45. Arabidopsis lines overexpressing *OsWRKY45* showed enhanced disease resistance and drought tolerance, possibly via ABA-mediated stomatal closure and induction of stress-related genes (Qiu and Yu, 2009). In Arabidopsis, *AtWRKY45* is induced by low-P, regulates *PSI* genes and is involved with changes in RSA that are apparently P-independent (see section "TFs Involved in Plant Low-P response/P Efficiency"; Devaiah et al., 2007). Also, AtWRKY45 participates in P responses by binding to the *PHT1;1* promoter and regulating its transcription, thereby enhancing P uptake (see section "TFs Involved in Plant Low-P response/P Efficiency"; Wang et al., 2014). These reports suggest that WRKY45 could have a pleiotropic effect, enhancing both drought tolerance and P acquisition in low-P conditions.

Ethylene Response Factors (ERFs)

Ethylene response factors are TFs in the AP2/ERF superfamily, which are involved in tolerance to multiple abiotic stresses (Debbarma et al., 2019). Well-known members of this family include the Dehydration Responsive Element Binding (DREB) factors, which are frequently involved in the ABA-independent regulation of drought responsive genes (Singh and Laxmi, 2015), possibly involving ethylene signaling (Leng and Zhao, 2019). However, in some cases, DRE elements on some promoters are involved in both ABA-dependent and ABA-independent abiotic stresses (Agarwal et al., 2017). Overexpression of DREB TFs has resulted in increased drought tolerance, but with occasional deleterious side-effects (Agarwal et al., 2017). Chen Y. et al. (2018) identified in *Jatropha curcas* a P starvation responsive AP2/ERF transcription factor, *JcERF035*, which was downregulated under -P conditions. Overexpression of this P-starvation responsive AP2/ERF in Arabidopsis resulted in enhanced root hairs but reduced number and length of lateral roots, which was apparently independent from P supply. Recently, overexpression of *ERF74* in Arabidopsis was shown to enhance tolerance to a variety of stress factors, including drought, high light, heat and Al toxicity, whereas the *erf74* mutant displayed higher sensitivity to these stresses. Like many other abiotic stresses, Al toxicity, generates ROS (see Section 2.2a), which may both be a toxic product and also can be a signal. Yao et al. (2017) showed that the *erf74* mutant lacked the reactive oxygen species burst often seen in the early stages of various stresses, which was due to lower expression level *of RESPIRATORY BURST OXIDASE HOMOLOG D (RbohD)* in the *erf74* mutant. Possibly this is part of a ROS signaling pathway conditioning tolerance to stresses such as Al toxicity, drought and temperature extremes, which may be related to ROS signals (Yao et al., 2017). While these studies with JcERF35 and AtERF74 suggest that some ERF transfactors might be involved in plant responses to P, Al and drought stress, this area awaits considerable further investigation. Also, given the involvement of ABA as a positive regulator of Al tolerance, including induction of *AtALMT1* and *AtMATE* expression (see Section 2.2b), other, yet unidentified factors may connect drought and Al tolerance via ABA-dependent pathways.

CONCLUSION

In this review, we examined the literature for common elements shared between the three major stresses that often co-occur on acidic soils: Al toxicity, low P availability and drought, with a focus on genes/proteins involved in signaling and/or regulatory pathways and networks controlling plant responses and adaptations to these three abiotic stresses. In general, research on crop adaptation to acidic soils has focused on one of the two primary stresses resulting from the unique chemistry of acidic soils, Al toxicity or P deficiency. But as this field has advanced and matured, we showed here that quite recently, a number of genes have been identified that are involved both in Al resistance and adaptation to P deficiency. The very broad field of research on crop adaptations to drought, on the other hand, has not focused much on the possible role of drought resistance mechanisms in adaptations to acidic soils. This is primarily because drought, by its very nature, occurs on all types of soils in many different eco-agricultural systems. Nonetheless, in this review we did identify several genes and the proteins they encode that could play a role in adaptation to all three of these abiotic stresses. At the beginning of this review, we speculated that genes involved in signaling/regulatory networks might be the best source for candidates involved in crop adaptation to all three stresses. This turned out to be the case as we identified transcription factors from several TF families that could play this pleiotropic role. These TF's are summarized here and a model of the function of the best candidate TF's in the three abiotic stresses is presented in **Figure 1**.

(1) STOP1, a C2H2 Zinc Finger Transcription Factor: This is probably the most interesting candidate as it has been shown to clearly be a key Al tolerance gene, regulating the Al-induced transcriptional activation of a number of Al tolerance genes in Arabidopsis, including the major Al tolerance gene, *ALMT1* (Iuchi et al., 2007). STOP1 also plays a key role in Arabidopsis root response to P deficiency, as under low P STOP1 again activates *ALMT1* expression, enhancing the production of the *ALMT1* anion channel that facilitates root malate release, which is central to the root apex Fe accumulation needed to exhaust the primary root RAM and root growth (Müller et al., 2015; Balzergue et al., 2017; Mora-Macías et al., 2017). The loss of primary root apical dominance then appears to lead to enhanced lateral root growth which could confer enhanced P acquisition in low P soil. Additionally, there is a reasonable amount of circumstantial evidence implicating STOP1 in several drought responses. This includes the recent finding that

stop1 knockout lines showed enhanced drought tolerance in Arabidopsis (Sadhukhan et al., 2019), suggesting that STOP1 is a negative regulator of drought tolerance. These authors also found that the STOP1 regulated the expression of the gene encoding the CBL-interacting protein kinase 23 (CIPK23), and complementation of the *stop1* mutant with CIPK23 reversed the drought phenotype (back to drought sensitivity). Furthermore, in a heterologous system, *Xenopus* oocytes, CIPK23 can activate via phosphorylation the guard cell PM anion channel, SLAC1 (SLOW ANION CHANNEL-ASSOCIATED 1) (Maierhofer et al., 2014), the direct link between this process and drought physiology involving stomatal closure is still unclear. Nevertheless, these findings all point toward the highly pleiotropic nature of the transcription factor, *STOP1*.

(2) WRKY Transcription factors: We found different members of the WRKY TF family that were involved in all three stresses, but did not identify a single WRKY member clearly influencing all stresses. One possibility is AtWRKY46, which is involved in Al tolerance where it represses *ALMT1* expression in the absence of Al (Ding et al., 2013). Additionally, it was shown to be involved in drought response, as it is expressed in guard cells and this expression is induced by drought, salt and oxidative stress (Ding et al., 2014). Additionally, AtWRKY46 was found to enhance lateral root development in response to osmotic and salt stress conditions, possibly via ABA-signaling and auxin homeostasis (Ding et al., 2015). It would be interesting to find out if this increased lateral root development and growth could also play a role in improved P efficiency.

(3) AtMYB2 Regulation of *miR399*: There is good evidence for this MYB transcription factor co-regulating P efficiency and drought tolerance via regulation of *miR399* (Baek et al., 2016, 2017). As detailed above, *miR399* is an important player in P homeostasis via a long distance systemic signaling system in the phloem translocating *miR399* from mature source leaves to the root, where it silences *PHO2*, the ubiquitin-conjugating E2 enzyme that negatively regulates P transporters under P sufficiency (Fujii et al., 2005; Baek et al., 2013). It was also shown that AtMYB2 regulation of *miR399* is also involved in plant responses to abscisic acid (ABA), and to salt and drought (Baek et al., 2016). Salt and ABA treatment induced the expression of *miR399*, and overexpression of *miR399* resulted in enhanced salt tolerance and interestingly, hypersensitivity to drought. Hence the pleiotropic nature of AtMYB regulation of *miR399* spanning low P and drought stress is apparent, although the functional basis of its impact on drought responses remains to be elucidated.

In conclusion, there are tantalizing links in the literature between the regulation of plant adaptation and responses to Al toxicity, P deficiency and drought stress. To provide the readers with a summary of the work reviewed in this paper, we have included **Supplementary Table 1** (Gene families possibly involved in pleiotropic mechanisms) resulting in multiple stress tolerances (tolerance to Al toxicity, P deficiency and drought) which contains lists of members of five gene families (*WRKY*, *STOP1*, *MYB*, *bHLH*, and *NAC*) involved in Al toxicity, drought stress and P deficiency. However more research is needed to say with certainty that the same factors can regulate tolerance to all three stresses. If that is the case, it will be quite intriguing to determine if these genes would be useful in a plant breeding program for multiple environmental stresses. Within a scenario where the same genes in some way influence tolerance to multiple stresses, instances of conflicting effects may be foreseen, as previously discussed. If the same gene acts simultaneously as positive and negative regulators of tolerance to different stresses, this may cancel its final effect in phenotypic expression or even be detrimental to crop performance on acidic soils. Within the context of molecular breeding strategies targeting quantitative traits such as genomic selection, these and other negative effects may be filtered out along the selection process via genomic estimation of breeding values. However, transgenic approaches may be more sensitive to this problem. In that regard, gene editing has emerged as a powerful approach to fine tune gene expression, which could help circumvent negative effects coming from pleiotropy or epistasis. This approach has proven useful in bypassing negative epistasis effects on yield in tomato (Soyk et al., 2017) and to tackle quantitative trait variation (Rodríguez-Leal et al., 2017). Particularly to deal with possible hurdles when exploring pleiotropy in crop adaption to acidic soils, it is more than certain that in-depth knowledge of the physiological and genetic underpinnings of multiple stress tolerance will be required.

AUTHOR CONTRIBUTIONS

JM and LK also handled the revising and editing of the full version of the manuscript. All authors contributed by conducting literature research and writing of different parts of the text.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.565339/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplemental table

Supplemental Table 1. Gene families possibly involved in pleiotropic mechanisms resulting in multiple stress tolerances (tolerance to Al toxicity, P deficiency and drought)

Family	Gene	Locus ID	Abiotic Stress	Function	Species	Reference
WRKY TFs	*AtWRKY18*	At4g31800	Drought	Negative regulator of ABA signaling	Arabidopsis	Shang et al. (2010)
	AtWRKY40	At1g80840	Drought	Negative regulator of ABA signaling	Arabidopsis	Shang et al. (2010)
	AtWRKY42	At4g04450	P Deficiency	Positively regulates *PHT1* and *PHO1* transcription under P starvation	Arabidopsis	Chen et al. (2009); Su et al. (2015)
	AtWRKY45	At3g01970	P Deficiency	Regulates *PHT1;1* expression under P starvation	Arabidopsis	Wang et al. (2014)
	AtWRKY46	At2g46400	Al Toxicity	Represses AtALMT1 expression	Arabidopsis	Ding et al. (2013)
	AtWRKY46	At2g46400	Drought	Positive role in drought response via stomatal regulation	Arabidopsis	Ding et al. (2014)
	AtWRKY47	At4g01720	Al Toxicity	Activates expression of *XTH17* and *ELP* which are responsible for cell wall modification in response to Al toxicity	Arabidopsis	Li et al. (2019a)
	AtWRKY6	At1g62300	P Deficiency	Negatively regulates Pi starvation response by modulating PHO1	Arabidopsis	Chen et al. (2009)
	AtWRKY60	At2g25000	Drought	Negative regulator of ABA signaling	Arabidopsis	Shang et al. (2010)
	AtWRKY63	At1g66600	Drought	Modulates ABA response (downstream of ABI5)	Arabidopsis	Ren et al. (2010)
	AtWRKY75	At5g13080	P Deficiency	Suppresses the transcription of *PSI* genes, including phosphatases, Mt4/TPS1-like genes and P transporters	Arabidopsis	Devaiah et al. (2007)
	OsWRKY22	Os01g60490	Al Toxicity	Activates *OsFRDL4* expression	Rice	Li et al. (2018)
	OsWRKY45	Os05g25770	Drought	Overexpression increases drought tolerance	Rice	Qiu and Yu (2009)
	OsWRKY74	Os09g16510	P Deficiency	Positively regulates miR399	Rice	Dai et al. (2016)
	SbWRKY1	Sb09g023500	Al Toxicity	Activates *SbMATE* expression	Sorghum	Melo et al. (2019)
	SbWRKY30	Sobic.010G045700	Drought	Enhances drought tolerance by regulation of *SbRD19*	Sorghum	Yang et al. (2020)
	TaWRKY33	Unigene22134_All	Drought	Reduces water loss by transpiration in transgenic Arabidopsis	Wheat	He et al. (2016)
C_2H_2 TFs	*AtSTOP1*	At1g34370	P deficiency	Regulates *AtALMT1* , *AtALS3* and *AtRAE1* expression and involved in P and Fe homeostasis	Arabidopsis	Balzergue et al. (2017); Mora-Macias et al. (2017)
	AtSTOP1	At1g34370	Drought	Regulates *CIPK23* expression, controlling stomatal opening	Arabidopsis	Sadhukhan et al. (2019)
	AtSTOP1	At1g34370	Al Toxicity	Regulates *AtALMT* expression	Arabidopsis	Iuchi et al. (2007)
	SbSTOP1	Sb03g041170	Al Toxicity	Regulates *SbSTAR2* and *SbMATE* expression	Sorghum	Huang et al. (2018)
	OsART1	Os12g0170400	Al Toxicity	Regulates expression of multiple Al tolerance genes	Rice	Yamaji et al. (2009)
	VuSTOP1	AKH61420	Al Toxicity	Regulates *VuMATE1* expression	*Vignia umbellata*	Fan et al. (2015)

Supplemental Table 1. Gene families possibly involved in pleiotropic mechanisms resulting in multiple stress tolerances (tolerance to Al toxicity, P deficiency and drought)

Family	Gene	Locus ID	Abiotic Stress	Function	Species	Reference
MYB TFs	*AtMYB2*	At2g47190	P Deficiency	Acts as a direct transcriptional activator of miR399	Arabidopsis	Baek et al. (2012)
	AtMYB60	AT1G08810	Drought	A null mutation results in the constitutive reduction of stomatal opening and decreased wilting under water stress	Arabidopsis	Oh et al. (2011)
	AtMYB96	AT5G62470	Drought	Its overexpression confers drought resistance when expressed in transgenic Camelina sativa	Arabidopsis	Baldoni et al. (2015)
	AtPHR1	At4g28610	P Deficiency	Activates Pi deficiency responsive genes	Arabidopsis	Miura et al. (2011)
	OsMYB2P-1		P Deficiency	Positively regulates P starvation signaling and rice lines overexpressing this gene have a longer primary root and more lateral roots compared to the wild type under low P conditions	Rice	Dai et al. (2012)
	SiMYB75		Drought	Ectopic exprression of this gene from sesame in Arabidopsis increases drought tolerance by stomatal closure, reducing leaf water loss	Arabidopsis	Dossa et al. (2020)
bHLH TFs	ZmPTF1		Drought	Regulates drought tolerance by promoting root development and abscisic acid synthesis	Maize	Li et al. (2019b)
	ZmPTF1		P Deficiency	Improves tolerance to low P by regulating carbon metabolism and root growth	Maize	Li et al. (2011)
	AtMYC2		Drought	Activates ABA-inducible genes under drought stress	Arabidopsis	Abe et al. (2003)
	AtMYB2		Drought	Activates ABA-inducible genes under drought stress	Arabidopsis	Abe et al. (2003)
NAC TFs	*ZmNAC30*		Drought	Upregulated by ZmPTF1 involved in auxin signaling	Maize	Li et al. (2019b)
	ATAF2/NAC081		Drought	Upregulated by ZmPTF1 involved in auxin signaling	Maize	Li et al. (2019b)
	TaRNAC1		Drought	Its overexpression increases root length, biomass, drought tolerance and grain yield under water limitation	Wheat	Chen et al. (2018a)
	OsSNAC1	DQ394702	Drought	Its overexpression increases drought tolerance	Rice	Hu et al. (2006)
	VuNAR1		Al Toxicity	Its overexpression in *Arabidopsis* improves Al resistance via Al exclusion	*Vigna umbellata*	Lou et al. (2020)
	OsNAC6		Drought	Controls root structural adaptions and nicotianamine biosynthesis for drought tolence	Rice	Lee et al. (2017)

CAPÍTULO 2

Transcription factor nuclear trafficking elicited by aluminum sensing in the plasma membrane plays a role in the regulation of the sorghum SbMATE transporter

Transcription factor nuclear trafficking elicited by aluminum sensing in the plasma membrane plays a role in the regulation of the sorghum SbMATE transporter

Abstract

Aluminum (Al) toxicity is a major constraint for crop production on acidic soils, which are widespread worldwide. In sorghum, the root citrate transporter, SbMATE, confers Al tolerance via Al-activated citrate release to the rhizosphere, where citrate binds and detoxifies Al^{3+}. Previous findings revealed that *SbMATE* is transcriptionally regulated by a WRKY and a zinc finger-DHHC transcription factor (TF), SbWRKY1 and SbZNF1, respectively, which bind to tandem repeats in the *SbMATE* promoter region to *trans*-activate *SbMATE*. The present study aimed at elucidating the mechanisms whereby SbWRKY1 and SbZNF1 transcriptionally regulate *SbMATE*. We found that SbWRKY1, a member of WRKY group III, contains a rather rare heptapeptide variant in a key region for WRKY/DNA recognition, WRKYGEK, and recognizes a novel *cis*-element for WRKY DNA-interaction. Localization studies in tobacco epidermal leaves and Arabidopsis roots established that SbZNF1 localizes to the plasma membrane, consistent with its four predicted transmembrane domains, whereas SbWRKY1 is located in the nucleus. SbZNF1 and SbWRKY1 were found to physically interact in the plasma membrane and, in response to Al^{3+}, SbZNF1 is mobilized to the nucleus in a SbWRKY1-dependent manner. This study shows that SbZNF1, a plasma membrane-localized zf-DHHC, senses Al^{3+} and transmits its signal to the nucleus, which may contribute to the previously observed synergistic action of SbZNF1 and SbWRKY1 on *SbMATE* expression.

INTRODUCTION

Acid soils comprise up to 50% of the potentially arable lands of the world, which are mainly located on tropical and subtropical regions (von Uexküll & Mutert, 1995), where food insecurity challenges many developing countries. In these soils, the rhyzotoxic form of aluminum (Al), Al^{3+}, becomes soluble into the soil solution, severely damaging the plant root system (Kochian et al., 2015). The site of Al toxicity is the root apex, where Al^{3+} ions perturb cellular structure and function, for example making the cell wall rigid, reducing permeability and fluidity of the plasma membrane, eliciting signal transduction disorders, and triggering oxidative stress and cell death (Kochian et al., 2005; Singh et al., 2017). Thus, Al toxicity impairs root growth, reducing water and nutrient uptake, resulting in significant yield losses for crops cultivated on acid soils (Kochian et al., 2015).

The most well-characterized mechanism employed by plants to withstand toxic levels of Al is the root Al exclusion, whereby organic acid anions, commonly malate and citrate, are released from the root apex via plasma membrane transporters. Once in the rhizosphere, both malate and citrate chelate the Al^{3+} ions forming stable, non-toxic complexes that prevent Al from entering the root (Kochian et al., 2015). The malate transporters involved with Al tolerance are members of the Al-activated malate transporter (ALMT) family. The *ALMT1* gene was firstly isolated in wheat (Sasaki et al., 2004) and, subsequently, its homologs were characterized in other plants, such *Arabidopsis*, rape, rye, soybean, and *Holcus lanatus* (Chen et al., 2013; Collins et al., 2008; Hoekenga et al., 2006; Liang et al., 2013; Ligaba et al., 2006). The Al-activated citrate transporters are members of the multidrug and toxic compound extrusion (MATE) family, which have been identified in many plants, including sorghum, barley, *Arabidopsis*, maize, rice, and wheat (Furukawa et al., 2007; Garcia-Oliveira et al., 2014; J. Liu et al., 2009; Magalhaes et al., 2007; Maron et al., 2008; Yokosho et al., 2011). In addition, some species can detoxify Al internally, for example, via Al uptake by plasma membrane transporters and subsequent sequestration into vacuoles. In rice, the plasma membrane-localized Al transporter, OsNrat1 (Nramp aluminum transporter 1), mediates Al uptake into the root cytosol and probably decreases Al^{3+} concentration in the cell wall (Xia et al., 2010). It is proposed that OsNrat1 acts in concert with OsALS1, a half-type ABC transporter localized in the tonoplast, which is supposed to sequester cytosolic Al into the vacuole (Huang et al., 2012). Moreover, OsSTAR1 and OsSTAR2, the nucleotide-binding domain and the transmembrane domain of a functional ABC transporter, respectively, release UDP-glucose into the apoplast, which possibly minimizes Al binding to the cell wall in rice (Huang et al., 2009). In Arabidopsis, AtSTAR1 and AtALS3, which are homologs of rice OsSTAR1 and

OsSTAR2, respectively, are required for Al tolerance (Huang et al., 2010; Larsen et al., 2005). Recently, it was demonstrated that AtSTAR1 and AtALS3 interact forming a tonoplast-localized ABC transporter, which plays a role in response to low phosphorous availability (Dong et al., 2017) and possibly also acts in Al tolerance (Dong et al., 2017; Huang et al., 2010). However, the exact substrate transported by AtSTAR1/AtALS3 remains to be elucidated.

Al induces a rapid transcriptional response in the root apex resulting in the expression of several genes involved with both external and internal Al detoxification (Kochian et. al, 2015). Most transcription factors (TFs), which regulate Al tolerance genes, belong to the zinc finger and WRKY TF families. Zinc finger TFs have zinc finger motif(s) in which cysteine and histidine residues coordinate one or more zinc atoms forming, in most cases, a DNA-binding domain, or are implicated in protein-protein interaction with other TFs (Tikatsuji, 1999). The C_2H_2-type zinc finger TF, AtSTOP1 (Arabidopsis sensitive to proton rhizotoxicity 1), positively regulates the expression of *AtALM1,* which is a major determinant of Arabidopsis Al tolerance, and also regulates *AtMATE1* and *ALS3* (Liu et al., 2009; Sawaki et al., 2009). OsART1 (Al Resistance Transcription factor 1), a homologous of AtSTOP1 in rice, activates the expression of several Al tolerance genes, including *OsNrat1*, *OsALS1*, *OsSTAR1*, *OsSTAR2*, and the MATE Al-activated citrate transporter, *FRDL4* (Yamaji et al., 2009; Xia et al., 2010; Yokosho et al., 2011; Huang et al., 2012;). WRKY TFs are characterized by possessing one or two WRKY domains, which contains a highly conserved WRKYGQK heptapeptide, followed by a zinc-finger motif. These TFs show a remarkable conservation of its DNA binding site, designated as W-box (TTGACC/T) element (Rushton et al., 2010). In Arabidopsis, AtWRKY46, a member of the WRKY family, represses the *AtALMT1* expression by binding to W-box elements on its promoter in the absence of Al, which possibly controls carbon losses , reducing malate exudation in the absence of Al (Ding et al., 2013). OsWRKY22, a WRKY TF from rice, works cooperatively with OsART1 in the activation of the *OsFRDL4* expression, binding to W-box elements in its promoter (Li et al., 2018).

In sorghum, the Alt_{SB} locus, where the plasma membrane-localized citrate transporter, SbMATE, resides, controls approximately 80% of the phenotypic variation for Al tolerance (Magalhaes et al., 2004, 2007). The *SbMATE* expression is strongly induced by Al in a time-dependent manner in root apices of Al-tolerant lines, and its expression is highly correlated with citrate secretion and Al tolerance. In turn, in Al-sensitive lines, the *SbMATE* expression is reduced without Al (Magalhaes et al., 2007). However, the introgression of Alt_{SB} locus from Al-tolerant lines into Al-sensitive recurrent parents results in the reduction of both *SbMATE* expression and Al tolerance, suggesting the influence of *trans*-acting factors in the regulation of *SbMATE* (Melo et al., 2013).

Recently, it was demonstrated that variation in *SbMATE* expression results from an interplay between factors acting both in *cis* and in *trans*, where a WRKY, SbWRKY1, and a DHHC-type zinc finger, SbZNF1, TFs bind to and *trans*-activate the *SbMATE* promoter (Melo et al., 2019). Similar to *SbMATE*, *SbWRKY1* and *SbZNF1* expression show a general trend for a time-dependent increase in response to Al in root apex from Al-tolerant lines, which is higher for *SbWRKY1* compared to *SbZNF1*. In turn, in Al-sensitive lines, the expression of both *TFs* decrease along time in response to Al (Melo et al., 2019).

SbWRKY1 and SbZNF1 bind to a *cis*-acting sequence flanking a Tourist-like miniature inverted-repeat transposable element (MITE; Wessler et al., 1995) found in the *SbMATE* promoter (Magalhaes et al., 2007; Melo et al., 2019). The MITE element and its flanking sequences, designated henceforth as MITE repeats, are repeated in tandem in different sorghum lines. Natural, allelic variation in the *SbMATE* promoter arises from variations in the number of MITE repeats, which is highly correlated with *SbMATE* expression, with Al-tolerant lines presenting, in general, more repeats than Al-sensitive lines (Magalhaes et al., 2007). Transactivation assay in *Arabidopsis* protoplasts showed that SbWRKY1 and SbZNF1 transactivation activity is correlated with the number of MITE repeats. Additionally, the effect on the *SbMATE* promoter activity of SbZNF1 combined with SbWRKY1 is higher than their individual effects, suggesting a synergistic mode of action of the TFs. Haplotype analyses in sorghum recombinant inbred lines (RILs) support the cooperative effect of SbZNF1 and SbWRKY1 in *SbMATE* expression. Lines carrying a haplotype containing *SbZNF1* and *SbWRKY1* alleles from Al-tolerant line shows higher *SbMATE* expression than lines with the alternate haplotype, containing either both TFs alleles from Al-sensitive line, or lines with haplotypes with only one “Al tolerant” TF allele (Melo et al., 2019).

It is proposed that, in Al-tolerant genotypes, MITE repeat expansion, combined with Al induction of *SbWRKY1* and *SbZNF1*, increase TF recruitment, resulting in higher *SbMATE* expression. In turn, in Al-sensitive lines, lower TFs expression and decreased number of MITE repeats result in reduced *SbMATE* expression (Melo et al., 2019). In the present work, we focused on the elucidation of the molecular mechanisms underlying the transcriptional regulation of *SbMATE* expression by SbWRKY1 and SbZNF1.

MATERIAL AND METHODS

Phylogenetic Analysis

Phylogenetic analyses were performed separately for proteins in the WRKY and zf-DHHC families. The amino acid sequences of maize, rice, sorghum, wheat and Arabidopsis proteins were downloaded from the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html). Accession numbers are provided in Supplemental Tables 1 (WRKY proteins) and 2 (zf-DHHC proteins). In total, 114 WRKY and 112 zf-DHHC proteins were selected from the literature so that representative members from each group and subgroup were included. Sequence alignments within each family were done with the Muscle software (Edgar, 2004) and were subsequently used for phylogenetic reconstructions. Unrooted phylogenetic trees were implemented in the Mega X software (Kumar et al., 2018) using the maximum likelihood statistical method with bootstrap values from 1000 replicates, and the Jones-Taylor-Thornton (JTT) +G (gamma rates) +F (empirical frequencies) amino acid substitution model. The phylogenetic trees were edited to generate a graphical display of phylogenetic relationships with the iTol (https://itol.embl.de) and Inkscape (https://inkscape.org/pt) tools. Consensus amino acid sequences identical at ≥80% level for each cluster identified in the phylogenetic trees were obtained using the CLC Genomics Workbench 20.0 software (https://digitalinsights.qiagen.com).

Vector constructs

The sequences of all primers used for amplification of *SbWRKY1* and *SbZNF1* coding regions are in Supplemental Table 3. For heterologous expression of TF proteins, primers were designed to amplify the SbZNF1 coding region lacking the two first transmembrane domains, and the full-length *SbWRKY1* from a commercially synthesized pBSK vector containing the TF genes. The amplicons were cloned into the Champion pETSUMO expression vector (Invitrogen) using T4 DNA Ligase (Promega) according to the manufacturer's instructions. The resulting vectors, designated His-SUMO::SbWRKY1 and His-SUMO::ΔSbZNF1, harbor the respective TF cDNAs C-terminally fused to a polyhistidine-tag (6xHis) followed by the Small Ubiquitin Modifier (SUMO), under the control of the T7 promoter.

For other experiments, recombinant plasmids were generated by the GATEWAY cloning system (Invitrogen) according to the manufacturer's instructions. The coding regions of *SbZNF1 and SbWRKY1* without the translational stop codon were also amplified from the pBSK vectors containing the TF genes and transferred by recombination to the entry vector, pDONR207. The resulting vectors were designated pDONR207_SbWRKY1 and pDONR207_SbZNF1.

For subcellular localization assays, *SbWRKY1* and *SbZNF1* cDNA were transferred via recombination, from the entry vectors, pDONR207_SbWRKY1 and pDONR207_SbZNF1, to the pK7FWG2 destination vector. The resulting vectors contained TF cDNA C-terminally fused to

green fluorescent protein (GFP) coding region (SbWRKY1::GFP and SbZNF1::GFP) under the control of the 35S promoter. The SbWRKY1::mCherry fusion was constructed in the destination vector, pH7m34GW, by triple recombination using pDONR207_SbWRKY1, pDONR-P4-R1R_2x35S and pDONR-P2R-P3_mCherry. The resulting vector harbored a translational fusion between SbWKRY1 and the fluorescent protein, mCherry (SbWRKY1::mCherry) under the control of the 35S promoter.

For co-immunoprecipitation assays, the SbZNF1::GFP construct was used in combination with a construct harboring the SbWRKY1 cDNA C-terminally fused to 6x influenza hemagglutinin tag (SbWRKY1::HA). SbWRKY1 was fused to HA in the destination vector, pH7m34GW, by triple recombination using pDONR207_SbWRKY1, pDONR-P4-R1R_2x35S and pDONR-P2R-P3_6xHA. In the resulting vector, SbWRKY1::HA expression is driven by the 35S promoter.

For bimolecular fluorescence complementation assay, the coding regions of *SbWRKY1* and *SbZNF1* were transferred from pDORN207_SbWRKY1 and pDONR207_SbZNF1 to the pSPYNE-GW and pSPYCE-GW destination vectors, respectively. The resulting vectors harbor SbWRKY1 and SbZNF1 cDNA C-terminally fused to N- and C- terminus fragments of the yellow fluorescent protein, respectively (SbWRKY1::nYFP and SbZNF1::cYFP), under the control of the 35S promoter.

SbZNF1 coding region was also amplified with translational stop codon and cloned via recombination into the entry vector pDONR207. Subsequently, *SbZNF1* cDNA was transferred via recombination, from the entry vector to the pEarleyGate104 destination vector. The resulting vector contained *SbZNF1* cDNA N-terminally fused to yellow fluorescent protein (YFP) driven by 35S promoter (YFP::SbZNF1).

The proteins AtWWP1 and AtFLS2 fused to mCherry (AtWWP1::mCherry and AtFLS2::mCherry, under 35S control) were used as nuclear and plasma membrane markers, respectively (Calil et al., 2018; Gouveia-Mageste et al., 2020).

Heterologous expression and purification of SbWRKY1 and SbZNF1

Escherichia coli strain BL21(DE3) was transformed with the constructs, His-SUMO::SbWRKY1 and His-SUMO::ΔSbZNF1, in addition to the pETSUMO empty vector (expressing only His-SUMO). The transformants were grown overnight at 37°C and, subsequently, re-inoculated in LB medium (1:100 proportion) and grown at 37°C. At mid-log phase, optical density at 600nm (OD_{600nm}) = 0.6, the protein expression was induced by adding 1 mM IPTG at 37°C for 18 hours.

Obtaining of His-SUMO::SbWRKY1 and His-SUMO protein extracts

Soluble protein extracts containing His-SUMO::SbWRKY1 and His-SUMO were obtained after cellular lysis using the BugBuster Master Mix Reagent (Merk) lysis buffer according to the manufacturer's instructions. Soluble recombinant proteins were purified using Ni-NTA agarose (Qiagen)**.** Briefly, soluble extracts were incubated with 2 mL of Ni-NTA agarose at 4°C for 1h under gentle rotation and, subsequently, loaded in the Poly-Prep Chromatography columns (Bio-Rad). The Ni-NTA agarose was washed 5 times with native wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0) and gradient elution was carried out by adding elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) containing 80 mM, 90 mM and 100 mM imidazole. Purified protein extracts eluted with 100 mM imidazole were quantified using the Bradford method (Bradford, 1976; Bradford reagent, Invitrogen) and were subsequently used in Electrophoretic Mobility Shift Assays (EMSA).

Obtaining of His-SUMO::ΔSbZNF1 protein extracts

After cellular lysis using the BugBuster Master Mix Reagent (Merk) lysis buffer, His-SUMO::ΔSbZNF1 was found in the insoluble protein fraction. Thus, the resulting pellet was washed 5 times in BugBuster 1/10x and resuspended in lysis buffer containing 6M urea. The denatured protein extract was recovered by centrifugation, resuspended in binding buffer (PBS 1X [150 mM NaCl, 50 mM Na_2HPO_4, 11,5 mM NaH_2PO_4; pH 7.5], 6M urea, 0.5 M NaCl) and applied to HisTrap High Performance (Cytiva) column containing 1 mL of Nickel Sepharose. The HisTrap columns, placed in an Akta Explorer 100 FPLC system, were washed with 10 mL of binding buffer at a flow-rate of 1 mL/min. Refolding of the recovered protein was performed on-column, by a linear decreasing gradient from 6 M to 0 M urea, starting by adding the binding buffer and finishing by adding the elution buffer (PBS 1X, 0.5 M NaCl, 300 mM imidazole). Protein extracts were subject to dialysis for 16 h at 4°C of with EMSA binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT; pH 7.5), and quantified using the Bradford method and used in EMSA.

SbWRKY1 Electrophoretic Mobility Shift Assay (EMSA)

The oligonucleotides used in EMSA (Supplemental Table 3) were synthesized and 3' end-labeled with biotin using the Pierce Biotin 3' End DNA Labeling Kit (Thermo Scientific), as recommended by the manufacturer's instructions. Subsequently, double-stranded DNA probes were prepared by annealing complementary oligonucleotide fragments in a thermal cycler heating at 95°C for 5 min, decreasing the annealing temperature at 1°C per cycle until the oligonucleotide

T_m for 30 min. Then, the annealing temperature was decreased by 1°C per cycle until 10°C. The number of cycles depends on the T_m of the oligonucleotides to be annealed. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific) according to the manufacturer's instructions. The SbWRKY1 binding reactions were composed by 1x Binding Buffer (10 mM Tris, 50 mM KCl, 1 mM DTT; pH 7.5), 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/µL Poly (dI.dC), 0.05% NP-40, 20 fmol of biotin labeled probe and 2 µg of recombinant protein extract (His-SUMO::SbWRKY1 or His-SUMO). The binding reactions were incubated for 20 min at room temperature and the DNA/protein complexes were resolved in DNA Retardation Gel 6% (Invitrogen). The protein/DNA complexes were transferred to a nylon membrane for detection by the streptavidin-conjugated horseradish peroxidase reaction, using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific). The chemiluminescent signals were detected using X-ray films (Thermo Scientific).

Subcellular localization assays

The subcellular localization of SbWRKY1 and SbZNF1 was firstly assessed in *N. benthamiana* epidermal cells. The *Agrobacterium tumefaciens*, GV3101 strain, harboring the SbZNF1::GFP, SbWRKY1::GFP or SbWRKY1::mCherry vectors, were infiltrated at $OD_{600nm} = 0.4$ into the abaxial surface of *N. benthamiana* leaves. For co-localization assays, *N. benthamiana* leaves were co-transformed with GV3101 harboring the vector pairs, SbZNF1::GFP and AtFLS2::mCherry; SbWRKY1::GFP and AtWWP1::mCherry; SbZNF1::GFP and SbWRKY1::mCherry. Co-infiltrations were performed mixing equal volumes of *Agrobacterium* suspensions at $OD_{600nm} = 0.8$, so that the final optical density at 600nm of each suspension amounted 0.4. After infiltration, the plants were maintained for 72h at 22°C and, subsequently, fluorescent images were captured using a Zeiss LSM510 META laser scanning microscope.

SbZNF1 and SbWRKY1 subcellular localization was also examined in transgenic Arabidopsis roots. The Columbia (Col-0) ecotype of *A. thaliana* was transformed with either SbZNF1::GFP or SbWRKY1::GFP vectors via *Agrobacterium tumefaciens* GV3101 strain by floral dip method (Zhang et al., 2006). Homozygous T3 families expressing SbZNF1::GFP and SbWRKY1::GFP were obtained. Seeds were surface sterilized and germinated in Murashige-Skoog agar plates supplemented with 1% sucrose. After three days at 22°C under 16h light/8h dark, Arabidopsis´ roots were photographed using a Zeiss AxioObserver Z1 Apotome 2.0 microscope.

Co-immunopreciptation assay

The interaction between SbZNF1 and SbWRKY1 was tested by co-immunoprecipitation using the μMACS Epitope Tag Protein Isolation Kit (MACS/Miltenyi Biotec), according to the manufacturer's instructions. *N. benthaminana* leaves were transiently co-transformed via *A. tumefaciens* GV3101 strain with SbWRKY1::HA and SbZNF::GFP, as previously described for subcellular localization studies. Leaf cells co-transformed with SbWRKY1::HA and an empty vector (expressing eGFP), and transformed with only SbWRKY1::HA, were used as controls. Total proteins from 1 g tissue/sample were extracted in buffer kept in ice (Tris-HCl at pH 8.0, 1% NP-40, 2 mM benzamidine, 2 mM PMSF). Protein extracts were incubated for 1h with magnetic beads conjugated with an antibody raised against HA (anti-HA magnetic beads, MACS-Miltenyi Biotec) at 4°C under gentle rotation. Subsequently, the protein extracts were loaded to the MACS column and washed five times for removal of unbound proteins. The immunoprecipitated proteins were eluted by adding 50 μL of elution buffer pre-heated at 95°C, which also denatures the immunopreciptated complexes. Immunoprecipitated protein and total protein extracts were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE gel) using Mini-PROTEAN TGX Stain-Free Precast gels, (4-15% w/v) (Bio-Rad), and transferred to nitrocellulose membranes. Immunoblotting was performed with anti-HA or anti-GFP polyclonal antibodies conjugated to horseradish peroxidase (Miltenyi Biotec), which were detected using Clarity Western ECL Substrate (Bio-Rad) according to the manufacturer's recommendations. The chemiluminescent signals were captured using a ChemiDoc XRS+ (Bio-Rad).

Bimolecular fluorescence complementation (BiFC) assay

The constructs, SbZNF1::cYFP and SbWRKY1::nYFP, were co-transformed in *N. benthamiana* via *A. tumefaciens* at OD_{600nm} = 0.4, as previously described for subcellular localization studies. As negative controls, SbZNF1::cYFP and SbWRKY1::nYFP were transformed combined with empty vectors expressing nYFP and cYFP (N- and C- terminus fragments of YFP), respectively. Seventy-two hours post infiltration, the YFP reconstituted signal was captured using a Zeiss inverted 510 META laser scanning microscope.

Al treatment and SbZNF1 mobilization

The SbZNF1 remobilization studies were performed in *N. benthamiana* leaves transiently transformed with either YFP::SbZNF1 vector or co-transformed with the SbWRKY1::mCherry and YFP::SbZNF1 vectors via *A. tumefaciens* infiltration (see "Subcellular localization assays" method). After 72 hours post transformation, plants were subject to Al treatment by infiltrating a

solution with (10uM $AlCl_3$, 200uM $CaCl_2$, pH 5.0; free Al^{3+} activity: 3 µM, calculated by GEOCHEM-EZ, Shaff et al., 2010) or without Al (200uM $CaCl_2$, pH 5.0). The Al treatment was imposed for 24 hours, and the fluorescence was observed in epidermal cells using a Zeiss AxioObserver Z1 Apotome 2.0 microscope. Nuclei were stained by infiltration of 5 µg/mL of DAPI (4′,6-diamidino-2-phenylindole, Thermo Scientific) diluted in PBS 1X for 20 min before image acquisition.

For SbZNF1 mobilization in Arabidopsis, seeds stably expressing SbZNF1::GFP were surfaced sterilized (see "Subcellular localization assays" section) and hydroponically germinated in magenta boxes containing MGRL nutrient solution one-fiftieth strength (0.08 mM $NaNO_3$; 0.06 mM KNO_3, 0.035 mM $NaPO_4$, 60 µM $MgSO_4$, 0.172 µM $FeSO_4$, 0.6 µM H_3BO_3, 0.48 nM $(NH_4)_6Mo_7O_{24}$, 0.206 µM $MnSO_4$, 0.02 µM $ZnSO_4$, 2.6 nM $CoCl_2$, 0.02 µM $CuSO_4$, 1.34 µM Na_2EDTA, 0.2 mM $CaCl_2$, pH 5.0) (Fujiwara et al., 1992; Kobayashi et al., 2007). Seedlings were grown for five days at 22°C under a 16h-light/8h-dark photoperiod, with light supplied at a photon flux density of 70 µmol m^{-2} s^{-1} and the MGRL nutrient solution was replaced every two days. Subsequently, the seedlings were transferred to MGRL nutrient solution with all nutrients except phosphorus and supplemented or not with 10 µM $AlCl_3$ (free Al^{3+} activity: 2.4 µM, calculated by GEOCHEM-EZ, Shaff et al., 2010). The fluorescent signals were examined in roots 24 hours post Al treatment using a Zeiss AxioObserver Z1 Apotome 2.0 microscope. For DAPI staining, seedlings were fixed in 4% paraformaldehyde solution for 1 hour and washed twice in PBS 1X. After fixation, the roots were stained with 5 µg/mL of DAPI solution diluted in PBS 1X for 20 min.

Microscopy

For acquiring fluorescent images, we used a Zeiss LSM 510 laser scanning microscope, which was equipped with an argon and helium lasers as excitation sources. GFP was excited at 488 nm wavelength, and emission was detected using 500 to 530 nm wavelength filters. mCherry and YFP were excited at 543 nm and 514 nm, and their emissions were detected using 608 to 680 nm and 560 to 615 nm filters, respectively.

For acquiring fluorescent images using the Zeiss AxioObserver Z1 Apotome 2.0 microscope, both GFP and YFP were excited at 493 nm wavelength filter, and the emission of both fluorescent proteins was detected using 500 to 550 nm wavelength filters. mCherry and DAPI were excited at 590 nm and 401 nm, respectively. The emissions of mCherry and DAPI were detected using 600 to 690 nm, and 420 to 470 nm filters, respectively.

RESULTS

Phylogenetic analysis of SbWRKY1 and SbZNF1

To explore the relationship of SbWRKY1 and SbZNF1 with WRKY and zf-DHHC proteins from other plant species, a phylogenetic tree for each protein family was inferred using maximum likelihood (Kumar et al., 2018). Our phylogenetic analysis was consistent with previous classification of WRKY proteins into the major groups I, II, and III, which reflect the number of WRKY domains and the structure of their zinc finger motif (Figure 1A), (Eulgem et al., 2000). Group I proteins harbor two WRKY domains and a C_2H_2-type zinc finger motif. Proteins in groups II and III have a single WRKY domain and C_2H_2 and C_2HC zinc fingers, respectively (Supplemental figure 1A). Based on the primary structure of the WRKY domain, group II was further divided into five subgroups (IIa to IIe, Eulgem et. al, 2000). Our analysis revealed that these subgroups cluster in three distinct clades, IIa/IIb, IIc and IId/IIe (Figure 1A), which is consistent with a possible paraphyletic origin of group II proteins (Rushton et al., 2008; Y. Zhang & Wang, 2005). Based on the number of amino acids separating the cysteine and histidine residues in the zinc finger motif, group III WRKY proteins were further allocated to subgroup IIIa (C-X_7-C-$X_{\overline{23}}$-HTC), IIIb and IIIc (C-X_7-C-$X_{\overline{24}}$-HTC). The WRKY domain of proteins within subgroup IIIc is highly conserved and differed of the subgroup IIIb (Supplemental figure 1A). According to its zinc finger structure and phylogeny, SbWRKY1 is a group IIIc WRKY protein (Figure 1A).

Interestingly, instead of the highly conserved WRKYGQK heptapeptide (Rushton et al., 2010), which was found in 88% of the WRKY TFs (Figure 1A), SbWRKY1 has a rather rare, WRKYGEK heptapeptide variant. In our analysis comprising 114 WRKY proteins, only nine proteins (8%) were found to possess the WRKYGEK heptapeptide, and all of them clustered into group III (Figure 1A, in orange color in the phylogenetic branches and the pie chart). In addition, other five members (4%) contain the variant WRKYGKK and were classified into group IIc (Figure 1A).

Zinc finger-DHHC (zf-DHHC) proteins have a conserved cysteine-rich region containing the DHHC motif (Putilina et al., 1999) (Supplemental Figure 1B). Our phylogenetic analysis classified the zf-DHHC proteins into eight groups, group I to VIII, as proposed by Yuan et al., (2013) (Figure 1B), which is consistent with the amino acid conservation among the cysteine residues in the zf-DDHC domain (Supplemental Figure 1B). SbZNF1 clustered into group I, which is the largest zf-DHHC group in plants (Yuan et. al, 2013).

SbWRKY1 recognizes a novel cis element for WRKY binding

The structure of the *SbMATE* promoter containing the MITE insertion is depicted in the Figure 2A. The MITE repeats are composed of the MITE element itself, "b" unit, and its flanked sequences, the 100-bp "a" unit and 20-bp "c" unit. This a-b-c triplet is followed by a single "a" unit containing an 8-bp deletion, resulting in 92-bp terminal, unrepeated sequence. SbWRKY1 and SbZNF1 were previously found to bind both to the 100- and 92-bp ~sequence, hence outside of the 8-bp deletion, but not to the 2010-bp downstream sequence (Melo et. al 2019) (Figure 2A). Thus, we performed Electrophoretic Mobility Shift Assays (EMSA) with His-SUMO tagged TFs and biotin-labeled overlapping probes covering the entire 92-bp sequence to more finely map the TF binding elements. The full-length 92-bp sequence and a 60-pb probe (F5) within the 2010-bp downstream fragment were used as positive and negative controls, respectively.

His-SbWRKY1 specifically bound to the 92-bp probe, and the binding signal was gradually suppressed by addition of an unlabeled competitor (non-labelled oligo with the same sequence of the labelled oligo, Figure 2B), consistent previous results with immunoprecipitated chromatin (ChIP, Melo et al, 2019). On the other hand, both the 92-bp probe incubated with only purified His-SUMO protein extract, as well as His-SbWRKY1 incubated with the F5 probe, did not produce a mobility shift (Supplemental Figure 2B). We could not detect a His-SbZNF1 binding signal, either alone or in combination with SbWRKY1, to the 92-bp probe.

Next, we set out to identify the region(s) within the 92-bp fragment recognized by SbWRKY1. His-SbWRKY1 specifically bound to the F3 and F4 probes, but neither to F1 nor F2, suggesting that SbWRKY1 binds to the 10-bp overlap between F3 and F4 (Figure 2C). Next, we designed probes for the F3 and F4 fragments flanking their overlap (F3' and F4', respectively), hence eliminating the GGCATCTATA binding sequence, which did not produce binding signal upon incubation with His-SbWRKY1 (Figure 2D). These results confirmed that SbWRKY1 binds to the GGCATCTATA sequence. Four mutated probes for the binding sequence harboring sequential, 2-bp deletions (M1 to M4) were tested for SbWKRKY1 recognition (Figure 2E). SbWRKY1 binding activity was dramatically reduced when the first dinucleotide (GG) was deleted (M1 probe), and was abolished upon further internal deletions in the binding sequence (Figure 2E, probes M2 to M4). These results suggest that the entire GGCATCTATA sequence is required for SbWRKY1 to fully occupy the *SbMATE* promoter, and established essentiality for the CATCTATA element in SbWRKY1/DNA interaction.

SbZNF1 localizes in the plasma membrane and SbWRKY1 is found predominantly in the nucleus

The knowledge of the subcellular localization of SbZNF1 and SbWRKY1 may help to understand their biological function on Al tolerance. To address this issue, both TFs were C-terminally fused to GFP and placed under the control of the CaMV 35S promoter for transient expression in *Nicotiana benthamiana* leaves. We co-expressed SbZNF1::GFP whit AtFLS2::mCherry, a plasma membrane marker (Mageste-Gouvea et. al, 2020), and SbWRKY1::GFP with the nuclear marker, AtWWP1::mCherry (Calil et. al, 2018). Consistent with the *in silico* prediction of transmembrane domains in SbZNF1, the SbZNF1::GFP signal co-localized with AtFLS2::mCherry in the plasma membrane (Figure 3A). In addition, the strong signal produced by SbWRKY1::GFP coincided with the mCherry signal of the AtWWP1 fusion, confirming that SbWRKY1 is predominantly localized in the nucleus (Figure 3B). To verify whether SbZNF1 and SbWRKY1 could influence each other's subcellular localization, next we co-expressed SbZNF1::GFP and SbWRKY1::mCherry in *N. benthamiana* leaves. There was no apparent difference in the fluorescence signals detected in cells individually expressing SbZNF1::GFP and SbWRKY1::mCherry compared to cells co-expressing both TFs (Figure 3C; Supplemental figure 3A and 3C). SbWRKY1 also shows a weak signal in the cytoplasm. Because of the large vacuole, in medial optical sections of the cells, the cytosolic signal is restrict to a band close to the plasma membrane (Supplemental figure 3B and 3C), and it is more evident in tangential sections at the cell surface (Supplemental figure 3C).

The subcellular localization of both TFs was further examined in transgenic *Arabidopsis* roots stably expressing SbZNF1::GFP and WRKY1::GFP translational fusions. In Arabidopsis roots, SbZNF1 localized exclusively in the plasma membrane whereas SbWRKY1 localized in the nucleus (Figure 4), which is consistent the subcellular results in *N. benthamiana* leaves.

SbWRKY1 and SbZNF1 physically interact in the plasma membrane

SbWRKY1 and SbZNF1 cooperate in the activation of *SbMATE* expression (Melo et al., 2019). Thus, we hypothesized that the TFs could physically interact, with such interact leading to an increased *SbMATE* expression. To test the interaction between the TFs, we initially performed a co-immunoprecipitation (Co-IP) assay with SbWRKY1::HA and SbZNF1::GFP fusions transiently co-expressed in *N. benthamiana* leaves. The immunoprecipitation of protein extracts was performed with magnetic beads coupled to anti-HA antibody. After denaturing of the immunoprecipitated complex, in plants expressing both TFs, an approximately 70 kDa protein band was detected by anti-GFP antibody in the immunoprecipitated fraction, consistent with the SbZNF1:::GFP molecular weight, indicating that SbWRKY1 specifically interacts with SbZNF1 (Figure 5A). In turn, no Co-IP protein was found in the controls. Next, we undertook Bimolecular Fluorescence

Complementation (BiFC) assay to further confirm the SbWRKY1/SbZNF1 interaction and to located the subcellular compartment harboring this TF complex. For that, the N- and C-terminal fragments of YFP (nYFP and cYFP) were respectively fused to SbWRKY1 and SbZNF1, generating 35S-driven SbWRKY1::YFP and SbZNF1::cYFP fusions, which were co-expressed in *N. benthamiana* leaves. The BiFC assay confirmed the interaction between SbWRKY1 and SbZNF1 and, interestingly, the reconstituted YFP fluorescent signal was located in the plasma membrane (Figure 5B). Taken together, these results show that SbZNF1 and SbWRKY1 physically interact, and the cellular site where this interaction occurs is the plasma membrane.

Al^{3+} induces SbZNF1 mobilization from the plasma membrane to the nucleus in a SbWRKY1-dependent manner in *N. benthamiana*

Given the plasma membrane-bound nature of SbZNF1, we investigated whether Al^{3+} could elicit SbZNF1 remobilization to the nucleus. *N. benthamiana* leaves transiently expressing YFP::SbZNF1, were subjected to Al treatment via infiltration of a solution with (+Al: 10uM $AlCl_3$, 200uM $CaCl_2$, pH 5.0) or without Al (-Al: 200uM $CaCl_2$, pH 5.0). Al treatment failed to elicit mobilization of SbZNF1 from the plasma membrane to the nucleus in cells transformed only with SbZNF1 (Figure 6A). However, Al treatment resulted in YFP::SbZNF1 to be detected not only in the plasma membrane but also in the nucleus in cells co-expressing both SbWRKY1 and SbZNF1, which co-localized with SbWKRY1 (Figure 6B, Supplemental figure 4A). The resulting SbZNF1 nuclear signal was present in 20.6% ± 2.5 of cells transfected with both TF constructs.

We also evaluated the SbZNF1 mobilization in *Arabidopsis* roots stably transformed with 35S::SbZNF1::GFP under Al stress. The fluorescent images revealed a low frequency of SbZNF1::GFP nuclear signal (5% ± 0.07) which was, nevertheless, detected exclusively upon Al^{3+} exposure (Figure 7). The subcellular localization of SbZNF1::GFP in the nucleus was confirmed by DAPI staining (Supplemental figure 4B).

DISCUSSION

WRKY transcription factors comprise a large protein family in plants whose members are implicated in the regulation of developmental processes and stress responses (Rushton et al., 2010). The number of WRKY domain(s) and its variable zinc finger motif underlies the phylogenetic relationships of WRKY proteins (Eulgem et. al, 2000; Supplemental figure 1A). SbWRKY1 possesses a single WRKY domain and a C_2HC-type zinc finger, which is a typical feature of group III WRKYs, some of which have been previously shown to be involved in the regulation of Al

tolerance transporters. Both positive and negative regulators are found in group III, which is considered more evolutionary active and adaptable to environmental clues (Huang et al., 2016; Wu et al., 2005). This is consistent with positive regulation of sorghum *SbMATE*, by SbWRKY1 (Melo et al., 2019) and of the rice MATE member, OsFRDL4 (Yokosho et al., 2011), by OsWRKY22 (Li et al., 2018), as well as the negative regulation of the Arabidopsis Al-activated malate transporter, *AtALMT1*, by AtWRKY46 (Ding et al., 2013).

The reason why closely related group III WRKY proteins are so functionally variable are not clear. However, existing evidence suggests the involvement of variations in the WRKY domain in this phenomenon. A remarkable characteristic of the WRKY domain is the presence of a highly conserved WRKYGQK heptapeptide (Eulgem et. al, 2000), which was found in 88% of the proteins used in our phylogenetic analysis. Nevertheless, SbWRKY1 has an heptapeptide variant, WRKYGEK, which has been found in only nine proteins (8%) (Figure 1A), which were all classified into subgroups IIIb and IIIc within group III (Figure 1A). Consistent with our findings, the WRKYGEK motif is rather rare and is found in a few group III proteins from monocots, such as sorghum (Baillo et al., 2019), rice (Zhang & Wang, 2005), maize (Zhang et al., 2017), barley (Liu et al., 2014), and wheat (Ning et al., 2017). However, it is absent in most of the dicots, suggesting a recent expansion of the WRKYGEK proteins (Liu et al., 2014).

We found that SbWRKY1 recognizes neither the canonical W-box (TTGACC/T), occupied by the vast majority of WRKY proteins (Chen et al., 2019) nor any known W-box variant (Figure 2). Instead, SbWRKY1 binds to a novel 10-bp *cis*-element in the *SbMATE* promoter, GGCATCTATA, located in the "a" unit that is part of the MITE repeats, with CATCTATA being essential for SbWRKY1/DNA interaction. All WRKYGQK-type TFs characterized thus far for DNA interaction have been shown to recognize the canonical W-box (Chen et al., 2019), with occasional promiscuous binding to W-box variants, as is the case for WRKY70, WRKY26 and WRKY41 in Arabidopsis that bind to the WT-box, YGACTTTT (Kanofsky et al., 2017; Machens et al., 2014). Importantly, dual recognition of W-box and different motifs occurs for WRKYGQK WRKY TFs, such as for SUSIBA2 in barley, which binds to the SURE element (Sun et al., 2003) and WRKY13 in rice, which binds to PRE4 element (Xiao et al., 2013). Previous studies indicated that amino acids in the WRKYGQK heptapeptide, including the amino acid glutamine (Q), directly contact the W-box element (Yamasaki et al., 2008), and variations in this amino acid might diversify WRKY recognition with respect to the canonical W-box element (Dong et al., 2003). For instance, WRKY12 from *Nicotiana tobacco* contains the WRKYGKK motif and interacts with the WK-box (TTTTCCAC), but not with the W-box. Moreover, mutations in NtWRKY12 replacing GKK by GQK or GEK suppressed its binding activity to the WK-box (Van Verk et al., 2008). SbWRKY1

is the first WRKYGEK-type TF characterized for DNA-interaction; hence, we hypothesize that the WRKYGEK motif is involved with the SbWRKY1 binding activity to a previously unidentified element for WRKY interaction. *In silico* analyses of the 92-bp sequence indicated that the SbWRKY1 binding sequence contains the predicted binding site of the *Arabidopsis thaliana* transcription factor, RELATED TO AP2 2 (RAP2.2, Figure 2A, Welsch et al., 2007). Based on *in vitro* assays, it was demonstrated that AtRAP2.2 binds to the ATCTA *cis*-element in the promoters of genes in the carotenoid biosynthetic pathway, phytoene synthase and phytoene desaturase (Welsch et al., 2007). A role for RAP2.2 on abiotic stress tolerance emerges from the Hinz et al., (2010) study, who showed that AtRAP2.2 is an ethylene-responsive TF required for hypoxia tolerance in *Arabidopsis*. Hence, these observations may hint on a possible functional convergence of Al tolerance and ethylene response pathways elicited by SbWKRY1 and RAP2.2, respectively.

SbZNF1 is a DHHC-like S-acyltransferase zinc finger protein (Melo et al., 2019). The members of the zf-DHHC family are characterized by promoting reversible S-acylation of target proteins, increasing their hydrophobicity (Hemsley, 2020). AtPAT10, a zf-DHHC protein, mediates the S-acylation of CBL10 (Calcineurin B-like 10), which triggers its tonoplast association required to salt tolerance in Arabidopsis (Chai et al., 2020). Structural predictions of the zf-DHHC domain suggest that it forms two C_2HC-type zinc fingers (Montoro et al., 2013), similar to other, non-DHHC-type zinc fingers and similar to some WRKY TFs, which are involved in protein/DNA interaction. However, the transcriptional activity of zf-DHHC proteins was only recently shown by Bass et al., (2015) in a study of potential human TFs and in plants, by Melo et al., (2019), who showed that SbZNF1 regulates *SbMATE* in sorghum . The domain prediction of zf-DHHC proteins consists of at least four transmembrane domains flanking the zf-DHHC domain (Stix et al., 2020), as is predicted for SbZNF1 (Figure 3A). We found that SbZNF1 is located to the plasma membrane, while SbWRKY1 is mainly localized in the nucleus, with a minor presence in the cytosol. Membrane-bound transcription factors are kept anchored to cellular membranes in a dormant stage and, upon stimuli, at a certain developmental phase or upon environmental stress, they are cleavage from the membrane and mobilized to the nucleus regulating gene expression (Seo, 2014; Liu et al., 2018). The location of SbZNF1 in the plasma membrane raises the hypothesis that SbZNF1 act as a receptor of either a direct or indicted signal elicited by the presence of Al^{3+} in the apoplast of root cells.

Next, we set out to investigate this hypothesis and demonstrated that, indeed, Al^{3+} post-translationally regulates SbZNF1 by inducing its mobilization from the plasma membrane to the nucleus in tobacco epidermal cells co-expressing SbZNF1 and SbWRKY1. No such mobilization was observed in cells expressing only SbZNF1 (Figure 6), indicating that Al^{3+} is required but not

sufficient to trigger SbZNF1 trafficking to the nucleus. Our co-IP results indicate that SbZNF1 and SbWRKY1 interact (Figure 5A). To elucidate the cellular compartment where the TF heterodimer complex is located, Bimolecular fluorescence complementation (BiFC) was undertaken, revealing that SbZNF1 and SbWRKY1 physically interact in the plasma membrane (Figure 5B). In Arabidopsis, retention of WRKY TFs away from the nucleus via protein-protein interaction has been shown. In response to high levels of ABA, AtWRKY40, a key negative regulator of ABA-responsive genes, and probably AtWRKY18 and AtWRKY60, are recruited from the nucleus to the cytosol via interaction with the chloroplast envelope-localized ABA receptor (ABAR) de-repressing the ABA signaling (Shang et al., 2010). Hence, we propose that Al^{3+}, via a yet unknown mechanism, triggers conformational changes in SbZNF1 that is complexed with SbWRKY1, activating proteolytic cleavage of SbZNF1, which is subsequently shuttled to the nucleus, either alone or complexed with SbWRKY1, to regulate *SbMATE*. SbWRKY1 and SbZNF1 synergistically regulate *SbMATE*, which was shown in transactivation assays in Arabidopsis protoplasts and via haplotype analysis in sorghum (Melo et al., 2019). The dependency of SbWRKY1 for SbZNF1 nuclear mobilization partially explain this cooperative effect. Additionally, SbZNF1/SbWRKY1 complex might be transcriptionally active, resulting, for instance, in an increase of DNA-binding affinity, as showed in rice for WRKY51, which interact with WRKY71 strongly enhancing the WRKY71 binding activity to its target gene (Xie et al., 2006). In turn, SbWRKY1 is found in the nucleus, independent from either Al^{3+} or its interaction with SbZNF1, which probably maintains *SbMATE* expression in the absence of Al (Magalhaes et al., 2007). Interestingly, a SbWRKY1 binding site is found 1.14 kb upstream of the *SbZNF1* coding sequence. We hypothesize that SbWRKY1 could activate the *SbZNF1* expression directly binding to its promoter, and, hence, forming a positive feedback loop between SbWRKY1 and SbZNF1. However, in the absence of Al, SbZNF1 appears to act to retain a fraction of the SbWRKY1 protein in the plasma membrane.

Functional redundancy is a common feature of WRKY TFs, as shown for resistance to *Pseudomonas syringae* regulated by WRKY18, WRKY40 and WRKY60 in Arabidopsis (Xu et al., 2006). Hence, the occurrence of SbZNF1 mobilization to the nucleus in the absence of SbWRKY1 that was observed in Arabidopsis roots may result from SbZNF1 interacting with a SbWRKY1-like protein in Arabidopsis, such as AtWRKY62 from group III, which is related to SbWRKY1 (Figure 1A).

In sorghum, *SbMATE* expression is temporally and spatially correlated with Al-induced reactive oxygen species (ROS) production in Al-tolerant near-isogenic lines leading Sivaguru et al., (2013) to propose that ROS accumulation could be involved in signaling mechanisms that result in the *SbMATE* expression. In Arabidopsis, NTL4, a plasma membrane-localized NAC

(NAM/ATAF1/2/CUC2) TF, undergoes proteolytic cleavage been translocated to the nucleus in response to ROS accumulation induced by heat stress and, possibly, by drought (Lee et al., 2014), which may support a more general role of ROS as a signaling molecule triggering TF remobilization in response to abiotic stresses. Another possible signal leading to proteolytic cleavage of SbZNF1 involves changes in the physicochemical properties of the plasma membrane caused by Al stress, leading to reduced membrane fluidity (Kochian et al., 2005). Cold-induced changes in membrane fluidity have been shown to induce the plasma membrane proteolytic cleavage and nuclear translocation of NTL6, a NAC TF considered a cold sensor in Arabidopsis (Seo et al., 2010a; Seo et al., 2010b).

In the current study, we provide the first report of Al acting as a remobilization signal, whereby the membrane-associated SbZNF1 is activated in response to Al by interaction with SbWRKY1 and transmits the stress signaling from the plasma membrane to the nucleus, where both TFs transcriptionally regulate *SbMATE*.

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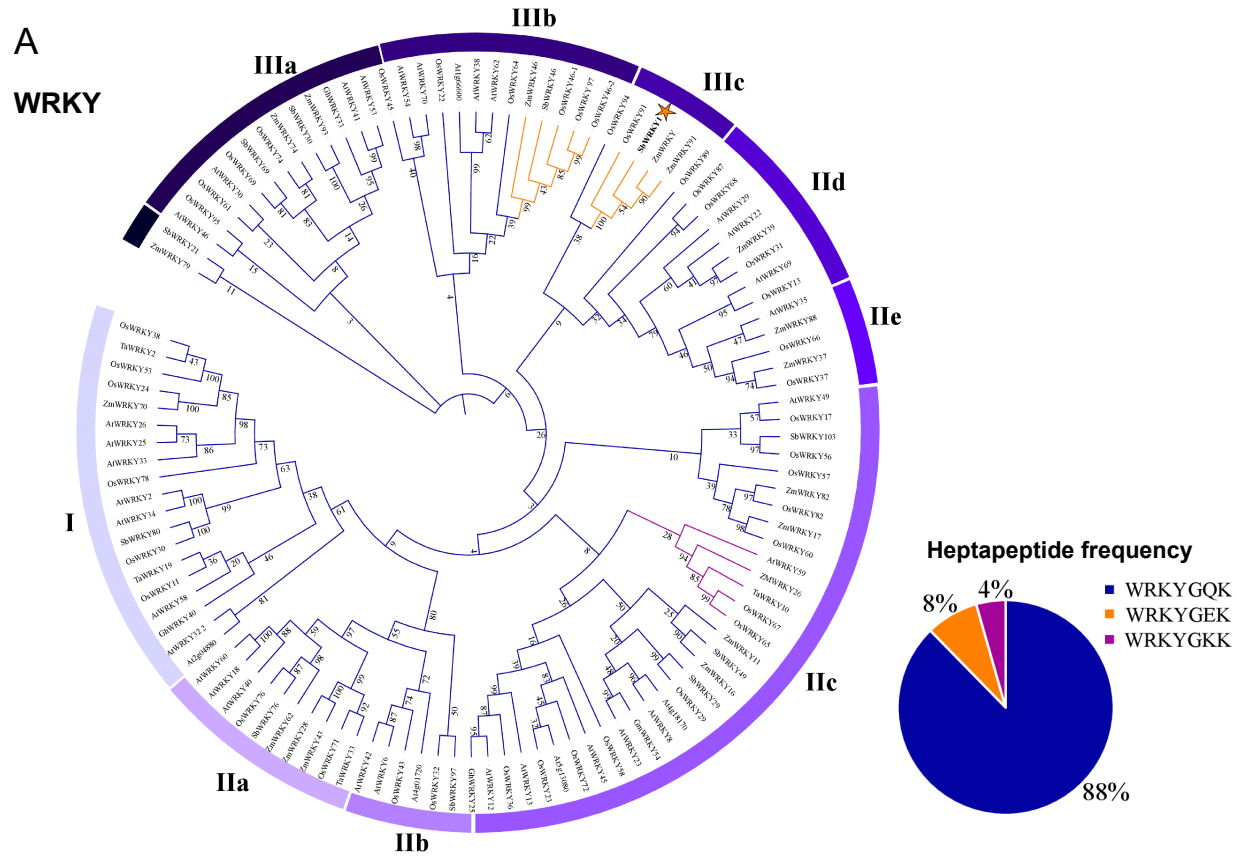
Main Figures



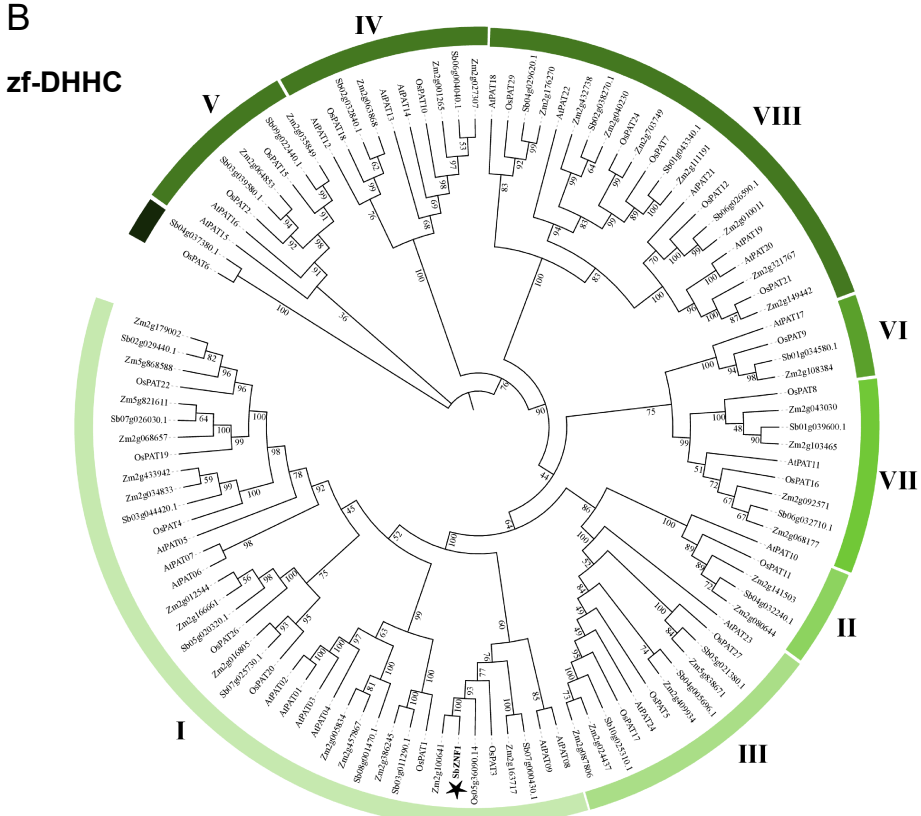
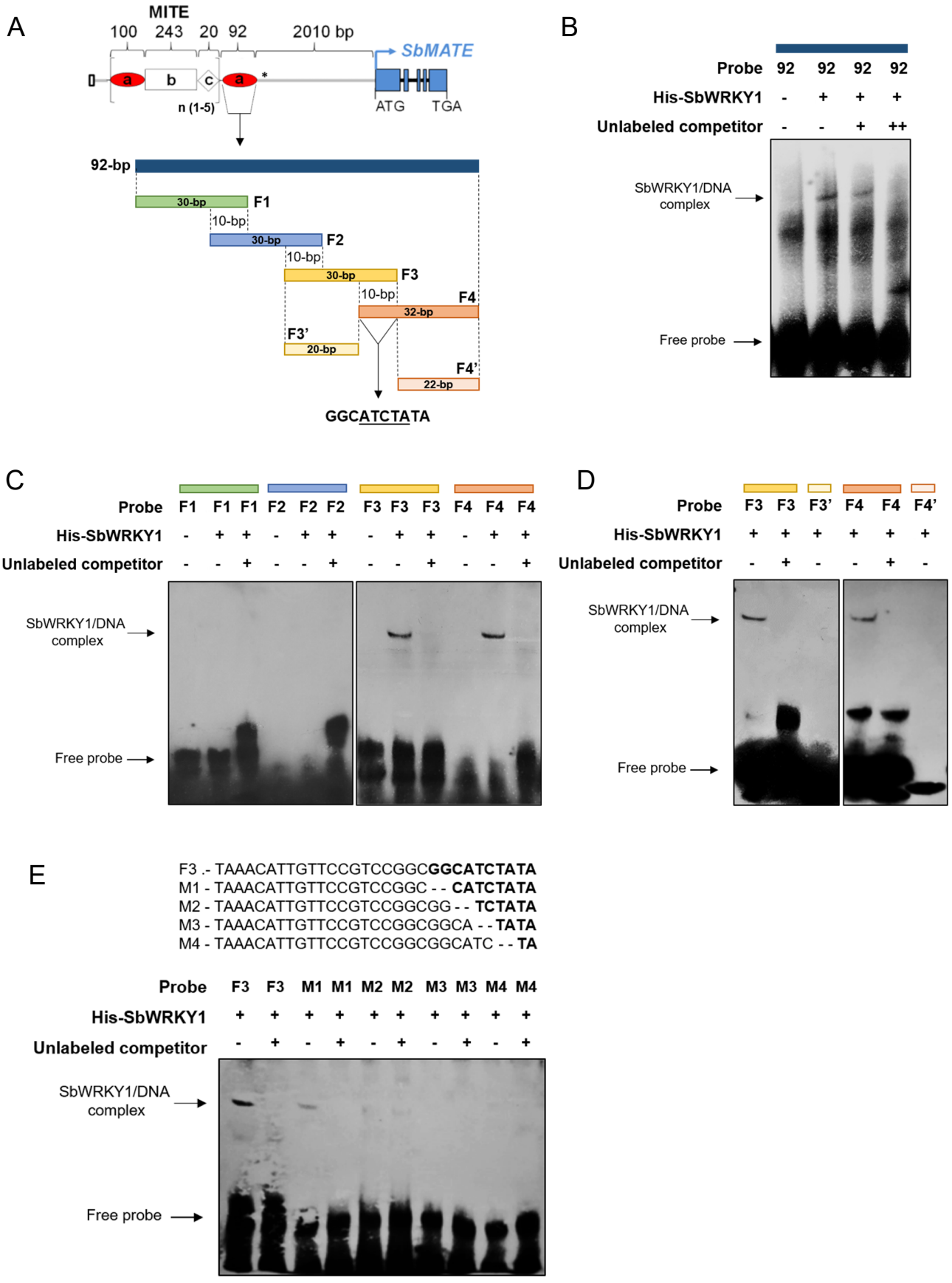


Figure 1: Phylogenetic analysis of WRKY and zf-DHHC proteins from sorghum, maize, rice and Arabidopsis.

The amino acid sequences of each protein family were aligned with the multiple sequence comparison by log-expectation algorithm (Muscle, Edgar, 2004). Unrooted phylogenetic reconstructions were implemented in the Mega X software (Kumar et al., 2018) using the maximum likelihood method and the Jones-Taylor-Thornton (JTT) +G (gamma rates) +F (empirical frequencies) amino acid substitution model. Bootstrap values from 1000 resampling steps are indicated in the branches. Accession numbers of all sequences are found in Supplemental Table 1 and 2.

(A) Phylogenetic tree of the WRKY proteins. WRKY proteins were grouped into the major groups I, II and III, as defined by Euglem et. al (2000). The group II was divided into five subgroups (IIa to IIe) whereas group III was divided into three subgroups (IIIa, IIIb, and IIIc). The outer ring in shades of purple depict WRKY groups and subgroups. The frequency of WRKY proteins containing variants of the WRKYGQK heptapeptide is shown in the pie chart and each of the three variants are represented by the respective colored branches in the phylogeny. SbWRKY1 has the WRKYGEK motif and it was classified into subgroup IIIc (depicted by an orange star). Amino acid sequences of 114 proteins were used.

(B) Phylogenetic tree of Zinc finger DHHC (zf-DHHC) domain-containing proteins. zf-DHHC proteins were grouped into I, II, III, IV, V, VI, VII, and VIII, as defined by Yuan et. al, (2013). The outer ring in shades of green indicates zf-DHHC groups as suggested by the phylogenetic analysis. SbZNF1 lies on group I (black star). Amino acid sequences of 112 proteins were used in the zf-DHHC phylogenetic reconstruction.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F4'
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GGCATCTATA
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His-SbWRKY1 - + + +
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Unlabeled competitor - + - - + -
SbWRKY1/DNA complex
Free probe
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F3 .- TAAACATTGTTCCGTCCGGCGGCATCTATA
M1 - TAAACATTGTTCCGTCCGGC - - CATCTATA
M2 - TAAACATTGTTCCGTCCGGCGG - - TCTATA
M3 - TAAACATTGTTCCGTCCGGCGGCA - - TATA
M4 - TAAACATTGTTCCGTCCGGCGGCATC - - TA
Probe F3 F3 M1 M1 M2 M2 M3 M3 M4 M4
His-SbWRKY1 + + + + + + + + + +
Unlabeled competitor - + - + - + - + - +
SbWRKY1/DNA complex
Free probe

Figure 2: Characterization of the SbWRKY1 binding site.

(A) Oligonucleotide probes used in SbWRKY1 electrophoretic mobility shift assays (EMSA) in the context of the MITE-containing repeats in the *SbMATE* promoter. The 243-bp MITE transposable element ("b" unit) is flanked by two sequences of 100-bp ("a" unit) and 20-bp ("c" unit), with the a-b-c triplet is repeated in tandem 1, 3, 4 and 5 times depending on the allele (n=1-5). The a-b-c triplet is followed by a single "a" unit with an 8-bp deletion, resulting in a 92-bp terminal sequence. SbWKRY1 has been previously found to bind both to the 100- and 92-bp "a" unit but not to the 2010-bp fragment between the *SbMATE* start codon and the MITE repeats (Melo et al. 2019). Four overlapping (10-bp) oligonucleotides each of 30- to 32-bp (F1 to F4) were designed covering the full-length 92-bp fragment, which was used as a positive control for SbWKRY1 binding. The asterisk downstream to the MITE insertion indicates the position of the negative control (60-bp, F5 probe within the 2010-bp sequence). Probes were also designed for fragments outside the 10-bp overlap (sequence shown) between probes F3 and F4 (F3'and F4'). EMSA was performed using biotin-labeled probes and a His-SUMO-SbWRKY1 fusion, designated as His-SbWRKY1 for simplicity. The nucleotides highlighted in the SbWRKY1 binding sequence represent a predicted *cis*-element for AtRAP2.2 DNA-interaction (Welsh et. al, 2007).

(B) SbWRKY1 specifically binds to the 92-bp probe. His-SbWRKY1 protein extract was incubated with the 92-bp probe, and the competitive binding assay with a molar excess of unlabeled 92-bp probe (50- "+" and 100-fold "++") revealed that SbWRKY1 binds specifically to the 92-bp fragment. His-SbWRKY1/DNA interaction is indicated (SbWRKY1/DNA complex). The experiment was repeated at least three times with similar results.

(C) SbWRKY1 binds to a *SbMATE* proximal fragment within the 92-bp sequence. His-SbWRKY1 protein extract incubated with probes F1, F2, F3, F4 revealed binding to both F3 and F4 probes but not with F1 and F2. 100-fold molar excess of the corresponding unlabeled probes were used as specific competitors in the binding reactions. The experiment was repeated three times with similar results.

(D) SbWRKY1 binds to the 10-bp sequence in the overlap between probes F3 and F4. Incubation of His-SbWRKY1 protein extract with probes eliminating the 10-bp sequence in the F3/F4 overlap, F3' and F4', did not yield a detectable binding signal, defining the SbWRKY1 binding sequence as GGCATCTATA. 100-fold molar excess of the corresponding unlabeled probes were used as specific competitors in the binding reactions. The experiment was repeated three times with similar results.

(E) Binding of SbWRKY1 to mutated probes. Oligonucleotides were designed to contain sequential, 2-bp deletions of the F3 probe (mutated, M1 to M4 probes, shown on top). The SbWRKY1 binding sequence is marked as bold letters within the F3 probe sequence and dashes (-) correspond to sequential

deletions of two adjacent nucleotides in each mutated probe. A 100-fold molar excess of the corresponding unlabeled probes were used as specific competitors in the binding reactions. The experiment was repeated twice with similar results.

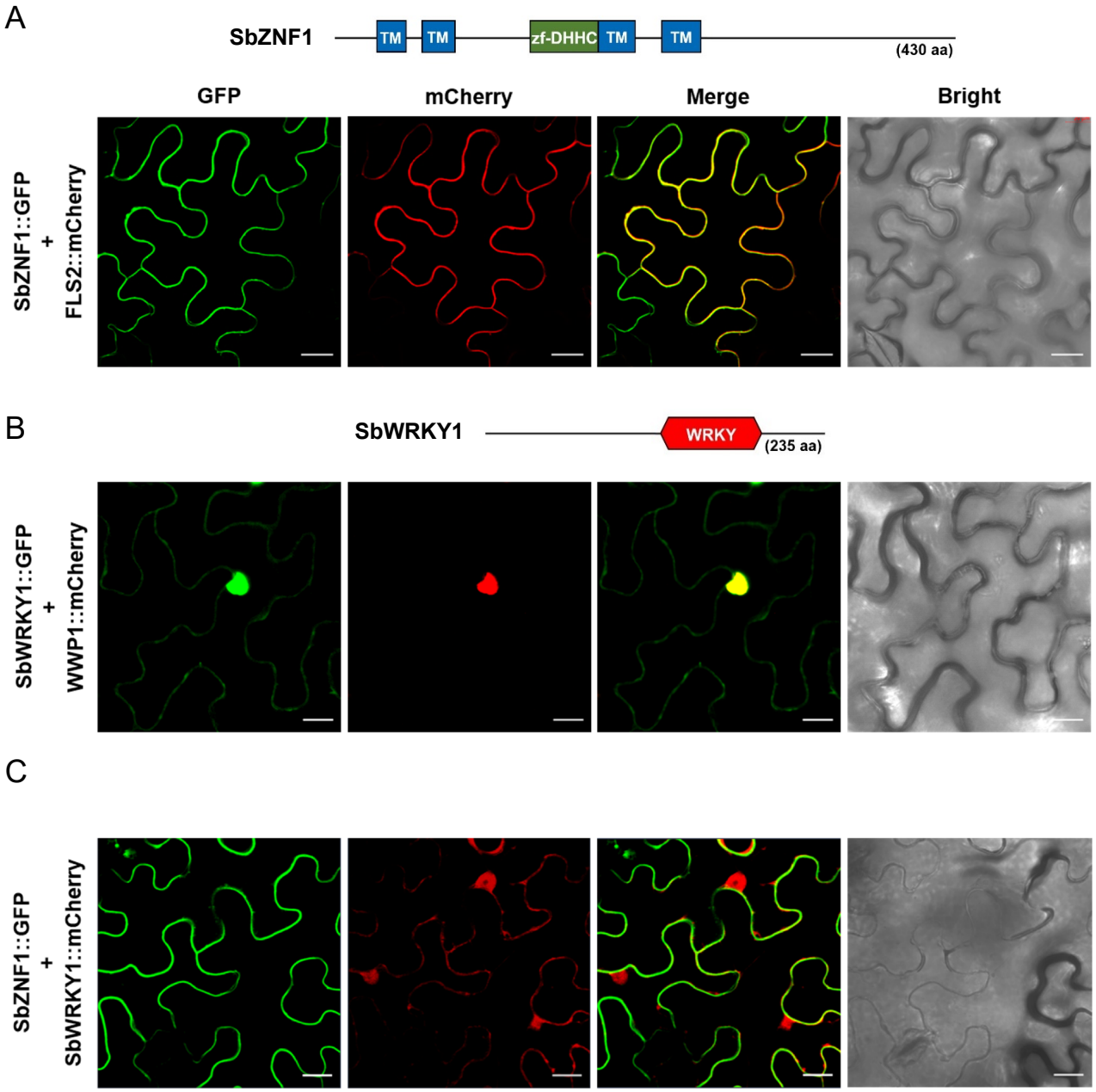


Figure 3: Subcellular localization of SbZNF1 and SbWRKY1 in *Nicotiana benthamiana* leaf epidermal cells.

N. benthamiana leaves were transiently co-transformed via *Agrobacterium tumefaciens* (GV3101) harboring translational reporter fusions with SbWRKY1 and SbZNF1 under the control of the CaMV 35S promoter. Images were captured by confocal scanning microscopy 72 hours after *Agrobacterium* infiltration. The schematics on top depict the domain prediction of SbZNF1 (A) and SbWRKY1 (B) obtained with InterPro (Mitchell et. al, 2019) and Phobius (Kall et. al, 2004) indicating predicted

transmembrane domains (TM) in SbZNF1. The experiments were repeated three times with similar results. Scale bars: 20 μm.

(A) Expression of SbZNF1::GFP and the plasma membrane marker, AtFLS2::mCherry. The subcellular localization of SbZNF1 in the plasma membrane, confirmed by its co-localization with AtFLS2::mCherry, is consistent with the presence of four predicted TM flanking the zf-DHHC domain. **(B)** Expression of SbWRKY1::GFP and the nuclear marker, AtWWP1::mCherry. SbWRKY1::GFP is co-localized in the nucleus with AtWWP1::mCherry and showed a weak signal in the cytosol.

(C) Co-expression of SbZNF1::GFP and SbWRKY1::mCherry. When both transcription factors were co-expressed, SbZNF1 persisted in the plasma membrane and SbWRKY1 remained predominantly in the nucleus and slightly in the cytosol.

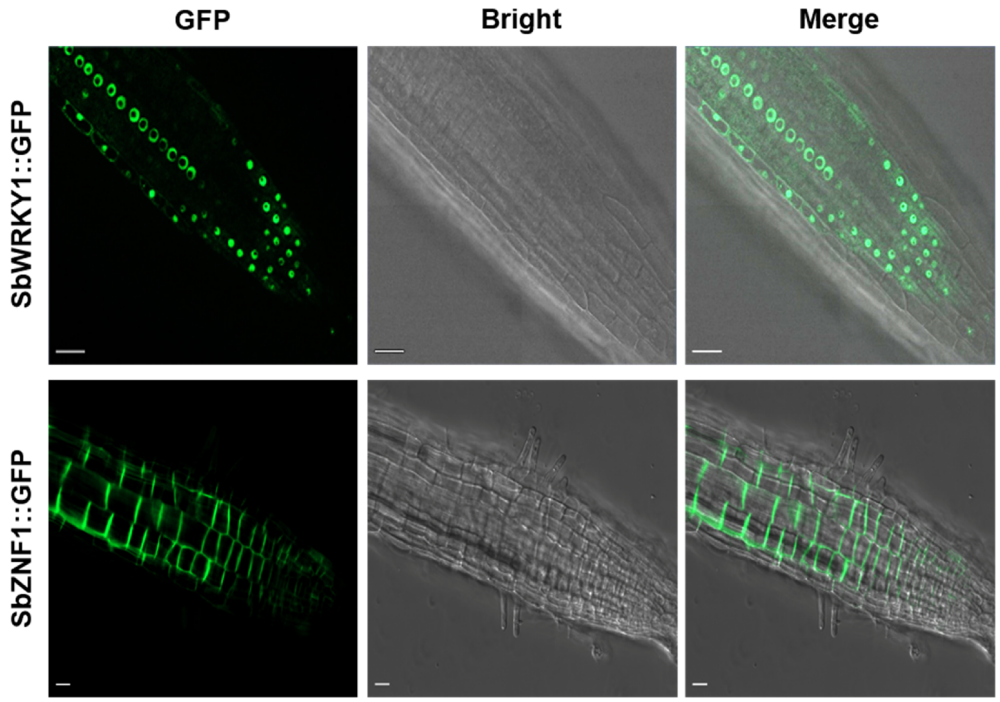
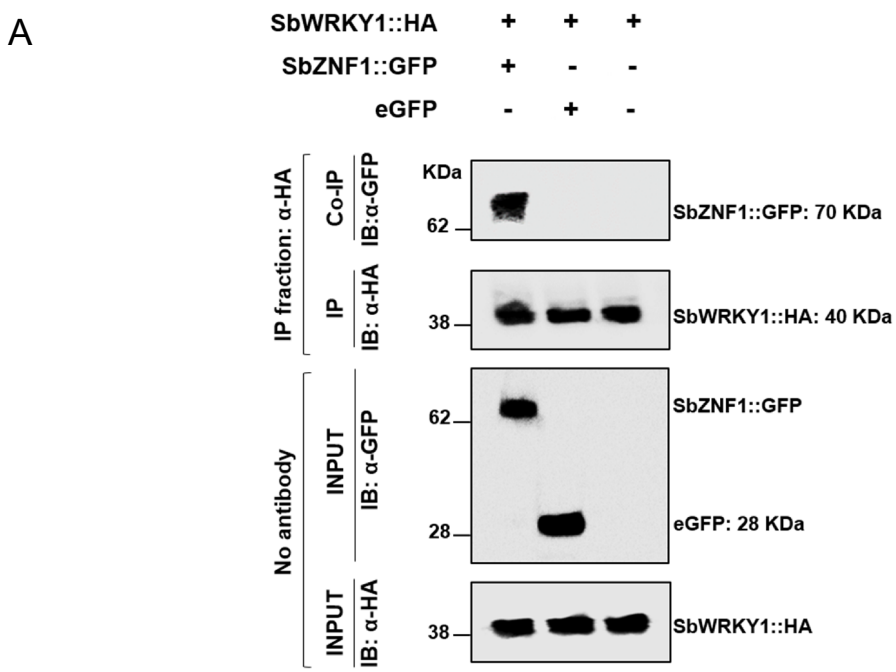


Figure 4: Subcellular localization of SbWRKY1::GFP and SbZNF1::GFP in Arabidopsis roots. Fluorescence images of 3-day old Arabidopsis seedlings stably transformed with either 35S::SbWRKY1::GFP (upper panel) or 35::SbZNF1::GFP (lower panel) confirmed the plasma membrane and nuclear localization of the respective TFs. Scale bars: 20 µm.


A
SbWRKY1::HA + + +
SbZNF1::GFP + - -
eGFP - + -
IP fraction: α-HA
Co-IP
IB:α-GFP
IP
IB: α-HA
No antibody
INPUT
IB: α-GFP
INPUT
IB: α-HA
KDa
62
38
62
28
38
SbZNF1::GFP: 70 KDa
SbWRKY1::HA: 40 KDa
SbZNF1::GFP
eGFP: 28 KDa
SbWRKY1::HA

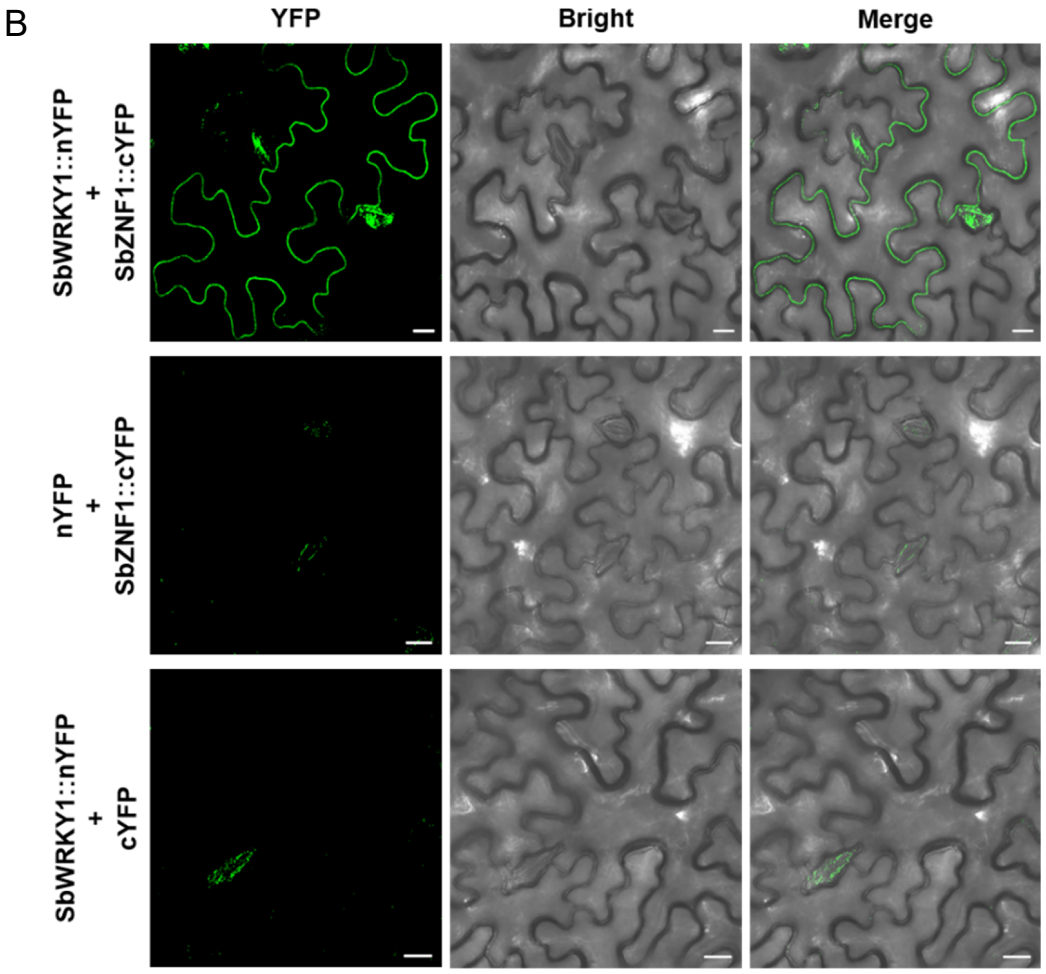

B
YFP
Bright
Merge
SbWRKY1::nYFP + SbZNF1::cYFP
nYFP + SbZNF1::cYFP
SbWRKY1::nYFP + cYFP

Figure 5: Molecular interaction between SbZNF1 and SbWRKY1.

(A) *In vivo* interaction assay between SbZNF1 and SbWRKY1 by co-immunoprecipitation (Co-IP). A C-terminal fusion between SbWRKY1 and human influenza hemagglutinin tag (SbWRKY1::HA) was co-expressed with either SbZNF1::GFP or an empty GFP vector (eGFP) in *Nicotiana benthamiana* leaves, via *A. tumefaciens* (GV3101) infiltration. Also, plants expressing only SbWRKY1::HA were used in the assay. Expression of all constructs was driven by the CaMV 35S promoter. Protein extracts from transformed plants were subject to immunoprecipitation with magnetic beads conjugated with an antibody raised against HA (anti-HA conjugated beads). After denaturing gel, the protein extracts were analyzed by immunoblotting (IB) with antibodies for α-GFP (anti-GFP), α-HA (anti-HA) conjugated to horseradish peroxidase (HRP). Total protein (input) was revealed by anti-HA and -GFP antibodies, and the immunoprecipitated (IP) was revealed by anti-HA antibody. The co-immunoprecipitated (Co-IP) was detected with anti-GFP revealing a band of molecular size of ~70 KDa, consistent with the molecular weight of SbZNF1::GFP, while no detectable signal was observed in plants lacking SbZNF1::GFP. This shows that SbZNF1 and SbWRKY1 interact. The experiment was repeated three times with similar results.

(B) *In vivo* interaction assay between SbZNF1 and SbWRKY1 by Bimolecular Fluorescence Complementation (BiFC). SbWRKY1 fused to the YFP N-terminus (SbWRKY1::nYFP), and SbZNF1 fused to YFP C-terminus (SbZNF1::cYFP) were transiently co-expressed in *N. benthamiana* leaves via *A. tumefaciens* (GV3101) infiltration. SbWRKY1::nYFP and SbZNF1::cYFP co-expressed with empty vectors expressing cYFP and nYFP, respectively, were used as controls (SbWRKY1::nYFP + cYFP and SbZNF1::cYFP + nYFP, respectively). Expression of all constructs was driven by CaMV 35S promoter. Images were captured 72h post transformation of tobacco epidermal cells via *A. tumefaciens* containing the constructs using a confocal laser scanning microscope. The YFP signal reconstituted by the interaction between SbWRKY1 and SbZNF1 (SbWRKY1::nYFP + SbZNF1::cYFP) was observed in the plasma membrane, while no interaction signal was detected in control transformations. The experiment was repeated at least three times with similar results. Scale bars: 20 μm.

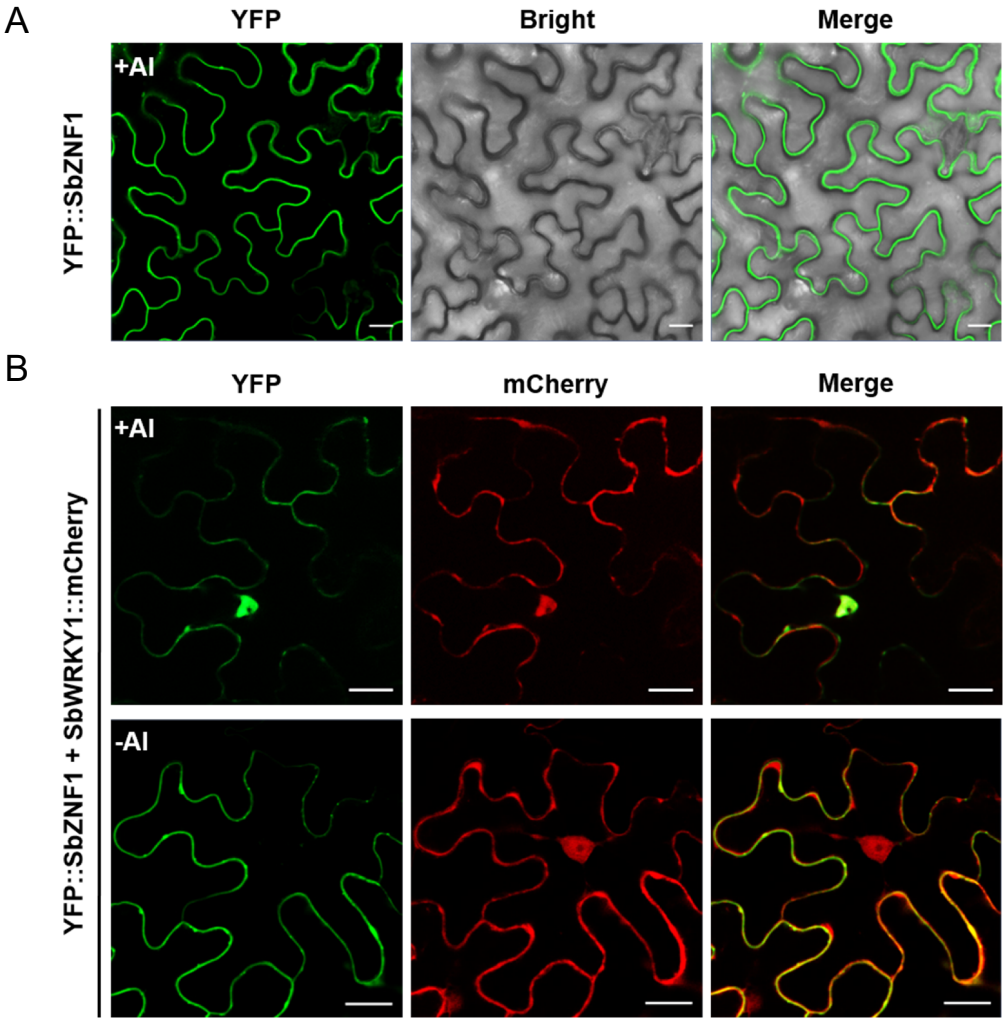


Figure 6: Subcellular localization of YFP::SbZNF1 in *N. benthamiana* leaves under Al^{+3} treatment. *N. benthamiana* leaves were transiently transformed via *A. tumefaciens* (GV3101) transformed with the constructs indicated in the left under the control of CaMV 35S promoter. Transfected leaves were infiltrated with a solution with (+Al: 10 µM $AlCl_3$, 100 µM $CaCl_2$; pH 5.0) and without (-Al: 100 µM $CaCl_2$; pH 5.0) Al. The images were captured by fluorescence microscopy 24h after Al treatment. The experiment was repeated twice with similar results. Scale bars: 20 µm.

(A) Subcellular localization of YFP::SbZNF1 under Al treatment. The canonical plasma membrane localization of YFP::SbZNF1 (Fig.3A) is not affected by Al treatment in plants expressing only YFP::SbZNF1.

(B) Subcellular localization of YFP::SbZNF1 in cells expressing both YFP::SbZNF1 and SbWRKY1::mCherry under Al treatment. The YFP::SbZNF1 signal in the nucleus is detected in plants co-expressing both TFs exposed to Al^{3+} (+Al), but not without Al (-Al).

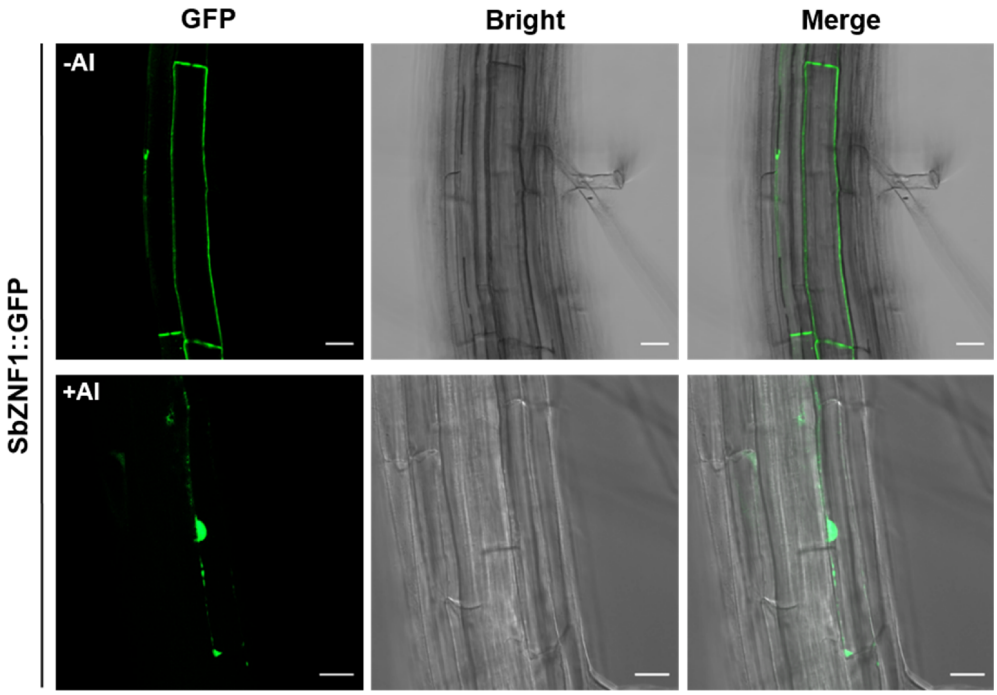
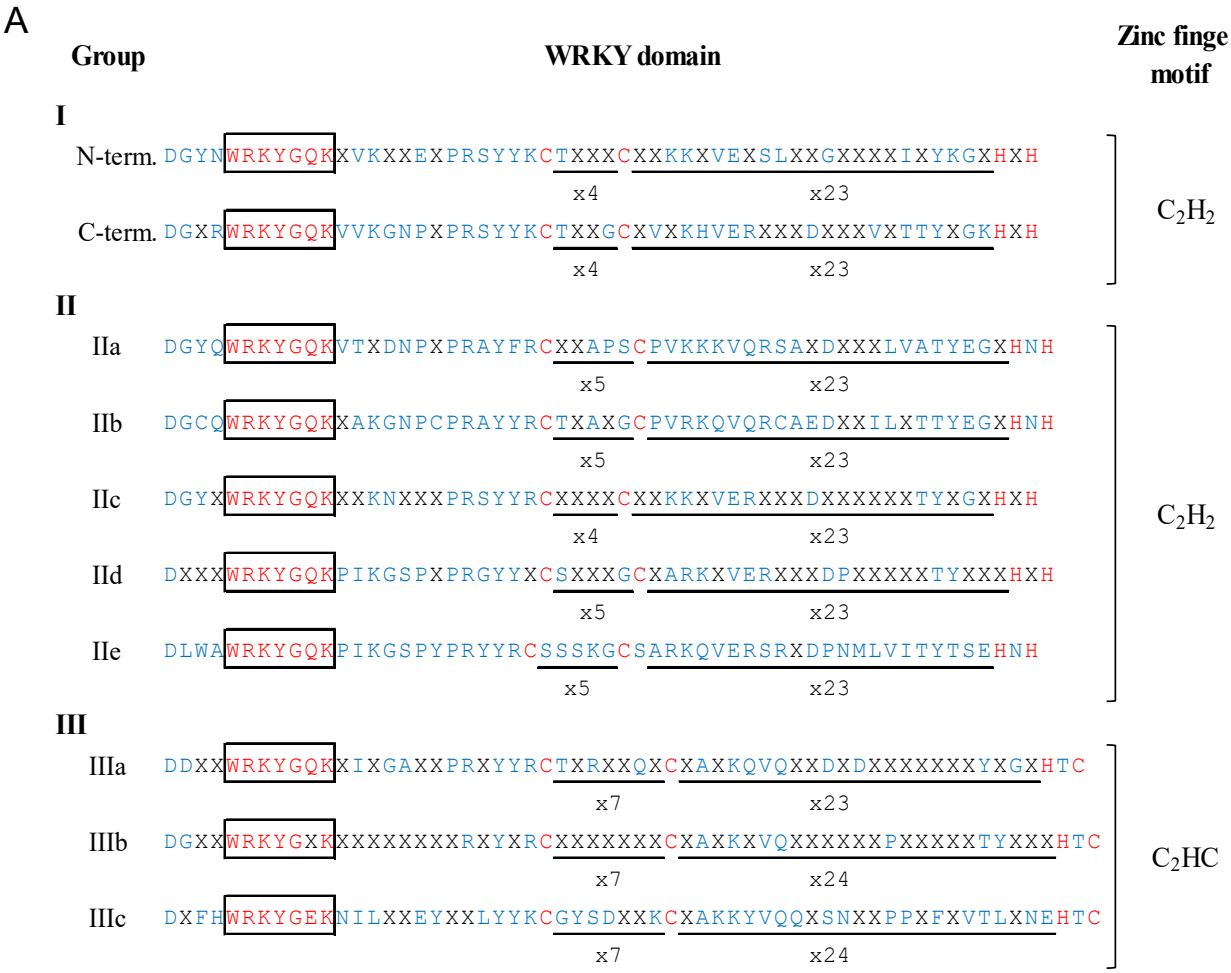
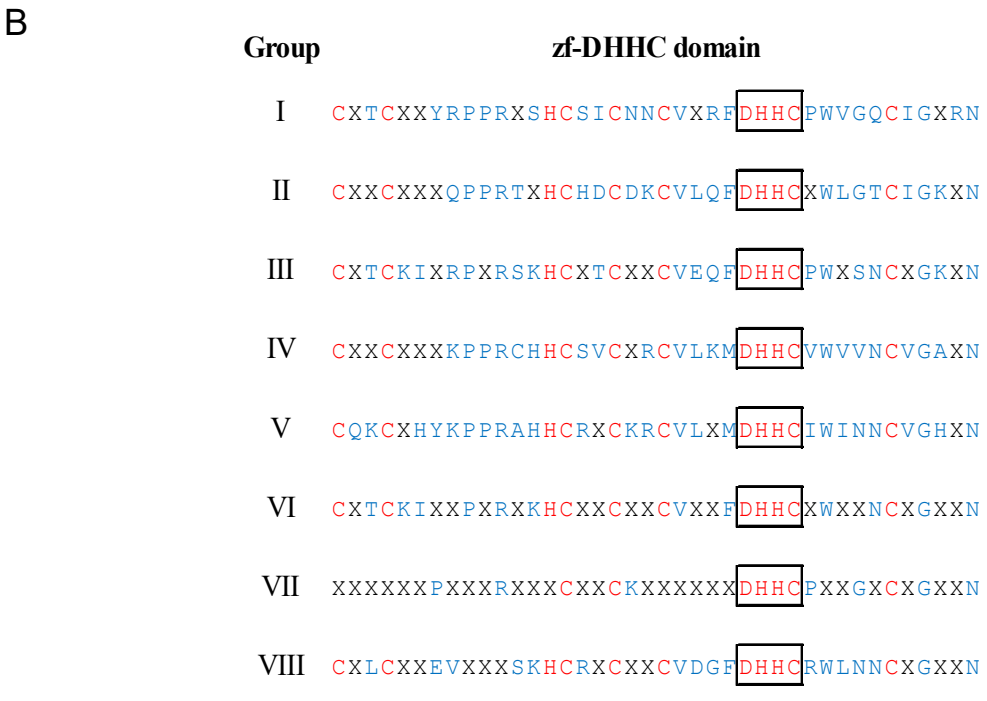


Figure 7: Subcellular localization of SbZNF1::GFP in Arabidopsis roots under Al^{3+} treatment. Arabidopsis seedlings stably transformed with 35S::SbZNF1::GFP were grown in hydroponic culture for 5 days and then transferred to nutrient solution with (+Al) or without Al (-Al). The images were captured by fluorescence microscopy 24h post Al treatment. SbZNF1::GFP was found in the nucleus only in plants exposed to 10 µM Al^{+3} (+Al), whereas the SbZNF1::GFP signal remained in the plasma membrane without Al (-Al). The experiment was repeated at least three times with similar results. Scale bars: 20 µm.

Supplemental figures



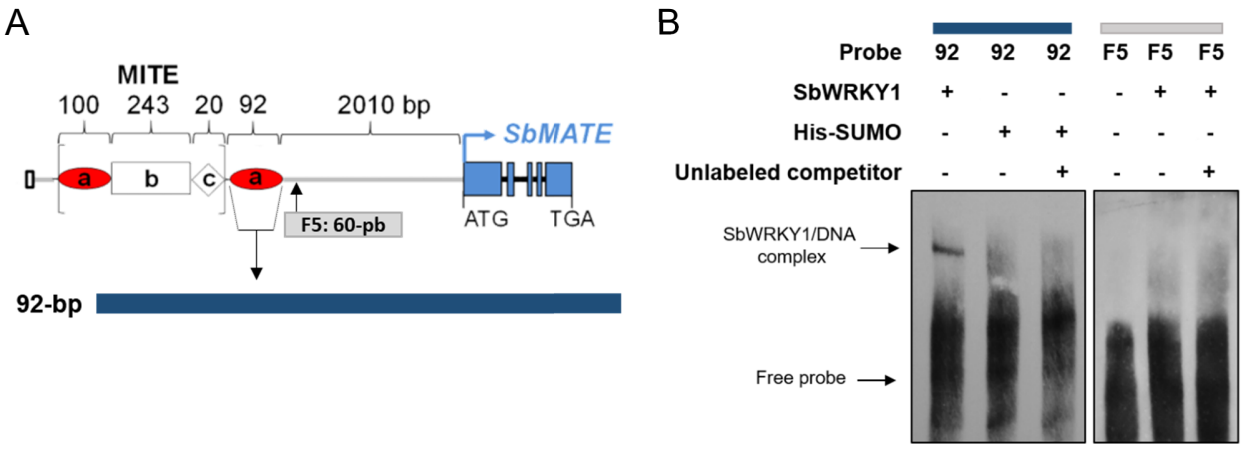


Supplemental Figure 1: Consensus amino acid sequence of the WRKY and zf-DHHC domains.

The WRKY and zf-DHHC domains from proteins used in phylogenetic analyzes were predicted *in silico* with the Motif software (https://www.genome.jp/tools/motif/). CLC Genomics Workbench 20.0 (https://digitalinsights.qiagen.com) was used to produce the consensus sequence from each phylogenetic groups and subgroups. The conserved amino acid residues (identity ≥ 80%) are represented in blue, and "X" indicates non-conserved amino acids (identity < 80%).

(A) Consensus sequence of the WRKY domain from each phylogenetic groups and subgroups. WRKY proteins from group I possess two WRKY domains and its consensus sequences are represented for the N-terminus (N-term.) and C-terminus (C-term.) domains. Proteins belonging to the group II and III harbor a single WRKY domain, whose position is shown for each subgroup. The WRKY-containing WRKYGQK heptapeptide and its variants are shown inside boxes. Cysteine and histidine residues that compose the zinc finger motif are marked in red, and the number of amino acids between these residues is indicated below the consensus sequences. On the right is depicted the type of zinc-finger motif found in each group.

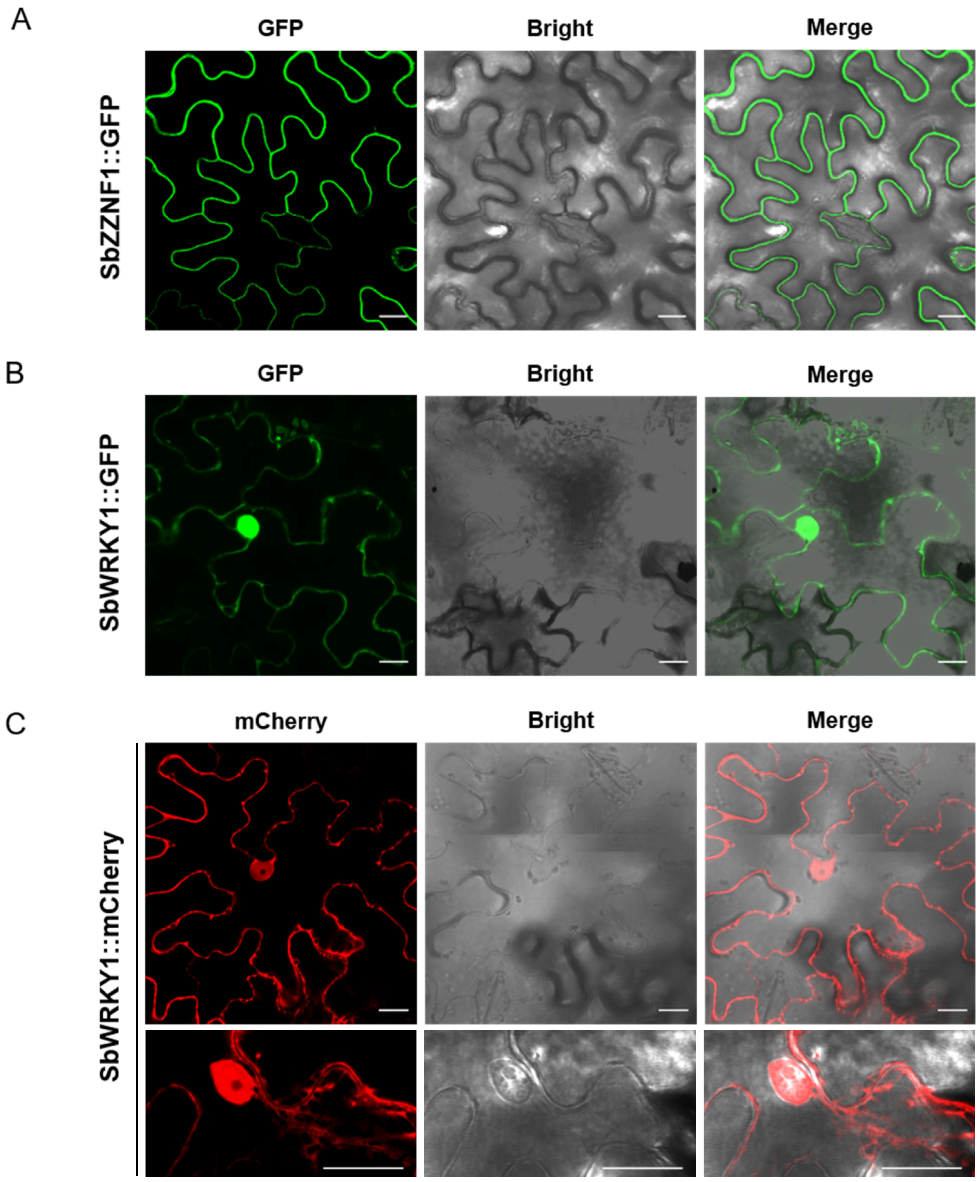
(B) Consensus amino acid sequence of the zf-DHHC domain from each phylogenetic group. The zf-DHHC motif (inside boxes) and the cysteine and histidine residues that comprise the zf-DHHC domain (marked in red) are shown. The canonical sequence of the zf-DHHC domain, C-X_2-C-X_9-HC-X_2-C-X_2-C-X_4-DHHC-X_5-C-X_4 (Putillina et. al, 2019), is conserved in all groups, except in group VII.



Supplemental Figure 2: Positive and negative controls used in the electrophoretic mobility shift assays.

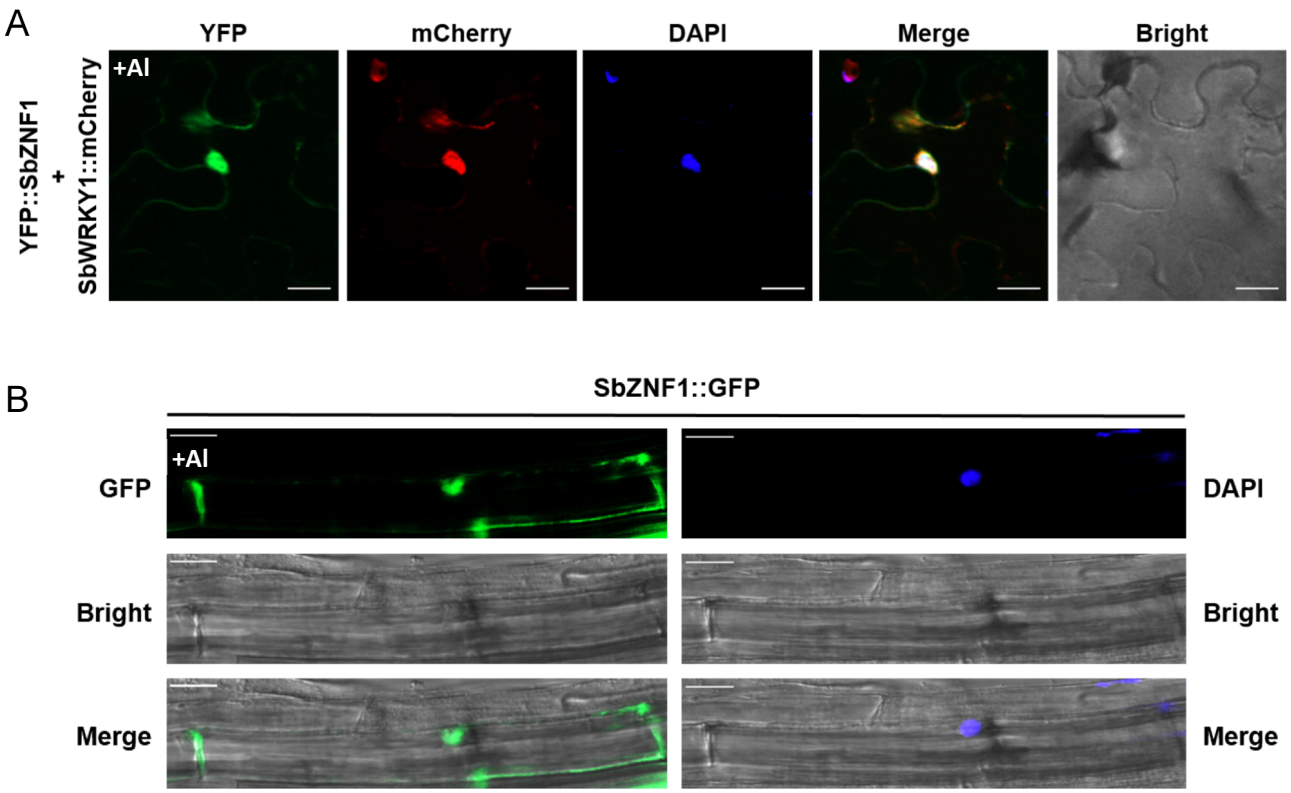
(A) Schematic design of the *SbMATE* promoter indicating the 92-bp probe and the 60-bp F5 probe, used as positive and negative controls for SbWRKY1 binding reactions, respectively. The 92-bp probe was previously shown to contain the SbWRKY1 binding site, and the F5 probe is located downstream to the MITE insertion, where SbWRKY1 did not show transactivation activity (Melo et. al, 2019).

(B) SbWRKY1 binding reactions with the positive and negative controls. 92-bp probe was incubated with either His-SbWRKY1 or His-SUMO protein extracts, and F5 probe was incubated with His-SbWRKY1. The binding signal produced by the interaction between His-SbWRKY1 and the 92-fragment probe, indicated as SbWRKY1/DNA complex, was not detected in the negative controls (His-SUMO incubated with the 92-bp probe, and His-SbWRKY1 incubated with the F5 probe). 100-fold molar excess of the corresponding unlabeled probes (unlabeled competitors) were used as specific competitor in the binding reactions.



Supplemental figure 3: Subcellular localization of SbZNF1 and SbWRKY1 in *N. benthamiana* epidermal cells.

N. benthamiana leaves were transiently transformed via *A. tumefaciens* (GV3101) harboring C-terminal, translational reporter fusions with SbWRKY1 and SbZNF1 under the control of the CaMV 35S promoter (indicated on left). The images were captured by confocal scanning microscopy 72h after *Agrobacterium* infiltration. **(A)** SbZNF1::GFP, **(B)** SbWRKY1::GFP and **(C)** SbWRKY1::mCherry. Medial optical section of leaf epidermal cells expressing SbWRKY1::mCherry (upper panel) and tangential optical section (lower panel), in which is more visible the weak cytosolic signal of SbWRKY1, and also its predominance in the nucleus. Scale bars: 20 µm.



Supplemental figure 4: DAPI staining of *N. benthamiana* and Arabidopsis subjected to Al treatment. **(A)** *N. benthamiana* leaves transiently co-transformed with 35S::YFP::SbZNF1 and 35S::SbWRKY1::mCherry fusions were subjected to Al treatment by infiltration of Al solution (10 µM $AlCl_3$, 100 µM $CaCl_2$; pH 5.0). After 24h of Al stress, the transfected leaves were stained with DAPI and visualized by fluorescence microscopy. Scale bars: 20 µm.

(B) Arabidopsis seedlings stably transformed with 35S::SbZNF1::GFP and grown in hydroponic culture for 5 days were transferred to nutritive solution containing Al. After 24h of Al stress, SbZNF1::GFP fluorescent signal was captured and the root was stained with DAPI to confirm the nuclear localization of SbZNF1.

Supplemental tables

Supplemental table 1: Genomic information for 114 WRKY proteins used in the phylogenetic reconstruction (Fig.1A)

Species			Access number	Gene Localization	Protein name	Group
Name	Genome Version	ID Phytozome[a]				
Arabidopsis thaliana	v13	167	AT2G38470.1	Chr2:16108361..16110766 forward	AtWRKY33	I
Arabidopsis thaliana	v13	167	AT2G30250.1	Chr2:12903236..12905198 reverse	AtWRKY25	I
Arabidopsis thaliana	v13	167	AT5G07100.1	Chr5:2204248..2205811 forward	AtWRKY26	I
Arabidopsis thaliana	v13	167	AT4G26440.1	Chr4:13357596..13359551 reverse	AtWRKY34	I
Arabidopsis thaliana	v13	167	AT2G30250.1	Chr2:12903236..12905198 reverse	AtWRKY2	I
Arabidopsis thaliana	v13	167	AT2G04880.1	Chr2:1717888..1720526 forward	AtWRKY1	I
Arabidopsis thaliana	v13	167	AT4G30935.1	Chr4:15051814..15054042 reverse	AtWRKY32	I
Arabidopsis thaliana	v13	167	AT3G01080.1	Chr3:25507..27449 forward	ATWRKY58	I
Gossypium hirsutum	v1.1	458	Gohir.D04G190600	D04:56442950..56446326 forward	GhWRKY40	I
Oryza sativa	v7.0	323	LOC_Os05g27730	Chr5:16150265..16152747 forward	OsWRKY38	I
Oryza sativa	v7.0	323	LOC_Os05g27730.1	Chr5:16150266..16152747 forward	OsWRKY53	I
Oryza sativa	v7.0	323	LOC_Os01g61080.1	Chr1:35347978..35350645 forward	OsWRKY24	I
Oryza sativa	v7.0	323	LOC_Os07g39480.1	Chr7:23654076..23659625 reverse	OsWRKY78	I
Oryza sativa	v7.0	323	LOC_Os08g38990.2	Chr8:24645880..24649829 reverse	OsWRKY30	I
Oryza sativa	v7.0	323	LOC_Os03g55164.1	Chr3:31391390..31399050 forward	OsWRKY11	I
Sorghum bicolor	v3.1.1	454	Sobic.002G242500.1	Chr02:63158788..63162729 reverse	SbWRKY80	I
Triticum aestivum	v2.2	296	Traes_1DS_A6733B734	ta_iwgsc_1ds_v1_750830:1463..3513 forward	TaWRKY2	I
Triticum aestivum	v2.2	296	Traes_2BS_380EC4D1E	ta_iwgsc_2bs_v1_5227909:9256..13095 reverse	TaWRKY19	I
Zea mays	Ensembl-18	-	GRMZM2G169966_T01	6:153458471..153461535 reverse	ZmWRKY70	I
Arabidopsis thaliana	v13	167	AT1G80840.1	Chr1:30383691..30385499 forward	AtWRKY40	II A
Arabidopsis thaliana	v13	167	AT4G31800.1	Chr4:15383201..15385029 forward	AtWRKY18	II A
Arabidopsis thaliana	v13	167	AT2G25000.1	Chr2:10629737..10631413 forward	AtWRKY60	II A
Oryza sativa	v7.0	323	LOC_Os02g08440.1	Chr2:4542762..4544983 forward	OsWRKY71	II A
Oryza sativa	v7.0	323	LOC_Os09g25060.1	Chr9:14975932..14977713 reverse	OsWRKY76	II A
Sorghum bicolor	v3.1.1	454	Sobic.002G202700.1	Chr02:59258884..59260622 reverse	SbWRKY76	II A
Triticum aestivum	v2.2	296	Traes_6AS_DA75BB1FD	ta_iwgsc_6as_v1_4428654:0..1588 reverse	TaWRKY33	II A
Zea mays	Ensembl-18	-	GRMZM2G111711_T01	9:104082393..104083964 forward	ZmWRKY43	II A
Zea mays	Ensembl-18	-	GRMZM2G111711_T01	9:104082393..104083964 forward	ZmWRKY28	II A
Zea mays	Ensembl-18	-	GRMZM2G169149_T01	7:115576479..115577711 reverse	ZmWRKY62	II A
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Arabidopsis thaliana	v13	167	AT4G04450.1	Chr4:2218379..2221114 forward	AtWRKY42	II B
Arabidopsis thaliana	v13	167	AT4G01720.1	Chr4:744921..748554 forward	AtWRKY47	II B
Oryza sativa	v7.0	323	LOC_Os05g49210.1	Chr5:28238562..28241041 forward	OsWRKY43	II B
Oryza sativa	v7.0	323	LOC_Os02g53100.1	Chr2:32489017..32495070 forward	OsWRKY32	II B
Sorghum bicolor	v3.1.1	454	Sobic.003G037500.1	Chr03:3520321..3522720 reverse	SbWRKY97	II B

Supplemental table 1: Genomic information for 114 WRKY proteins used in the phylogenetic reconstruction (Fig.1A)

Species			Access number	Gene Localization	Protein name	Group
Name	Genome Version	ID Phytozome[a]				
Arabidopsis thaliana	v13	167	AT5G43290.1	Chr5:17371838..17373201 reverse	AtWRKY49	II C
Oryza sativa	v7.0	323	LOC_Os08g17400.1	Chr8:10633195..10639603 reverse	OsWRKY82	II C
Oryza sativa	v7.0	323	LOC_Os03g45450.1	Chr3:25651039..25652125 reverse	OsWRKY60	II C
Oryza sativa	v7.0	323	LOC_Os01g74140.1	Chr1:42946753..42948750 forward	OSWRKY17	II C
Oryza sativa	v7.0	323	LOC_Os01g62514.1	Chr1:36193840..36194611 reverse	OSWRKY56	II C
Oryza sativa	v7.0	323	LOC_Os12g01180.1	Chr12:100799..104008 reverse	OSWRKY57	II C
Sorghum bicolor	v3.1.1	454	Sobic.002G008600.2	Chr02:791719..795938 forward	SbWRKY29	II C
Sorghum bicolor	v3.1.1	454	Sobic.003G353000.1	Chr03:67192266..67198503 reverse	SbWRKY103	II C
Zea mays	Ensembl-18	-	GRMZM2G549512_T01	4:58356325..58374672 forward	ZmWRKY82	II C
Zea mays	Ensembl-18	-	GRMZM2G149219_T01	1:259014756..259016110 forward	ZmWRKY17	II C
Arabidopsis thaliana	v13	167	AT4G18170.1	Chr4:10061373..10062841 forward	AtWRKY28	II C
Arabidopsis thaliana	v13	167	AT5G46350.1	Chr5:18801218..18804043 reverse	AtWRKY8	II C
Arabidopsis thaliana	v13	167	AT2G47260.1	Chr2:19404820..19407084 reverse	AtWRKY23	II C
Arabidopsis thaliana	v13	167	AT2G44745.1	Chr2:18447273..18449009 reverse	AtWRKY12	II C
Arabidopsis thaliana	v13	167	AT4G39410.1	Chr4:18332878..18334789 reverse	AtWRKY13	II C
Arabidopsis thaliana	v13	167	AT3G01970.1	Chr3:326397..327412 reverse	AtWRKY45	II C
Arabidopsis thaliana	v13	167	AT5G13080.1	Chr5:4149740..4151150 reverse	AtWRKY75	II C
Arabidopsis thaliana	v13	167	AT2G21900.1	Chr2:9334149..9336222 reverse	ATWRKY59	II C
Glycine max	v1	275	Glyma.10G011300.3	Chr10:1066745..1068878 reverse	GmWRKY54	II C
Gossypium hirsutum	v1.1	458	Gohir.D08G160500	D08:52685837..52688495 forward	GhWRKY25	II C
Oryza sativa	v7.0	323	LOC_Os05g09020.1	Chr5:4998210..4999626 reverse	OsWRKY67	II C
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Oryza sativa	v7.0	323	LOC_Os01g53260.1	Chr1:30604295..30608077 forward	OsWRKY23	II C
Sorghum bicolor	v3.1.1	454	Sobic.009G234100.1	Chr09:57342319..57345920 forward	SbWRKY49	II C
Triticum aestivum	v2.2	296	Traes_3B_7F3B35623	ta_iwgsc_3b_v1_2094363:1034..2153 reverse	TaWRKY10	II C
Zea mays	Ensembl-18	-	GRMZM2G101405_T01	3:203155290..203156282 forward	ZmWRKY26	II C
Zea mays	Ensembl-18	-	GRMZM2G151763_T01	3:211059226..211062376 forward	ZmWRKY16	II C
Zea mays	Ensembl-18	-	GRMZM2G145554_T01	8:145062095..145064464 forward	ZmWRKY11	II C

Supplemental table 1: Genomic information for 114 WRKY proteins used in the phylogenetic reconstruction (Fig.1A)

Species			Access number	Gene Localization	Protein name	Group
Name	Genome Version	ID Phytozome[a]				
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Arabidopsis thaliana	v13	167	AT4G11070.1	Chr4:6759303..6760794 forward	AtWRKY41	III A
Arabidopsis thaliana	v13	167	AT4G23810.1	Chr4:12392370..12393982 reverse	AtWRKY53	III A
Arabidopsis thaliana	v13	167	AT2G46400.1	Chr2:19043414..19044826 reverse	AtWRKY46	III A
Gossypium hirsutum	v1.1	458	Gohir.A12G235400	A12:99111544..99113491 reverse	GhWRKY33	III A
Oryza sativa	v7.0	323	LOC_Os09g16510.1	Chr9:10128825..10131136 forward	OsWRKY74	III A
Oryza sativa	v7.0	323	LOC_Os08g29660.1	Chr8:18220041..18222408 forward	OsWRKY69	III A
Oryza sativa	v7.0	323	LOC_Os11g45850.1	Chr11:27740142..27741375 forward	OsWRKY61	III A
Oryza sativa	v7.0	323	LOC_Os07g27670.1	Chr7:16147913..16149483 forward	OsWRKY95	III A
Sorghum bicolor	v3.1.1	454	Sobic.010G045700.1	Chr10:3566332..3570889 forward	SbWRKY30	III A
Sorghum bicolor	v3.1.1	454	Sobic.007G118301.1	Chr07:51192936..51194468 forward	SbWRKY69	III A
Zea mays	Ensembl-18	-	GRMZM2G163418_T01	2:176398481..176400987 forward	ZmWRKY74	III A
Zea mays	Ensembl-18	-	GRMZM2G003551_T01	9:18108182..18111635 forward	ZmWRKY93	III A
Arabidopsis thaliana	v13	167	AT2G40750.1	Chr2:17000454..17002468 reverse	AtWRKY54	III B
Arabidopsis thaliana	v13	167	AT3G56400.1	Chr3:20908928..20910481 reverse	AtWRKY70	III B
Arabidopsis thaliana	v13	167	AT1G66600.1	Chr1:24848320..24849364 forward	AtWRKY63	III B
Arabidopsis thaliana	v13	167	AT5G22570.1	Chr5:7495539..7496784 reverse	AtWRKY38	III B
Arabidopsis thaliana	v13	167	AT5G01900.1	Chr5:351008..352069 reverse	AtWRKY62	III B
Oryza sativa	v7.0	323	LOC_Os12g02420.2	Chr12:802489..806097 reverse	OsWRKY97	III B
Oryza sativa	v7.0	323	LOC_Os05g25770.1	Chr5:14991579..14993800 forward	OsWRKY45	III B
Oryza sativa	v7.0	323	LOC_Os12g02450.1	Chr12:824302..825793 reverse	OsWRKY64	III B
Oryza sativa	v7.0	323	LOC_Os01g60490.1	Chr1:34981468..34985447 forward	OsWRKY22	III B
Oryza sativa	v7.0	323	LOC_Os11g02480.1	Chr11:759587..763334 reverse	OsWRKY46_1	III B
Oryza sativa	v7.0	323	LOC_Os12g02420.1	Chr12:802489..806097 reverse	OsWRKY46_2	III B
Sorghum bicolor	v3.1.1	454	Sobic.008G029400.2	Chr08:2628801..2632242 forward	SbWRKY46	III B
Zea mays	Ensembl-18	-	GRMZM2G063216_T01	4:184812978..184816591 reverse	ZmWRKY46	III B
Oryza sativa	v7.0	323	LOC_Os07g17230.1	Chr7:10155452..10159775 reverse	OsWRKY94	III C
Oryza sativa	v7.0	323	LOC_Os05g40070.1	Chr5:23536113..23539013 reverse	OsWRKY91	III C
Sorghum bicolor	v3.1.1	454	Sobic.009G174300.1	Chr09:52963804..52966801 reverse	SbWRKY1	III C
Zea mays	Ensembl-18	-	GRMZM2G034421_T01	8:118496550..118498937 reverse	ZmWRKY	III C
Zea mays	Ensembl-18	-	GRMZM2G034421_T01	8:118496550..118498937 reverse	ZmWRKY91	III C
Sorghum bicolor	v3.1.1	454	Sobic.003G337900.1	Chr03:66104667..66107290 forward	SbWRKY21	-
Zea mays	Ensembl-18	-	GRMZM2G127064_T01	:135889651..135892588 forward	ZmWRKY79	-

[a] The data were downloaded from the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html)

Supplemental table 2: Genomic information zf-DHHC proteins used in the phylogenetic reconstruction (Fig.1B)

Species			Access number	Gene Localization	Protein name	Group
Name	Genome Version	ID Phytozome[a]				
Arabidopsis thaliana	v13	167	AT3G56920.1	Chr3:21070648..21072702 forward	AtPAT01	I
Arabidopsis thaliana	v13	167	AT2G40990.1	Chr2:17105097..17106773 reverse	AtPAT02	I
Arabidopsis thaliana	v13	167	AT5G05070.1	Chr5:1496855..1498544 forward	AtPAT03	I
Arabidopsis thaliana	v13	167	AT3G56930.1	Chr3:21073495..21076314 forward	AtPAT04	I
Arabidopsis thaliana	v13	167	AT3G48760.1	Chr3:18075794..18078291 forward	AtPAT05	I
Arabidopsis thaliana	v13	167	AT5G41060.1	Chr5:16435322..16437699 forward	AtPAT06	I
Arabidopsis thaliana	v13	167	AT3G26935.1	Chr3:9932600..9935499 reverse	AtPAT07	I
Arabidopsis thaliana	v13	167	AT4G24630.1	Chr4:12714624..12717111 forward	AtPAT08	I
Arabidopsis thaliana	v13	167	AT5G50020.1	Chr5:20351342..20354436 forward	AtPAT09	I
Oryza sativa	v7.0	323	LOC_Os01g17160.1	Chr1:9866424..9870864 forward	OsPAT1	I
Oryza sativa	v7.0	323	LOC_Os01g64810.1	Chr1:37615955..37622095 reverse	OsPAT3	I
Oryza sativa	v7.0	323	LOC_Os01g70100.1	Chr1:40566660..40569777 reverse	OsPAT4	I
Oryza sativa	v7.0	323	LOC_Os05g36090.1	Chr5:21388694..21392697 forward	Os05g36090.14	I
Oryza sativa	v7.0	323	LOC_Os08g42370.1	Chr8:26744794..26748485 reverse	OsPAT19	I
Oryza sativa	v7.0	323	LOC_Os08g42620.2	Chr8:26938776..26942169 reverse	OsPAT20	I
Oryza sativa	v7.0	323	LOC_Os09g33450.1	Chr9:19702022..19706618 reverse	OsPAT22	I
Oryza sativa	v7.0	323	LOC_Os11g32960.1	Chr11:19481652..19484228 reverse	OsPAT26	I
Sorghum bicolor	v3.1.1	454	Sobic.009G151400.1	Chr09:50778337..50784906 forward	SbZNF1	I
Sorghum bicolor	v3.1.1	454	Sobic.002G259400.1	Chr02:64465689..64471473 reverse	Sb02g029440.1	I
Sorghum bicolor	v3.1.1	454	Sobic.003G132400.1	Chr03:12462060..12464589 reverse	Sb03g011290.1	I
Sorghum bicolor	v3.1.1	454	Sobic.003G409600.1	Chr03:71705833..71709037 reverse	Sb03g044420.1	I
Sorghum bicolor	v3.1.1	454	Sobic.005G138900.1	Chr05:59284736..59286829 reverse	Sb05g020320.1	I
Sorghum bicolor	v3.1.1	454	Sobic.007G002600.2	Chr07:245788..250267 reverse	Sb07g000430.1	I
Sorghum bicolor	v3.1.1	454	Sobic.007G187400.1	Chr07:62029386..62032817 forward	Sb07g025730.1	I
Sorghum bicolor	v3.1.1	454	Sobic.007G191100.1	Chr07:62379819..62395454 forward	Sb07g026030.1	I
Sorghum bicolor	v3.1.1	454	Sobic.008G016800.1	Chr08:1373530..1377736 reverse	Sb08g001470.1	I
Zea mays	Ensembl-18	-	GRMZM2G068657_T01	1:195026666..195030944 reverse	Zm2g068657	I
Zea mays	Ensembl-18	-	GRMZM2G179002_T03	2:193070917..193077187 reverse	Zm2g179002	I
Zea mays	Ensembl-18	-	GRMZM2G166661_T01	2:224815007..224816975 reverse	Zm2g166661	I
Zea mays	Ensembl-18	-	GRMZM2G386245_T01	3:45389414..45391791 forward	Zm2g386245	I
Zea mays	Ensembl-18	-	GRMZM2G034833_T01	3:157689786..157693067 forward	Zm2g034833	I
Zea mays	Ensembl-18	-	GRMZM2G457867_T01	3:230007030..230008371 forward	Zm2g457867	I
Zea mays	Ensembl-18	-	GRMZM2G005834_T01	3:230084075..230087764 reverse	Zm2g005834	I
Zea mays	Ensembl-18	-	GRMZM5G821611_T01	4:46647187..46652116 reverse	Zm5g821611	I
Zea mays	Ensembl-18	-	GRMZM2G012544_T01	4:192281003..192283039 forward	Zm2g012544	I
Zea mays	Ensembl-18	-	GRMZM2G016805_T01	4:193529418..193532950 reverse	Zm2g016805	I
Zea mays	Ensembl-18	-	GRMZM2G163717_T01	4:195964866..195968913 reverse	Zm2g163717	I
Zea mays	Ensembl-18	-	GRMZM2G100641_T01	6:150498189..150502572 reverse	Zm2g100641	I
Zea mays	Ensembl-18	-	GRMZM5G868588_T01	7:136050602..136057068 forward	Zm5g868588	I
Zea mays	Ensembl-18	-	GRMZM2G433942_T01	8:159987625..159991190 forward	Zm2g433942	I

Supplemental table 2: Genomic information zf-DHHC proteins used in the phylogenetic reconstruction (Fig.1B)

Species			Access number	Gene Localization	Protein name	Group
Name	Genome Version	ID Phytozome[a]				
Arabidopsis thaliana	v13	167	AT3G51390.1	Chr3:19075576..19078120 forward	AtPAT10	II
Oryza sativa	v7.0	323	LOC_Os04g47410.1	Chr4:28131106..28136550 forward	OsPAT11	II
Sorghum bicolor	v3.1.1	454	Sobic.004G286500.2	Chr04:62885138..62890137 reverse	Sb04g032240.1	II
Zea mays	Ensembl-18	-	GRMZM2G080644_T01	4:154449829..154456617 reverse	Zm2g080644	II
Zea mays	Ensembl-18	-	GRMZM2G141503_T01	5:193965292..193970084 forward	Zm2g141503	II
Arabidopsis thaliana	v13	167	AT2G14255.1	Chr2:6036974..6040978 forward	AtPAT23	III
Arabidopsis thaliana	v13	167	AT5G20350.1	Chr5:6876589..6881270 forward	AtPAT24	III
Oryza sativa	v7.0	323	LOC_Os02g09130.1	Chr2:4681347..4689703 reverse	OsPAT5	III
Oryza sativa	v7.0	323	LOC_Os06g43680.1	Chr6:26296679..26303260 forward	OsPAT17	III
Oryza sativa	v7.0	323	LOC_Os11g34860.1	Chr11:20425839..20432265 forward	OsPAT27	III
Sorghum bicolor	v3.1.1	454	Sobic.004G068100.1	Chr04:5538726..5545216 reverse	Sb04g005696.1	III
Sorghum bicolor	v3.1.1	454	Sobic.005G149500.1	Chr05:61781935..61788849 forward	Sb05g021380.1	III
Sorghum bicolor	v3.1.1	454	Sobic.010G205800.1	Chr10:54888681..54896125 forward	Sb10g025310.1	III
Zea mays	Ensembl-18	-	GRMZM5G838671_T01	2:225763933..225765876 reverse	Zm5g838671	III
Zea mays	Ensembl-18	-	GRMZM2G409934_T01	5:95002877..95010724 forward	Zm2g409934	III
Zea mays	Ensembl-18	-	GRMZM2G024437_T01	6:97038589..97045549 reverse	Zm2g024437	III
Zea mays	Ensembl-18	-	GRMZM2G087806_T01	9:95033444..95044390 reverse	Zm2g087806	III
Arabidopsis thaliana	v13	167	AT4G00840.1	Chr4:355258..357279 reverse	AtPAT12	IV
Arabidopsis thaliana	v13	167	AT4G22750.1	Chr4:11949180..11951424 reverse	AtPAT13	IV
Arabidopsis thaliana	v13	167	AT3G60800.1	Chr3:22467173..22469592 reverse	AtPAT14	IV
Oryza sativa	v7.0	323	LOC_Os03g58960.1	Chr3:33570513..33576145 reverse	OsPAT10	IV
Oryza sativa	v7.0	323	LOC_Os07g28460.1	Chr7:16650377..16657246 forward	OsPAT18	IV
Sorghum bicolor	v3.1.1	454	Sobic.002G297600.1	Chr02:67378582..67383696	Sb02g032840.1	IV
Sorghum bicolor	v3.1.1	454	Sobic.006G034000.1	Chr06:8976562..8982383 forward	Sb06g004040.1	IV
Zea mays	Ensembl-18	-	GRMZM2G027307_T01	2:86977315..86991334	Zm2g027307	IV
Zea mays	Ensembl-18	-	GRMZM2G063868_T03	2:199307780..199316619 reverse	Zm2g063868	IV
Zea mays	Ensembl-18	-	GRMZM2G001265_T01	10:103214136..103232644 forward	Zm2g001265	IV
Arabidopsis thaliana	v13	167	AT5G04270.1	Chr5:1182718..1184765 reverse	AtPAT15	V
Sorghum bicolor	v3.1.1	454	Sobic.003G353400.1	Chr03:67225322..67229166 reverse	Sb03g039580.1	V
Arabidopsis thaliana	v13	167	AT3G09320.1	Chr3:2861743..2864245 reverse	AtPAT16	V
Oryza sativa	v7.0	323	LOC_Os01g62620.1	Chr1:36257435..36261729 reverse	OsPAT2	V
Oryza sativa	v7.0	323	LOC_Os05g38360.2	Chr5:22488533..22492117 forward	OsPAT15	V
Sorghum bicolor	v3.1.1	454	Sobic.009G162100.1	Chr09:51904009..51907488 forward	Sb09g022440.1	V
Zea mays	Ensembl-18	-	GRMZM2G064853_T01	3:180553262..180560928 forward	Zm2g064853	V
Zea mays	Ensembl-18	-	GRMZM2G035849_T02	6:151669428..151677880 forward	Zm2g035849	V
Zea mays	Ensembl-18	-	GRMZM2G108384_T01	9:128116054..128129443 reverse	Zm2g108384	VI
Arabidopsis thaliana	v13	167	AT3G04970.1	Chr3:1376175..1378500 forward	AtPAT17	VI
Oryza sativa	v7.0	323	LOC_Os03g24900.1	Chr3:14183628..14188181 forward	OsPAT9	VI
Sorghum bicolor	v3.1.1	454	Sobic.010G205800.1	Chr10:54888681..54896125 forward	Sb01g034580.1	VI

Supplemental table 2: Genomic information zf-DHHC proteins used in the phylogenetic reconstruction (Fig.1B)

Species			Access number	Gene Localization	Protein name	Group
Name	Genome Version	ID Phytozome[a]				
Oryza sativa	v7.0	323	LOC_Os03g16790.1	Chr3:9307510..9312680 forward	OsPAT8	VII
Oryza sativa	v7.0	323	LOC_Os06g20400.5	Chr6:11718878..11722902 reverse	OsPAT16	VII
Sorghum bicolor	v3.1.1	454	Sobic.001G420800.2	Chr01:70178887..70185315 reverse	Sb01g039600.1	VII
Sorghum bicolor	v3.1.1	454	Sobic.006G264500.1	Chr06:59871647..59875192 forward	Sb06g032710.1	VII
Zea mays	Ensembl-18	-	GRMZM2G068177_T01	2:1973624..1977075 reverse	Zm2g068177	VII
Zea mays	Ensembl-18	-	GRMZM2G103465_T01	9:139913720..139922927 reverse	Zm2g103465	VII
Zea mays	Ensembl-18	-	GRMZM2G092571_T03	10:147332167..147334201 forward	Zm2g092571	VII
Arabidopsis thaliana	v13	167	AT3G18620.1	Chr3:6408318..6411064 forward	AtPAT11	VII
Zea mays	Ensembl-18	-	GRMZM2G043030_T01	1:40649116..40654273 forward	Zm2g043030	VII
Arabidopsis thaliana	v13	167	AT4G01730.1	Chr4:749574..752034 forward	AtPAT18	VIII
Arabidopsis thaliana	v13	167	AT3G22180.1	Chr3:7827036..7830813 forward	AtPAT20	VIII
Arabidopsis thaliana	v13	167	AT2G33640.1	Chr2:14239213..14242365 forward	AtPAT21	VIII
Arabidopsis thaliana	v13	167	AT1G69420.2	Chr1:26093196..26096907 forward	AtPAT22	VIII
Oryza sativa	v7.0	323	LOC_Os03g11110.1	Chr3:5710186..5715012 reverse	OsPAT7	VIII
Oryza sativa	v7.0	323	LOC_Os04g49560.1	Chr4:29556866..29563246 reverse	OsPAT12	VIII
Oryza sativa	v7.0	323	LOC_Os08g44230.1	Chr8:27842259..27847633 forward	OsPAT21	VIII
Oryza sativa	v7.0	323	LOC_Os10g19180.1	Chr10:9770755..9776538 forward	OsPAT24	VIII
Sorghum bicolor	v3.1.1	454	Sobic.001G461800.1	Chr01:73654364..73659162 forward	Sb01g043340.1	VIII
Sorghum bicolor	v3.1.1	454	Sobic.006G193100.1	Chr06:54661163..54667555 reverse	Sb06g026590.1	VIII
Zea mays	Ensembl-18	-	GRMZM2G111191_T01	1:20989320..20994288 forward	Zm2g111191	VIII
Zea mays	Ensembl-18	-	GRMZM2G149442_T01	1:205605815..205610074 reverse	Zm2g149442	VIII
Zea mays	Ensembl-18	-	GRMZM2G010011_T01	2:14450181..14459904 forward	Zm2g010011	VIII
Zea mays	Ensembl-18	-	GRMZM2G321767_T01	2:167126682..167132106 reverse	Zm2g321767	VIII
Zea mays	Ensembl-18	-	GRMZM2G040230_T01	2:209860388..209888481 reverse	Zm2g040230	VIII
Zea mays	Ensembl-18	-	GRMZM2G432738_T01	7:162993180..162999021 reverse	Zm2g432738	VIII
Arabidopsis thaliana	v13	167	AT4G15080.1	Chr4:8608700..8612693 reverse	AtPAT19	VIII
Oryza sativa	v7.0	323	LOC_Os12g16210.1	Chr12:9274091..9276999 reverse	OsPAT29	VIII
Sorghum bicolor	v3.1.1	454	Sobic.002G362700.1	Chr02:72370223..72375420 reverse	Sb02g038270.1	VIII
Sorghum bicolor	v3.1.1	454	Sobic.004G257600.1	Chr04:60342867..60345829 reverse	Sb04g029620.1	VIII
Zea mays	Ensembl-18	-	GRMZM2G703749_T01	1:138022108..138030200 forward	Zm2g703749	VIII
Zea mays	Ensembl-18	-	GRMZM2G176270_T01	5:203852846..203861024 forward	Zm2g176270	VIII
Oryza sativa	v7.0	323	LOC_Os02g57370.1	Chr2:35155661..35158722 forward	OsPAT6	-
Sorghum bicolor	v3.1.1	454	Sobic.004G347100.1	Chr04:67661968..67664722 forward	Sb04g037380.1	-

[a] The data were downloaded from the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html)

Supplemental Table 3: Primers and probe sequences

Primer/Probe	Identification	Primer/Probe sequence (5'-3')	Aplication
Primers	SbWRKY1_1Fw	ATGACGCTGTCTCCACCGCA	SbWRKY1 cloning into pETSUMO
	SbWRKY1_1Rv	CTAGTACCCATCATACGAAA	SbWRKY1 cloning into pETSUMO
	SbZNF1_1Fw	ATGGCGCGTCCGCAGCGAGT	SbZNF1 cloning into pETSUMO
	SbZNF1_1Rv	CTAGTGAGTCTCTGCTAGAG	SbZNF1 cloning into pETSUMO
	SbWRKY1_2Fw	AAAAAGCAGGCTTCATGACGCTGTCTCCAC	SbWRKY1 cloning into pDONR207 - without stop codon
	SbWRKY1_2Rv	AGAAAGCTGGGTCGTACCCATCATATCGAAAACG	SbWRKY1 cloning into pDONR207 - without stop codon
	SbZNF1_2Fw	AAAAAGCAGGCTTCATGGCGCGTCCGCAG	SbZNF1 cloning into pDONR207 - without stop codon
	SbZNF1_2Rv	AGAAAGCTGGGTCGTGAGTCTCTGCTAGAGATG	SbZNF1 cloning into pDONR207 - without stop codon
	SbZNF1_3Fw	GGGGACAAGTTTGTACA	SbZNF1 cloning into pDONR207 - with stop codon
	SbZNF1_3Rv	GGGGACCACTTTGTACA	SbZNF1 cloning into pDONR207 - with stop codon
Probes	92-bp	GGATCCAGTGAGCTACCGGTGAAGGTGCTCGTT ATGCGTTTAAACATTGTTCCGTCCGGCGGCATCT ATACTCCTACAGACTATTAAAGTTG	Electrophoretic Mobilit Shift Assay (EMSA)
	F1	GGATCCAGTGAGCTACCGGTGAAGGTGCTC	
	F2	GAAGGTGCTCGTTATGCGTTTAAACATTGT	
	F3	TAAACATTGTTCCGTCCGGCGGCATCTATA	
	F4	GGCATCTATACTCCTACAGACTATTAAAGTTG	
	F5	CAAACACCAAAGCATGCCTGGTTAATTTCAGTTA ATAAGAGGGCCTTCAAAGGCAGTCTC	
	M1	TAAACATTGTTCCGTCCGGCCATCTATA	
	M2	TAAACATTGTTCCGTCCGGCGGTCTATA	
	M3	TAAACATTGTTCCGTCCGGCGGCATATA	
	M4	TAAACATTGTTCCGTCCGGCGGCATCTA	

CONCLUSION

In this work, we identified from the literature regulatory proteins, including transcription factors (TFs) from several families, which could mediate pleiotropic control of plant response leading to adaptation to co-existing abiotic stresses on acidic soils, namely Al tolerance, P deficiency and drought stress. We found that AtSTOP1 possibly has a highly pleiotropic nature. STOP1 is a key TF controlling both Al tolerance and root response to P deficiency via regulation of *ALMT1* expression. Additionally, AtSTOP1 may negatively regulate drought tolerance. Moreover, AtWRKY46 is involved in Al tolerance repressing *ALMT1* expression, and it is implicated in the regulation of drought responses. In response to osmotic and salt stress, AtWRKY46 was found to enhance lateral root development, and it would be interesting to investigate whether this response also improves P efficiency. Finally, AtMYB2, via regulation of *miR399*, is a key component of P homeostasis and controls drought responses. The possible unifying role of these and other TFs on abiotic stress tolerance makes them potentially useful in breeding programs targeting sorghum adaptation to tropical soils.

We also functionally characterized SbWRKY1 and SbZNF1, which transcriptionally regulate *SbMATE* expression in sorghum. We found that SbWRKY1 belongs to WRKY group III and contains a rather rare, WRKYGEK, heptapeptide variant. Moreover, we demonstrated that SbWRKY1 recognizes a novel *cis*-element for WRKY DNA-interaction. Subcellular localization assays in tobacco epidermal cells and Arabidopsis showed that SbZNF1 is located in the plasma membrane, whereas SbWRKY1 is found mainly in the nucleus. We observed that both TFs physically interact in the plasma membrane and, under Al^{3+} exposure, SbZNF1 is mobilized to the nucleus in a process that requires SbWRKY1. We propose that Al^{3+} induces proteolytic cleavage of SbZNF1 when complexed with SbWRKY1, with subsequent SbZNF1 mobilization to the nucleus. In turn, in the absence of Al, SbZNF1 appears to retain a fraction of the SbWRKY1 protein in the plasma membrane. The present study shows that the plasma membrane-localized TF, SbZNF1, senses Al^{3+} and transmits its signal to the nucleus, which may contribute to the previously observed synergistic action of SbZNF1 and SbWRKY1 on *SbMATE* expression.

FUTURE PERSPECTIVES

- *Electrophoretic Mobility Shift Assay (EMSA).*

To confirm the exact binding site of SbWRKY1, we will conduct the EMSA assay using the 10-bp SbWRKY1 binding site in an intermediary position of a 30-bp probe and perform mutations (deletions or substitutions) in each nucleotide. Moreover, it would be interesting to test whether SbWRKY1 shows binding activity to a W-box element.

Additionally, to determine the SbZNF1 binding site, we will perform heterologous expression of SbZNF1 lacking all four transmembrane domains (SbZFN1ΔTMD) so that the native protein remains in the soluble fraction, which would avoid the renaturing step. Subsequently, the SbZNF1 protein extract will be used in EMSA with the probes designed for 92-bp sequence.

- *Analysis of the SbWRKY1 dependency to the SbZNF1 nuclear shuttling*

SbZNF1 nuclear shuttling might be driven by its partner interaction, SbWRKY1. To gain insights into this issue, we extensively tried detect mobilization of the SbWRKY1/SbZNF1 complex using BiFC system (*N. benthamiana* leaves co-expressing SbWRKY1::nYFP and SbZNF1::cYFP). However, there are two points to be considered about this assay. First, the frequency of reconstituted YFP by the interaction between SbZNF1 and SbWRKY1 in BiFC was approximately 50%. Second, we observed a low frequency (20%) of cells in which the SbZNF1 was relocated from the plasma membrane to the nucleus in tobacco epidermal cells co-expressing SbZNF1 and SbWRKY1 under Al^{+3} exposure. These may make the SbZNF1/SbWRKY1 mobilization in the BiFC system difficult to detect. As an alternative, we propose the expression of fluorescent protein-tagged SbZNF1, lacking all their transmembrane domains (SbZNF1ΔTMD), and perform a subcellular localization assay in *N. benthamiana* leaves. We hypothesize that, in the absence of SbWRKY1, SbZNF1ΔTMD could locate in the cytosol. In turn, when co-expressed with fluorescent protein-tagged SbWRKY1, SbZNF1ΔTMD could produce nuclear signals, giving us strong evidence that the interaction with SbWRKY1 mediate the nuclear localization of SbZNF1. Moreover, using SbZNF1ΔTMD and SbWRKY1 in *N. benthamiana* BiFC analysis, we could determine whether SbZNF1 and SbWRKY1 further interact in the nucleus.

- *Analysis of SbWRKY1 binding site enrichment in Al-inducible genes*

We will conduct an analysis of SbWRKY1 binding site enrichment in sorghum Al-inducible genes from previous RNA-Seq data. The analyzes will be performed based on the relative appearance ratio (RAR) of octamers (the frequency of a particular octamer in the grouped genes relative to that in the genome-wide genes).

- *Characterization of the SbWRKY1 and SbZNF1 promoter regions in different sorghum lines*

Al^{3+} differentially regulates *SbWRKY1* and *SbZNF1* alleles from different Al-tolerant lines, where Al-induced *SbWRKY1* expression is higher in SC566 compared with SC283, and SbZNF1 expression is higher in SC283 compared with SC566. On the other hand, in the Al-sensitive lines, BR007 and BR012, both TFs are down-regulated. The cDNA sequences of *SbWRKY1* and *SbZNF1* alleles in Al-tolerant and Al-sensitive lines are identical. Therefore, we hypothesized that an allelic variation on its promoters might determine the differential regulation by Al^{3+} of the TF alleles. Thus, we generated maize transgenic events stably expressing different *SbWRKY1* and *SbZNF1* promoter alleles from Al-tolerant, SC283 and SC566, and Al-sensitive lines, BR007 and BR012, transcriptionally fused to the reporter gene,*GUS*. For clarity, the plants were individually transformed with the constructs: pSbWRKY1(SC566)::GUS (where "p" stands for promoter), pSbWRKY1(SC283)::GUS, pSbWRKY1(BR012)::GUS, pSbZNF1(SC566)::GUS, pSbZNF1(SC283)::GUS, pSbZNF1(BR007)::GUS. Transgenic plants in the T1 generation and will be selected to characterize the SbWRKY1 and SbZNF1 promoter regions by histochemical and quantitative analysis of *GUS* expression.

- *Chromatin Immunoprecipitation-sequencing (ChIP-Seq) assay*

SbWRKY1 and SbZNF1 might be involved in the regulation of other sorghum Al-responsive genes and genes related to other abiotic and biotic stresses. Thus, we will conduct ChIP-Seq in sorghum root protoplasts to identify the SbWRKY1 and SbZNF1 genome-wide target genes.