The Potential of Transcription Factor-Based Genetic Engineering in Improving Crop Tolerance to Drought

Roel C. Rabara,^{1*} Prateek Tripathi,² and Paul J. Rushton^{1*}

Abstract

Drought is one of the major constraints in crop production and has an effect on a global scale. In order to improve crop production, it is necessary to understand how plants respond to stress. A good understanding of regulatory mechanisms involved in plant responses during drought will enable researchers to explore and manipulate key regulatory points in order to enhance stress tolerance in crops. Transcription factors (TFs) have played an important role in crop improvement from the dawn of agriculture. TFs are therefore good candidates for genetic engineering to improve crop tolerance to drought because of their role as master regulators of clusters of genes. Many families of TFs, such as CCAAT, homeodomain, bHLH, NAC, AP2/ERF, bZIP, and WRKY have members that may have the potential to be tools for improving crop tolerance to drought. In this review, the roles of TFs as tools to improve drought tolerance in crops are discussed. The review also focuses on current strategies in the use of TFs, with emphasis on several major TF families in improving drought tolerance of major crops. Finally, many promising transgenic lines that may have improved drought responses have been poorly characterized and consequently their usefulness in the field is uncertain. New advances in high-throughput phenotyping, both greenhouse and field based, should facilitate improved phenomics of transgenic lines. Systems biology approaches should then define the underlying changes that result in higher yields under water stress conditions. These new technologies should help show whether manipulating TFs can have effects on yield under field conditions.

Introduction

HUMANS HAVE DOMESTICATED and improved wild progenitor species of plants to create the crops that are cultivated and eaten today. This domestication has typically involved the manipulation of just a small number of genes that have large effects. As noted by Doebley et al. (2006), many of the major genes controlling morphological and structural changes during the domestication are transcriptional regulators, which show that transcription factors (TFs) play a central role in the domestication. Examples include: maize *tb1* (a TCP transcription factor) for plant and inflorescence structure and *tga1* (SBP) for seed casing; rice *qSH1* (homeodomain) and *sh4* (Myb) for panicle shattering, and *Rc* (bHLH) for seed color; and wheat *Q* (AP2) for inflorescence structure. Drought tolerance is a more complex trait than simple morphogenesis but the capacity of TFs as tools to improve drought responses remains high.

The world population is growing at a staggering rate and is estimated to reach 9 billion by 2050 (Godfray et al., 2010). In order to feed the growing population, studies have suggested

that a 70%–100% increase in food production is needed to feed the world by 2050 (Godfray et al., 2010). However, several factors affect food production, and one of the limiting factors is changing environment. An estimate of the effect of abiotic stress suggests that it limits production by as much as 70% (Boyer, 1982). Water deficit or drought, which is considered to be the single greatest abiotic factor that limits global food production (Araus et al., 2002), is significantly affecting 64% of the global land area (Cramer et al., 2011). In 2012, the United States experienced one of the worst droughts to hit the country that resulted in 57% of total cropland experiencing severe drought (USDA-ERS, 2012). Therefore, it is important to have a detailed understanding of major environmental problems such as drought, to maintain a balance in supply and demand. The development of more drought tolerant crops would be a major step in raising food production.

Drought Tolerance: A Complex Trait

Drought tolerance as defined by Turner (1979) is the capability of plants to thrive in a condition with limited water

¹Texas A&M AgriLife Research and Extension Center, Dallas, Texas.

²Molecular and Computational Biology Section, University of Southern California, Los Angeles, California.

*Authors contributed equally.

supply or in sporadic conditions of water deficit (Turner, 1979). It is a quantitative trait that exhibits complex genetic control (McWilliam, 1989). The complexity of this trait explains why there is slow progress in crop improvement in drought-prone areas (Cattivelli et al., 2008). Relying on conventional breeding as an approach for yield stability under drought conditions is labor-intensive and time-consuming, primarily because of the complicated genetics that control yield traits (Yang et al., 2010). Due to the intricacy in genetic control of drought tolerance, marker-assisted selection has not contributed significantly to crop improvement for dry environments (Fleury et al., 2010). Hence, plant breeding has to rely on direct phenotypic selection (Venuprasad et al., 2007). However, studies show that direct selection for grain yield under water-stress conditions has also been hindered by the trait's low heritability, its multigenic nature, significant genotype-by-environment (GxE) interactions, and quantitative trait loci-by-environment (QTLx E) interactions (Cattivelli et al., 2008; Serraj et al., 2011). Large GxE interactions for drought traits are partly due to the wide differences in environmental variables such as weather patterns and soil composition (Serraj et al., 2011). Hence, a drought trait that had been identified to be of substantial benefits in a particular environment could exhibit a negative effect in another (Sinclair and Muchow, 2001).

Major Families of Transcription Factors as Tools to Improve Crop Responses to Drought

Genetic engineering provides a complementary approach to conventional breeding to improve crop tolerance to drought. Many genetically engineered plants have shown phenotypes with improved tolerance under water-limited conditions (Umezawa et al., 2006; Vinocur and Altman, 2005). Most of these published results were based on the analysis of transgenic model plants such as *Arabidopsis* and were further extended to some major crop species such as rice (Karaba et al., 2007), soybean (Seo et al., 2012), and maize (Zhang et al., 2004). Because of the complexity of the trait involved, understanding the genetic basis of drought tolerance is a necessary step in many strategies to develop a drought-tolerant crop. Deciphering how plants sense and acclimatize to an abiotic stress and using this knowledge to develop crops with enhanced tolerance to abiotic stresses is the "central dogma of abiotic research" (Mittler and Blumwald, 2010).

Transcription factor-based genetic engineering

Current commercially available genetically-modified (GM) crops are based on single-gene traits such as expression of Cry proteins (δ -endotoxins) derived from *Bacillus thuringiensis* for insect resistance (Romeis et al., 2006) or expression of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; 2.5.1.26) gene derived from *Agrobacterium tumefaciens* strain CP4 for herbicide resistance (Green, 2009).

However, trait stacking (herbicide and insect resistance) is gaining popularity among farmers with 42 M ha (26% of global biotech crop area) already planted in 2011 with GM crops having stacked traits (James, 2011). TF-based genetic engineering provides an alternative route for crop improvement because of the role of TFs in gene regulation. In some cases, genetic engineering of transcriptional networks promises to be

a more practical approach than conventional breeding methods because stress tolerance is inherently multigenic in nature (Tran et al., 2010). Several studies have shown the promise of using TFs as transgenes in improving crop responses to various stresses under greenhouse and field conditions, as summarized in Tables 1 and 2.

TFs are DNA-binding proteins that interact with other transcriptional regulators, including chromatin remodeling/modifying proteins, to recruit or block access of RNA polymerase to the DNA template (Udvardi et al., 2007). They act naturally as master regulators of many cellular processes (Century et al., 2008). In the *Arabidopsis* genome, around 7% of their coding sequences are devoted to encoding TFs (Iida et al., 2005) because of the complexity in transcriptional regulation in plants (Udvardi et al., 2007).

TFs are excellent candidates for modifying complex traits in crop plants (Century et al., 2008) and could be an important component in the next generation of successful biotechnology crops (Century et al., 2008). Table 1 summarizes the diverse functions characterized from various members of TF families that include regulation of plant development and responses to biotic and abiotic stresses. TFs play an important role in the regulation of plant response to abiotic stress. Expression profile of 7000 *Arabidopsis* genes showed that 40 genes belonging to AP2/ERF, WRKY, MYB, NAC, HD, and bZIP families of TFs were upregulated during various biotic stresses (Seki et al., 2002). Large families of TFs, such as NAC, AP2/ERF, bZIP, and WRKY, are involved in regulating plant responses to various stresses (Mizoi et al., 2012; Nakashima et al., 2012; Rushton et al., 2010; Singh et al., 2002). These four families of TFs were selected for discussion in this review due to studies that have shown their capability as tools for improving plant responses to drought. The role of these four families of TFs is reviewed below. An overview of the role of TFs for crop improvement (Table 2) shows members of these families of TFs that have been used to give drought-tolerant phenotypes in the important crops of rice, wheat, and tomato.

NAC TF Family

The NAC family proteins are plant-specific TFs and one of the largest families among plant-specific TFs (Nakashima et al., 2012; Rushton et al., 2008). It has a DNA-binding domain known as the NAC domain, named after the *No Apical Meristem* (*NAM*) gene from petunia, and *ATAF1/ATAF2* and *Cup-Shaped Cotyledon2* (*CUC2*) genes from *Arabidopsis*, located in the N-terminal region (Aida et al., 1997). The NAC domain is typically 150 amino acids in length, and also possesses a highly variable C-terminal transcriptional regulatory region that can act as either a transcriptional activator or repressor (Tran et al., 2010). The NAC DNA-binding domain consists of five subdomains labeled A to E. In tobacco, a novel clade of NAC genes (TNACS) represents a difference between the Solanaceae and other plants (Rushton et al., 2008). NAC TFs have been reported during drought in rice (Rabbani et al., 2003; Zhou et al., 2007) and *Arabidopsis* (Matsui et al., 2008; Tran et al., 2010). The use of NAC TFs as tools to improve drought tolerance in rice (Hu et al., 2006; Jeong et al., 2010; Zheng et al., 2009), tobacco (Ramegowda et al., 2012), wheat (Xue et al., 2011), and *Arabidopsis* (Gao et al., 2010) has also been reported.

TABLE 1. NUMBER OF GENES IDENTIFIED IN SELECTED TF FAMILIES WITH EXAMPLE OF A SPECIFIC MEMBER CHARACTERIZED FROM EACH FAMILY

Family	Rice	Arabidopsis	Brassica rapa	Populus trichocarpa	Jatropha curcas	Gene	Evidence	Reference
ABI3/VP1	7	6	135	108	54	<i>OsIDEF1</i>	Regulates response to iron deficiency in rice	(Kobayashi et al., 2010)
AP2/ERF	161	146	289	212	126	<i>SlERF5</i>	Drought and salinity tolerance in tomato	(Pan et al., 2012)
bHLH	180	164	241	148	87	<i>PbFAMA</i>	Regulates stomatal development in poplar during drought	(Hamanishi et al., 2012)
bZIP	94	76	127	85	136	<i>AtABF3</i>	Drought and heat tolerance in bentgrass	(Choi et al., 2013)
C3H-type 1	9	11	106	78	41	<i>AtSZF1</i> and <i>AtSZF2</i>	Regulate salt stress responses in Arabidopsis	(Sun et al., 2007)
CCAAT	37	36	57	38		<i>AtNFA5</i>	Regulate drought response	(Li et al., 2008)
Homeobox	84	90	156	106	32	<i>AtEDT1/HDG11</i>	Confers drought tolerance in transgenic rice	(Yu et al., 2013)
HSF	27	23	52	31	21	<i>AtHsfA6a</i>	Improve tolerance to salt and drought	(Hwang et al., 2013)
MYB	182	189	368	216	115	<i>TaMYB30-B</i>	Improve drought tolerance in Arabidopsis	(Zhang et al., 2012)
NAC	149	105	188	172	97	<i>OsNAC10</i>	Drought tolerance and increased grain yield	(Jeong et al., 2010)
Trihelix	32	38	48	47	33	<i>AtGT2L</i>	Involved in plant responses to cold and salt stress	(Xi et al., 2012)
WRKY	100	72	147	104	61	<i>OsWRKY30</i>	Drought tolerance in rice	(Shen et al., 2012)

TF families from rice, Arabidopsis (Xiong et al., 2005), Brassica (Cheng et al., 2011), Poplar (Zhu et al., 2007) and Jatropha curcas (Lin et al., 2014). Table 1 is the authors' new original synthesis from the works of Kobayashi et al. (2010), Pan et al. (2012), Hamanishi et al. (2012), Choi et al. (2013), Sun et al. (2007), Li et al. (2008), Yu et al. (2013), Hwang et al. (2013), Zhang et al. (2012), Xi et al. (2012), Shen et al. (2012), and Jeong et al. (2010).

Testing of transgenic plants under field conditions show promising results for NAC gene overexpression to enhance drought tolerance. For example, overexpression of *SNAC1* (*Stress-Responsive NAC 1*) shows increased tolerance to drought and salt stress in transgenic rice (Hu et al., 2006). The transgenic rice also resulted in an increase in seed setting (22%–34% higher than control) in the field under severe drought stress conditions during the reproductive stage of rice plants (Hu et al., 2006). Overexpression of another rice *SNAC* gene, *SNAC2*, showed tolerance to polyethylene glycol (PEG), cold, and salinity treatments in the rice seedlings (Hu et al., 2008). In a separate field trial, there was a 42% increase in grain yield in *OsNAC10*-overexpressing transgenic rice under drought conditions (Jeong et al., 2010). Heterologous expression of rice *SNAC1* in transgenic wheat resulted in improved drought tolerance of transgenic rice under controlled growth conditions (Saad, 2013). These results show that relevant factors involved in *SNAC1* regulation and signaling pathways in rice are also present in the wheat genome and are potentially conserved in cereals. Overexpressing *OsNAC045* in rice not only improved drought tolerance but also salinity tolerance of transgenic rice grown in a hydroponic system (Zheng, 2009). These results show the promise of NAC TFs in improving drought responses.

bZIP TF family

The basic region leucine zipper (bZIP) family of TFs is exclusively present in eukaryotes and regulates diverse functions such as plant development and stress responses (Deppmann et al., 2004; Ying et al., 2012). This family of TFs is characterized by a 40–80 amino acid long conserved bZIP domain, hence its name (Correa et al., 2008; Nijhawan et al., 2008). The bZIP domain is composed of two motifs: the basic region that functions as the DNA binding domain and a leucine zipper that is responsible for the TF dimerization (Correa et al., 2008). Deppman et al. (2004) reported that dimerization specificity of the leucine zipper region of bZIP TFs in Arabidopsis is influenced by length of the leucine zipper, the position of a charged amino acid residue, as well as the occurrence of interhelical electrostatic interactions. bZIP TFs DNA-binding domain normally recognize *cis*-element with an ACGT core motif (Foster et al., 1994; Nijhawan et al., 2008). The potential of bZIPs as tools to improve crop responses to drought was shown by heterologous expression of soybean *GmbZIP1* in Arabidopsis: the recovery of transgenic plants was improved when re-watered for 12 days after subjecting the plants to drought for 30 days (Gao et al., 2011). It also improved tolerance to other abiotic stresses with a survival rate as high as 45% and 52% under freezing (−6°C for 6 h) and high salinity (250 mM NaCl for 30 days) stresses, respectively (Gao et al., 2011). Overexpressing the same gene improved drought tolerance of transgenic wheat grown in liquid medium with 8% PEG for 3 weeks.

Several members of rice bZIP TFs had shown the capability of improving drought response of transgenic rice. *OsbZIP16*-overexpression in rice seedlings grown in Yoshida solution showed improved recovery after 9 h of dehydration (Chen et al., 2012a). The same response was observed for the same transgenic lines grown in soil media and subjected to 5 days of drought. A similar phenotype was also observed with overexpression of *OsbZIP23* and *OsbZIP46* in rice (Tang

TABLE 2. SUMMARY OF GENETIC ENGINEERING USING TRANSCRIPTION FACTORS OF IMPORTANT CROPS TO IMPROVE RESPONSES TO DROUGHT

Gene	Promoter	Gene source	Transgenic host	Approach	Positive phenotype	Negative phenotype	Target downstream gene(s)	Test condition	References
NAC Family <i>SNAC1</i>	CaMV35S	Rice	Rice	Overexpression (OE)	Enhanced drought resistance	None observed	Induced several TFs like MYB, HB-2, Zn finger containing protein, NAC, C2H2; enzyme-encoding genes like ATPase, SD3, glycosyl transferase	Plants at reproductive stage were stressed in 3 drought conditions: refined paddy field, drought-prone field and PVC tubes.	(Hu et al., 2006)
<i>OsNAC10</i>	Constitutive GOS2; root-specific RCc3	Rice	Rice	OE	Drought tolerance and increased grain yield	–	34 root- and 40-leaf specific up-regulated (≥ 3 -fold)	Field trial for 2 years	(Jeong et al., 2010)
<i>SNAC2</i>	Ubiquitin1	Rice	Rice	OE	Enhanced tolerance to PEG treatment, cold and salinity	None observed	36 up-regulated and 9 down-regulated (2.5 fold)	MS medium supplemented either 150 mM salt or 15%PEG or placed at 4-8°C	(Hu et al., 2008)
<i>SNAC1</i>	Ubiquitin	Rice	Wheat	OE	Drought and salinity tolerance	–	Induced <i>P13K</i> , <i>SPS</i> , <i>RCAR</i> ; repressed <i>PP2C3</i>	4-week old plants in growth chamber	(Saad et al., 2013)
<i>ONAC045</i>	CaMV35S	Rice	Rice	OE	Drought and salt tolerance	None observed	Induced <i>OsLEA3-1</i> ; <i>OsPMI</i>	15-18 d plants hydroponically grown	(Zheng et al., 2009)
<i>OsNAC5</i>	Ubiquitin	Rice	Rice	OE	Drought and salinity tolerance	None observed	Induced <i>OsLEA3</i> , plastocyanin-like protein	Grown in soil for 14d then either grown in 250 mM NaCl or withheld water for 4 d	(Takasaki et al., 2010)
bZIP Family <i>OsbZIP16</i>	Actin1	Rice	Rice	OE	Drought tolerance	–	Induced <i>LEA7</i> , <i>LEA3-1</i> , <i>RAB16C</i> and <i>D</i> , <i>RAB21</i>	2-week old plant grown in Yoshida solution (seedling stage); 25 d old plants grown in soil in greenhouse (tillering stage)	(Chen et al., 2012a)
<i>GmbZIP1</i>	Ubiquitin	Soybean	Wheat	OE	Drought tolerance	–	Induced <i>ABI1</i> , <i>ABI2</i>	Immature embryos grown in MS medium with 8% PEG	(Gao et al., 2011)
<i>OsbZIP23</i>	Ubiquitin	Rice	Rice	OE	Drought and salinity tolerance	Sensitive to exogenous ABA during germination and postgermination stages	–	Grown in PVC pipes	(Xiang et al., 2008)
<i>OsbZIP46CA1</i>	Ubiquitin	Rice	Rice	OE	Drought tolerance	Increased sensitivity to ABA; reduced drought tolerance for native <i>OsZIP46</i> overexpressor	–	4-leaf stage plant grown in sandy soil and water withheld for 1 wk (seedling); PVC pipes (reproductive)	(Tang et al., 2012)
<i>ABF3</i>	CaMV35S	Arabidopsis	Bentgrass	OE	Drought and heat tolerance	None observed	–	Vegetatively propagated plants grown in soil mixture withheld of water for 2 wk; for heat treatment, plants were subjected to 2, 4 and 6 h in 48°C growth chamber	(Choi et al., 2013)
<i>AREB1</i>	CaMV 35S	Arabidopsis	Soybean	OE	Drought tolerance	Reduced plant height and internode length	–	Plants grown in the greenhouse; at V2 stage water was withheld for 5 d	(Barbosa et al., 2013)

(continued)

TABLE 2. (CONTINUED)

Gene	Promoter	Gene source	Transgenic host	Approach	Positive phenotype	Negative phenotype	Target downstream gene(s)	Test condition	References
AP2/ERF Family <i>GmDREB1A</i>	rd29A and CaMV35S	Soybean	Arabidopsis	OE	Drought and salt tolerance	–	–	Drought for 19 d and salt treatment for 22 d withheld for 9d	(Chen et al., 2007)
<i>JERF1</i>	CaMV35S	Tomato	Rice	OE	Drought tolerance	–	Induced <i>OsP5CS</i> , <i>OsSPDS2</i> , <i>OsLTP1</i> , <i>OsCDPK13</i>	10d old seedlings grown in soil and water withheld for 9d	(Zhang et al., 2010b)
<i>S1ERF5</i>	CaMV35S	Tomato	Tomato	OE	Drought and salinity tolerance	Stunted growth and have more side shoots	–	4 week old plants grown in compost with various NaCl concentration or water withheld for 7d.	(Pan et al., 2012)
<i>GmERF3</i>	CaMV35S	Soybean	Tobacco	OE	Drought, salinity and disease tolerance	–	Induced <i>PR1</i> , <i>Osmotin</i> , <i>SAR8.2</i> and <i>PR2</i>	For drought, shoot tips of seedlings were rooted in MS media with 2% PEG; For salinity, leaf discs floated in MS solution with 400 mM NaCl for 5 d; for disease	(Zhang et al., 2009)
WRKY Family <i>HvWRKY38</i>	Ubiquitin	Barley	Turf grass (<i>Paspalum notatum</i>)	OE	Drought tolerance	Reduced seed production	–	Plants with 3 tillers grown in hydroponics for 2 wk and dehydrated in 5 timepoints	(Xiong et al., 2010)
<i>OsWRKY11</i>	HSP101	Rice	Rice	OE	Heat and drought tolerance	Dwarf phenotype; bent leaves	–	2 wk old seedlings grown in greenhouse at 37/22°C for 2 wk with water and then water withheld for 2 d	(Wu et al., 2009)
<i>OsWRK30</i>	CaMV35S	Rice	Rice	OE	Drought tolerance	–	Induced <i>WS176</i> , <i>WRKY11</i> , <i>OsSAP1</i> , <i>Oshox7</i> , <i>HSP</i>	2 wk old seedlings in hydroponics with PEG for 5 d	(Shen et al., 2012)
<i>TaWRKY2</i>	CaMV 35S	Wheat	Arabidopsis	OE	Salt and drought tolerance	–	Induced <i>STZ</i> and <i>RD29B</i>	7 d old seedlings grown in 0.5X MS with 300mM sorbitol for 20d or 150 mM NaCl for 15 d	(Niu et al., 2012)
<i>TaWRKY19</i>	CaMV 35S	Wheat	Arabidopsis	OE	Salt, drought and freezing tolerance	–	Induced <i>DREB2A</i> , <i>RD29A</i> , <i>RD29B</i> <i>Cor6.6</i>	7 d old seedlings grown in 0.5X MS with 300 mM sorbitol for 20d or 150 mM NaCl for 15d; freezing 3 wk old	(Niu et al., 2012)

et al., 2012; Xiang et al., 2008). Expression of *OsbZIP23* in transgenic lines were predominantly in leaves and showed significantly lower water loss than the wild type (WT) (Xiang et al., 2008). However, lower water loss rate was not due to stomatal density and aperture since no significant difference was observed between overexpressed and knockout lines under normal and drought conditions (Xiang et al., 2008). Heterologous expression of Arabidopsis *bZIP* genes also showed a phenotype with increased drought tolerance in bentgrass (Choi et al., 2013) and soybean (Barbosa et al., 2013).

AP2/ERF TF family

One of the large families of TFs involved in plant responses to abiotic stress is the *APETALA 2*/ethylene-responsive element binding factor (AP2/ERF) family. In the Arabidopsis genome, the AP2/ERF family comprises 9% of the total 1600 TFs annotated (Feng et al., 2005; Perez-Rodriguez et al., 2010; Riano-Pachon et al., 2007). The AP2/ERF TFs are a group of plant-specific TFs involved in regulating diverse developmental and regulatory processes (Dietz et al., 2010). The AP2/ERF family is composed of four to five subfamilies: the AP2, RAV, ERF, the dehydration-responsive element binding protein (DREB), and others (AL079349) (Mizoi et al., 2012). Among the subfamilies, member of the DREB subfamily are the major factors involved in plant responses to abiotic stress through regulation of gene expression via the *cis*-regulatory element DRE/C-repeat (DRE/CRT) (Abe et al., 2003; Lata and Prasad, 2011; Shinozaki and Yamaguchi-Shinozaki, 2007). An increasing number of studies have reported the potential of DREB TFs to engineer enhanced tolerance to drought (Liu et al. 1998; Zhao, 2010). The summary of characterized members of AP2/ERF family members are shown in Table 2. Overexpression of *AtDREB1A* improved drought tolerance in Arabidopsis and also resulted in dwarf phenotypes (Liu et al., 1998). Thus for a successful strategy to improve drought tolerance, correct promoter selection is important.

Overexpression of the same gene in wheat using the *rd29A* promoter resulted in improved drought tolerance in wheat. A similar phenotype was observed when *AtDREB1A* was overexpressed in tobacco (Kasuga et al., 2004) and chrysanthemum (Hong et al., 2006). Heterologous expression of *GmDREB2* resulted in improved tolerance to drought and high-salinity stresses in transgenic Arabidopsis (Chen et al., 2007). Similarly, *GhDREB* from cotton expressed in wheat conferred enhanced tolerance to drought, high salt, and freezing stresses in transgenic wheat (Gao et al., 2009). ERF TFs have also been used as a tool to improve plant responses to drought. Heterologous expression of tomato *JERF1* in transgenic rice improved its drought tolerance (Zhang et al., 2010b). Overexpression of *GmERF3* improved drought tolerance in transgenic tobacco (Zhang et al., 2009). This indicates that several subfamilies of the AP2/ERF family may contain TFs that could improve drought responses.

WRKY TF family

WRKY TFs are a large family of TFs that are present across the green lineage and beyond (Rushton et al., 2010). The WRKY TFs are defined by highly conserved 60 residues long DNA binding domain, the WRKY domain. The WRKY domain at the N-terminus is almost invariant within the

WRKY signature sequence with just a few variants reported that replace the WRKY amino acid sequences with WIKY, WRRY, WKRY, WSKY, or WKKY (Rushton et al., 2010; Zhang and Wang, 2005). The WRKY DNA binding domain has high affinity for the W-box *cis*-regulatory element (TTGACC/T) (Rushton et al., 1996; 2010). WRKY TFs play important roles in plant response to stresses. In particular, many studies have shown the role played by WRKY TFs in plant responses to biotic stresses (Eulgem, 1999; 2006; Eulgem and Somssich, 2007; Ishihama and Yoshioka, 2012; Pandey and Somssich, 2009; Rushton et al., 2010). However, compared to the knowledge on the role of WRKY TFs in plant responses to biotic stress, the information available on its role in plant responses to abiotic stress is still limited (Chen et al., 2012b; Rushton et al., 2010). An increasing number of reports show the potential of WRKY TFs as tools for engineering abiotic stress tolerance such as drought in plants (Chen et al., 2012b; Jiang et al., 2012; Rabara et al., 2013; Rushton et al., 2010; Tripathi et al., 2013). For example, constitutive expression of *AtWRKY57* conferred drought tolerance in Arabidopsis (Jiang et al., 2012). Fourteen days drought stressed T₂ transgenic lines of 35S::*WRKY57* showed recovery when re-watered compared to WT (Jiang et al., 2012). In rice, drought tolerance was conferred by overexpression of *OsWRKY30* through activation by a mitogen-activated protein kinase (MAPK) (Shen et al., 2012). Examples of multiple stress tolerance in transgenic plants were also observed when a single WRKY gene was overexpressed. Heterologous overexpression of *OsWRKY45* in Arabidopsis resulted in enhanced resistance to the bacterial pathogen *Pseudomonas syringae* and improved tolerance to salt and drought stresses in transgenic plants (Qiu and Yu, 2009). Overexpression of *OsWRKY11* driven by an *HSP101* promoter conferred enhanced tolerance to heat and drought in seedlings after heat pretreatment at 37/22°C (12 h/12 h) for 2 weeks (Wu et al., 2009). This again illustrates the importance of promoter choice in strategies that use transgenes to confer drought tolerance.

Molecular Tools for Deciphering the Role of TFs

In order to develop strategies in genetic engineering of drought tolerance in plants, one must understand the molecular mechanisms involved in these particular traits. Drought triggers a wide variety of plant responses that include perturbations in gene expression. Hence, to be able to understand the plant's response to stress at the molecular level, detailed knowledge of the mechanisms involved in regulating transcription is required. Several tools are available to identify and manipulate TFs involvement in plant responses to drought (Fig. 1). Identifying the TFs involved in regulating the plant responses to the stress is the first logical first step, followed by functional analyses of these genes. Put simply, manipulating the regulators will likely manipulate the response. In this section, recent techniques and strategies that can be employed to discover and manipulate TF expression in order to improve drought tolerance are discussed.

Gene discovery

Transcriptome profiling is used to determine perturbation in gene expression in plants during drought. Transcriptome analyses have been conducted on many plants including

FIG. 1. Techniques for the manipulation of TF genes to improve drought responses in plants.

drought-tolerant plants such as resurrection plants (Dinakar and Bartels, 2013). Identifying genes from these drought-tolerant plants is important to provide insights into genes that are involved in regulating drought stress responses. Expression profiling using microarray platforms is one of the most common molecular tools for large-scale expression profiling because it allows the simultaneous detection of tens of thousands of transcripts at a reasonable cost (Busch and Lohmann, 2007). For small- and medium-scale expression profiling, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is also useful. Depending on the choice of primers and detection technologies used, qRT-PCR can profile a single gene or several hundreds of genes in a single experiment (Busch and Lohmann, 2007; Gachon et al., 2004). Because of recent advances in high-throughput sequencing-based methods, the current state-of-the-art in transcriptome analyses is RNA sequencing (RNA-Seq). The advantages of using RNA-Seq compared to other techniques are its ability to identify the exons and introns and their boundaries, as well as the accurate transcript quantification and identification of alternative splicing (Nagalakshmi et al., 2010; Wang et al., 2009). Also, RNA-Seq provides a transcriptome in the

absence of a sequenced genome, something that is not possible when using microarrays.

Analysis of cis-acting elements

The regulation of gene expression is mediated by the binding of TFs to *cis*-regulatory elements present in the promoter region of their target genes (Priest et al., 2009). Hence, identification and prediction of *cis*-regulatory elements through promoter analysis is an important aspect in the functional analysis of TFs and signaling networks. The analyses have the potential to identify novel elements that have not been reported to be involved in drought responses. There are several publicly available software programs for identification and *in silico* analysis of putative *cis*-regulatory elements. The word “putative” should be stressed here as all of these analyses are *in silico* and none of the programs actually identifies any *cis*-acting elements. Only functional analyses can demonstrate that a promoter sequence is a transcription factor binding sites/ *cis*-acting element. Nevertheless, these programs can suggest potential *cis*-acting elements and help guide subsequent functional analyses. Additionally, statistical over-representation of sequences can also suggest the sites of *cis*-acting elements.

In silico analysis programs include Multiple Em Motif Elicitation (MEME; <http://meme.sdsc.edu/meme/cgi-bin/meme.cgi>) (Bailey et al., 2009), a web-based software tool used to identify and predict conserved DNA motifs in promoter sequences. Other programs such as PlantCARE, PlantPAN, and AGRIS are also available. However, PlantCARE and PlantPAN only locate sequences that are similar to known *cis*-elements, and there is no functional data to support these sequences as being functional. AGRIS has more functionality and is useful for Arabidopsis and similar plants. There are several good techniques, both *in vitro* and *in vivo* approaches, which can be employed to provide evidence of the binding of TFs to *cis*-regulatory elements. Techniques such as chromatin immunoprecipitation followed by sequencing (ChIP-seq) (Park, 2009), electrophoretic mobility shift assay (EMSA) (Hellman and Fried, 2007), and DNase I footprinting can be used to find TF binding sites in promoters. This information can then be used to isolate the putative TFs that bind to these elements using techniques such as South Western screening and yeast one-hybrid screening.

Mechanisms of regulation of different classes of TFs

There are thousands of TFs in every plant species and the list of possible interacting partners is long. They include co-activators and co-repressors, different classes of kinases, R proteins, 14-3-3 proteins, histone deacetylases and histone acetyltransferases, protein phosphatases, ubiquitinationases, enzymes such as E3 ligases, calcium calmodulin, SUMO, VQ proteins, and TFs. TFs also bind to their cognate DNA-binding sites on promoters. Many different mechanisms of activation or repression of transcription have been reported in plants, and most TFs require protein complexes for activity. One core component of signaling network in plant responses to water stress is mitogen-activated protein (MAP) kinase cascades. It is a highly conserved mechanism that plays a vital role in signal relay in plant responses to both biotic and abiotic stresses (Fujita et al., 2006). Another mechanism involved in abiotic stress tolerance in plants is protein ubiquitination. Proteins for degradation are tagged by ubiquitin in the ubiquitin pathway through a three-enzyme system: ubiquitin-acting (UBA; E1) enzyme, ubiquitin-conjugating (UBC; E2) enzyme, and ubiquitin ligase (E3) (Lyzenga and Stone, 2012). The E3 ligases regulate stress-responsive TFs by promoting their degradation in the absence or presence of a stress signal. The complexity in plant signaling networks underlines the importance of identification of other proteins that interact with the TF proteins to explain their mechanisms of regulation. The information gathered from protein–protein interaction can be used to generate protein–protein interaction networks and give clues to the mechanisms of regulation (Zhang et al., 2010a). Techniques such as yeast two-hybrid screens and affinity purification mass spectrophotometry (AP-MS) can be utilized to find interacting protein partners. Yeast two-hybrid (Y2H) assays (Fields and Song, 1989) are a popular tool and Shang et al. (2010) used this approach to show the interaction between an ABA receptor (ABAR) and AtWRKY40 using the ABAR C-terminus as the bait. BiFC is a noninvasive fluorescent-based technique that allows visualization of protein–protein interactions in living cells and is most commonly used to investigate known interactions between two proteins (Ohad et al., 2007). It is based upon the

interaction of proteins that are fused to nonfluorescent GFP deletion proteins, usually from the yellow fluorescent protein (YFP). Interaction of the proteins recreates a complete fluorescent fluorophore and YFP signal is detected (Hu and Kerppola, 2003; Ohad et al., 2007). These techniques help to find interacting partners of TFs, elucidate their mechanisms of regulation, and provide new targets for crop improvement.

Techniques for the manipulation of TF genes to improve drought responses

The two most common current approaches in the study of functional genomics of TFs are the generation of mutant lines through knockouts/knockdowns and the overexpression of the target genes. RNA interference (RNAi) is one of the most used contemporary approaches for targeted gene knockdown to analyze gene function because it is rapid and inexpensive. However, the limitation of this approach is that its inhibition of gene function is incomplete and could exhibit unpredictable off-target effects affecting the interpretation of linking the phenotype to the gene being knocked down (Gaj et al., 2013). New approaches are emerging, such as the use of sequence-specific nucleases as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and homing endonucleases or meganucleases to create targeted double-strand breaks that would promote recombination at a specific locus (Voytas, 2013). Another approach is the use of clustered regulatory interspaced short palindromic repeats (CRISPRs) to target the engineering of any genes from a wide range of organisms for researchers to establish gene function and to produce improved plants (Gaj et al., 2013).

Targeting-induced local lesions in genomes (TILLING) is another approach that can be used for functional genomics in plants. It offers the advantage of identifying the function of genes without generating transgenic plants. Ecotilling, a TILLING approach to study the variation in natural population, was successfully used to identify transcription factors associated with drought tolerance in rice (Yu et al., 2012).

Another popular tool for manipulating TFs and establishing phenotypes is through overexpressing the gene. Overexpression (OE) is a popular tool used by researchers for establishing gene function in TFs, as shown in Table 2. It is an alternative option to the knockout (KO) approach that is sometimes unable to show a phenotype because of functional redundancy in the plant genome (Zhang, 2003). It is common to use both the OE and KO approaches to obtain a better idea of TF function. Zhang outlined different strategies to employ when overexpressing TFs, which include the use of strong constitutive promoters such as 35S, multiple TFs, modified TFs, induced expression of TFs, and tissue-specific expression of TFs (Zhang, 2003). The advantages and disadvantages of all these techniques are summarized in Table 3.

One approach to improving crop tolerance to abiotic stresses is to identify candidate genes that play a role in plant responses to these stresses and manipulate their expression *in planta*. These candidate genes can be expressed in several ways, depending on the approach employed by the researcher to improve crop tolerance to stress. The most common approach is to constitutively overexpress the transgenes using strong promoters (Gurr and Rushton, 2005). However, constitutively overexpressing transgenes to improve a target phenotype (e.g., drought tolerance) sometimes comes with a

TABLE 3. MOLECULAR APPROACHES USED IN IDENTIFYING AND ELUCIDATING THE FUNCTIONS OF TFs

<i>Technology</i>	<i>Advantages</i>	<i>Disadvantages</i>	<i>References</i>
<i>Expression profiling</i>			
Microarray	Low cost and large scale profiling	Rely on genomic annotations	(Busch and Lohmann, 2007)
qPCR	Real-time gene expression analyses	Require prior sequence data of target genes	(Smith and Osborn, 2009)
RNA-Seq	High throughput, More Robust. Good for even un annotated species; identify alternative splicing forms	Expensive and limited	(Wang et al., 2009)
<i>Molecular functional analyses</i>			
Overexpression	Visible Phenotype, Target Identification; Less affected by functional redundancy	Unwanted expression. Sometime hard to correlate with the real situation	(Prelich, 2012)
Knockdown by RNAi	Visible Phenotype, Target Identification; rapid and high-throughput	Unpredictable off-target effect; temporary inhibition of gene function	(Gaj et al., 2013)
Y2H	Easy to Perform	High rate of false positives; interactions often limited in the nucleus	(Zhang et al., 2010a)
BIFC	Simple and sensitive to identify interaction	Background noise due to auto-fluorescence	(Ohad et al., 2007)
EMSA	Highly Robust, simple and efficient	Mobility shift does not provide molecular weight or identities of the proteins	(Hellman and Fried, 2007)
ChIP-seq	High Throughput	Expensive, loss of sensitivity when insufficient number of reads are generated; needs good antibody; requires transgenic line	(Park, 2009)
TILLING/EcoTILLING	Non-transgenic, cost-effective and high-throughput reverse genetic approach	False positives and false negatives due to gel scoring can be an issue	(Barkley and Wang, 2008)
ZFNs	Site-specific alteration	Immediately feasible to crops with reference genome sequence	(Curtin et al., 2012)
TALENs	Site-specific custom alteration at specific genomic location	Delivery may be limited by size-restricted vectors	(Gaj et al., 2013)
CRISPR/Cas system	Does not require time-consuming assembly of DNA-binding proteins; robust approach	Relatively high off-target rate	(Belhaj et al., 2013)

penalty in terms of unwanted phenotypes (e.g., stunted growth, low yield). Because key regulatory proteins, like TFs, regulate the expression of many downstream genes, several different phenotypic effects may be observed when these regulators are mutated (Des Marais and Juenger, 2010). The potential of TFs to involve multiple downstream target genes that control a single phenotype provides a good argument for their potential as tools for improving multigenic traits such as drought tolerance. Pleiotropic effects might be observed when specific genes regulate various genes or when altered levels of the TF protein or other proteins (for example, downstream targets) cause an altered phenotype. For example, although overexpression of a wheat dehydrin in Arabidopsis improves drought tolerance of the transgenic plants, the plants also exhibit an additional phenotype, being less sensitive to jasmonic acid compared to wild-type plants (Brini

et al., 2011). Table 2 shows some undesired phenotypes observed when TF-encoding genes were constitutively expressed. Stunted growth is the common phenotype observed when a TF gene is overexpressed in transgenic plants. This has been observed in soybean (Barbosa et al., 2013), tomato (Pan et al., 2012), rice (Wu et al., 2009), tobacco (Kasuga et al., 2004), and chrysanthemum (Hong et al., 2006).

With the selection of different promoters, it is possible to express transgenes in defined temporal and spatial manners. One of these is through constitutive ectopic overexpression of the genes (Gurr and Rushton, 2005). Other options include induced expression (e.g., chemical-induction or stress-induction), or tissue/cell type-specific expression of the transgenes. Either of these approaches required specific promoters to drive the expression of the transgenes. Promoters can be constitutive (e.g., CaMV35S, Actin, ubiquitin),

inducible (e.g., RD29A), or tissue/cell type-specific (root specific promoter *RCc3*) (Jeong et al., 2010), or combinations of these, for example leaf specific and ABA inducible. Promoters can also be native or synthetic (for example, 4xW and 4xS; (Rushton et al., 2002). With constantly expanding lists of available promoters for use in gene expression, the right choice of promoter could answer phenotypic problems concerning pleiotropy. The use of constitutive promoters for overexpression of transgenes fits some strategies to improve drought tolerance as shown in Table 2 without exhibiting negative effect on plant development. However, when there is a case of an unwanted phenotype as a result of constitutive expression, the use of an inducible promoter (e.g., drought-inducible promoter) has the potential to alleviate the problem on pleiotropic effects (Kasuga et al., 1999). Using promoters that are induced by stress could provide a potential solution to undesirable traits if there is low background expression of the transgene under normal growth (Umezawa et al., 2006; Zhang et al., 2004). Overexpression of the *Arabidopsis* DREB1 gene resulted in improved tolerance to drought, cold, and salinity, but also resulted in growth retardation under normal growth conditions (Liu et al., 1998). This undesired phenotypic component was overcome when the stress-inducible RD29A promoter was used to drive expression of the DREB1A gene in tobacco (Kasuga et al., 2004) and chrysanthemum (Hong et al., 2006) and resulted in greater tolerance to environmental stresses (Kasuga et al., 1999).

Phenomics, High Throughput Phenotyping, and Field Trials

One of the major criticisms of transgenic (often incorrectly called GM—incorrect because all cultivated plants are genetically modified) plant lines that claim to show improved drought tolerance is that they are ill-defined, neglect physiology, and that the phenotypes are unspecific in their definitions (Lawlor, 2013). This is often true. However, new advances in phenomics promise to address this problem. High-throughput phenotyping is rapidly gaining popularity, and major universities, research institutes, and companies are all investing in these precision phenotyping systems. Many physiological parameters such as leaf area, chlorophyll content, stem diameter, plant height or width, growth rate, stress pigment level, tip burn, biomass, internode length, color, leaf rolling, and leaf tracking can be determined noninvasively and in real time. In this way, a large amount of good quality phenomics data can be produced for many promising transgenic lines; this also facilitates the comparison of experiments from different research groups. Mutant populations, such as EMS or T-DNA insertion lines can be phenotyped in addition to transgenic lines. In this way, the phenotypes of mutants and transgenic lines can be more specifically and reliably defined.

There remains, however, one major problem. Drought tolerance is a complex quantitative and multigenic trait with a significant environmental component (Langridge and Fleury, 2011; Mir et al., 2012). As a result, the genetic control of traits associated with tolerance to drought often shows low heritability. Consequently, water stress responses from hydroponics, growth chambers, greenhouses, and different field conditions vary. Drought tolerance results from high throughput phenotyping experiments under greenhouse conditions are unlikely to

be similar to results obtained under field conditions. For this reason, field phenotyping systems that are currently being developed may give us our best opportunity to determine whether the manipulation of TFs can produce plants with increased drought tolerance. It seems that one of the major hurdles in using TF genes to improve drought responses lies not in producing these plants but in accurately defining them. The development of new phenomics technologies will greatly facilitate the characterization of transgenic lines, especially under field conditions.

Conclusion and Future Perspectives

This review emphasizes the potential of TFs, using four large families of TFs as examples, as an important part in the next generation of biotech crops. In the last 10 years, a total of 194 articles were published (based on PubMed database searches) that focused on functional analysis of TFs to improve plant responses to drought tolerance. The majority of these (130 articles) were published during the last 5 years. This trend shows that there is increasing interest in TFs and acknowledges the promising roles of TFs as tools to improve plant responses to drought. TFs are strong candidates for the development of next generation of transgenic crops because of their natural role in plant growth and development. The recent advances in high throughput sequencing technologies have provided new methods for mapping and quantification of plant transcriptomes. RNA-Seq is now becoming the method of choice for gene expression profiling. This is because of the inherent advantages of sequencing-based approaches compared to hybridization-based approaches of quantifying the level of transcripts.

The area of functional genomics also has its share of advances. Recent developments in mutagenesis technology have shown the application of site-specific nucleases such as ZFNs and TALENs to induce targeted mutation. For genome-wide manipulation, CRISPR/Cas9 technology is also a recommendable preference. Although RNAi is still the preferred approach for knockdown of target genes, its limitations such as off-target effects and incomplete silencing of target genes still need to be considered when using this approach. T-DNA insertion lines may be a good alternative to RNAi. However, depending on the target genes, RNAi may be sufficient to determine the functions of a gene or produce an improved plant. To avoid issues of off-target effect and inefficacy, the use of site-specific nucleases for targeted mutation provides an alternative approach.

The rapid advancement in next generation sequencing technologies, as well as the availability of mutagenesis approaches, provides efficient tools for functional analyses of TFs. The challenge now is to identify desired phenotypes under field conditions. Most of the screening for drought tolerance in transgenic crops is performed in greenhouses and controlled environment conditions. This could be partly due to regulatory policies regarding screening of transgenic plants in the field. Although many countries have national biosafety guidelines in place concerning field trials of transgenic crops, this does not assure approval for field-testing of transgenic plants. Regulation policies will surely evolve in the future as there are transgenic events that are now being deregulated. The change in regulatory policy landscape may result in more field trials of transgenic plants

for screening for improved response to environmental stresses such as drought. Finally, new advances in precision/high throughput phenotyping will provide much better characterized phenotypes of promising lines under both greenhouse and field conditions, and this in turn will provide much better evidence as to whether lines containing manipulated transcription factors display improved drought tolerance.

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Address correspondence to:

Dr. Roel C. Rabara

Texas A&M AgriLife Research and Extension Center

17360 Coit Road

Dallas, TX 75252

E-mail: roel.rabara@tamu.edu

or

Dr. Paul J. Rushton

Texas A&M AgriLife Research and Extension Center

17360 Coit Road

Dallas, TX 75252

E-mail: paul.rushton@tamu.edu