Group VII Ethylene Response Factors in Arabidopsis: Regulation and Physiological Roles[1][OPEN]

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The ethylene response factor (ERF) family of plant-specific transcription factors (TFs) comprises a large number of elements with diversified functions in terms of hormone responses, development, and biotic and abiotic stress responses (Dey and Corina Vlot, 2015; Licausi et al., 2013). Of these, group VII ERFs form a phylogenetic cluster (Nakano et al., 2006), which is conserved across angiosperms (Licausi et al., 2011a). One universal function attributed to ERF-VII TFs in higher plants is to coordinate their signature response to oxygen deficiency, which consists of the accumulation and enhanced selective translation of a core set of transcripts (Mustroph et al., 2009, 2010; Branco-Price et al., 2008). These transcripts are responsible for reshaping cell metabolism for sustained energy production, energy saving, the protection of subcellular components, and the detoxification of harmful anaerobic metabolism products.

The relationship between ERF-VII members from various plant species and their tolerance to low-oxygen (hypoxia) stresses has been widely reported. Different rice types rely on *ERF-VII* genes to develop contrasting strategies of underwater growth arrest (Xu et al., 2006) or shoot elongation (Hattori et al., 2009), which equally ensure stress endurance. ERF-VIIs are exploited to (1) convert the signal arising from ethylene entrapment in submerged tissues into the gibberellin-mediated regulation of carbohydrate metabolism (Fukao and Bailey-Serres, 2008; Hattori et al., 2011; van Veen et al., 2013), (2) protect plants from concurrent redox stress, and (3) prepare them for postsubmergence dehydration by enhancing ABA sensitivity (Bailey-Serres et al., 2012). Additionally, the over-expression and stabilization of ERF-VII proteins can enhance hypoxia survival in Arabidopsis (*Arabidopsis thaliana*) and barley (*Hordeum vulgare*; Hinz et al., 2010; Licausi et al., 2010; Gibbs et al., 2011; Mendiondo et al., 2016).

The molecular mechanisms by which varying oxygen levels regulate the activity of the ERF-VII factors have been most extensively investigated in Arabidopsis (Fig. 1), where the subfamily is composed of three highly expressed (*AtRAP2.2/2.3/2.12*, RELATED TO AP-2, "RAP-type") and two hypoxia-inducible *ERF-VII* genes (*AtHRE1/2*, HYPOXIA RESPONSIVE ERF, "HRE-type"; Licausi et al., 2010). The feedback repression of RAP2.12 by the anaerobic transcription factor HRA1 suggests that the ERF-VII activity is tightly modulated to grant transcriptional flexibility in response to fluctuations in oxygen availability (Giuntoli et al., 2014). With the noticeable exception of OsSub1A, ERF-VIIs are directly regulated by oxygen, in that their protein half-life is determined by an oxygen-dependent mechanism of proteasomal degradation, which prevents their nuclear accumulation in the presence of oxygen (Gibbs et al., 2011; Licausi et al., 2011a). RAP-type factors seem to operate as redundant activators of the anaerobic response (Bui et al., 2015; Papdi et al., 2015; Gasch et al., 2016). One of them, RAP2.12, has been shown to be stored at an inactive site (the plasma membrane) under aerobic conditions and to move to the nucleus after short-term hypoxia (Kosmacz et al., 2015). This mechanism is believed to ensure plant cells a fast response to oxygen shortages.

Although the direct regulation of the ERF-VIIs by oxygen has been revealed, submergence is a complex

ADVANCES

- N-terminal cysteine oxidation of ERF-VII proteins is enzymatically controlled by specific cysteinyl dioxygenases, the PCO family enzymes, as a prerequisite for substrate protein processing via the Arg-Cys/N-end rule pathway.
- The ERFVIIs bind anaerobic gene promoters through a novel cis-acting element, HRPE, which is different from the canonical GCC-box element recognized by other AP-2 domain-containing ethylene response factors.
- The first ERF-VII partner proteins have been identified and are involved in transcription (Med25, BRM), hormone signaling (GAI, PP2C), and proteolysis (SINAT2). The investigation of these interactions broadens the perspective of ERF-VII regulation beyond their direct dependence upon O^2 availability.

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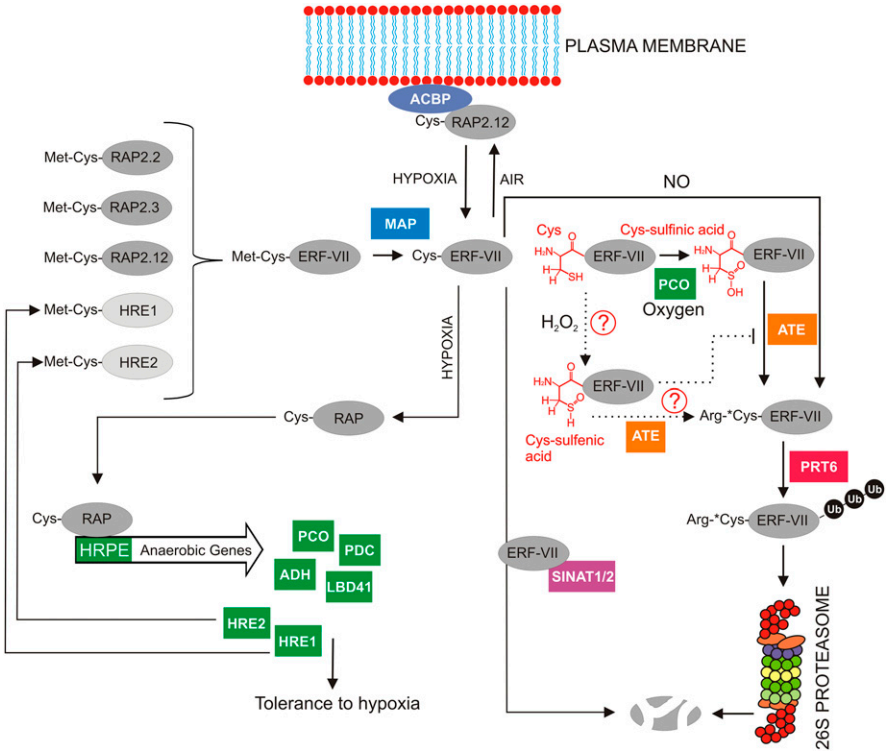

Figure 1. Overview of the regulation of group-VII ERF factor stability in Arabidopsis. The stability of plant ERF-VII proteins is controlled by intracellular O_2 and NO levels by means of the Arg-Cys/N-end rule pathway (NERP). The N-terminal Cys, exposed upon Met cleavage by MAP (Met aminopeptidase) enzymes, is susceptible to oxidation. Arginyl transferase (ATE) enzymes conjugate oxidized Cys (*Cys) to Arg, which in turn recruits the Arg-specific N-recognin PRT6 (Garzón et al., 2007), an N-end rule pathway-specialized E3 ubiquitin ligase that labels the substrate for degradation through the 26S proteasome (see Box 1 for additional details of the pathway). Cys oxidation can be promoted by specific thiol oxygenases called plant Cys oxidases (PCOs): In the presence of oxygen, PCOs convert Cys into Cys-sulfinic acid, which acts as an ATE substrate (White et al., 2017). Therefore, PCOs target ERF-VII proteins to the proteasome in an oxygen-dependent fashion. Besides oxygen, nitric oxide (NO) also promotes ERF-VII turnover via the Arg-Cys/NERP, through a still-undetermined Cys-dependent mechanism (Vicente et al., 2017; Gibbs et al., 2014). Finally, although N-terminal Cys reactivity to hydrogen peroxide (H_2O_2) has not been assessed in ERF-VII proteins, *Cys forms generated in relation to H_2O_2 concentration (García-Santamarina et al., 2014) could in principle play a role in the pathway, by either working as an alternative ATE substrate or interfering with Cys-sulfinic acid catalysis. Plasma membrane localization has been observed for RAP2.3 (Abbas et al., 2015) and RAP2.12 (Giuntoli et al., 2017; Kosmacz et al., 2015). As the latter has been found to be associated with peripheral membrane proteins belonging to the ACBP (Acyl-CoA binding protein) family, it has been proposed that this interaction is useful to maintain an inactive pool of RAP2.12 factor at the plasma membrane (Licausi et al., 2011a). ERF-VIIs have a primary role as master activators of the hypoxic metabolism. ERF-VII transcription factors (represented in this figure by the five subfamily members from Arabidopsis, AtRAP2.2/2.3/2.12 and AtHRE1/2) exert direct control on the hypoxia-inducible expression of plant anaerobic genes by binding an HRPE (hypoxia response promoter element) motif present in their promoters (e.g. *ADH*, *PDC*, *LBD41*, *HRE1* and *HRE2*, and *HRA1*; Gasch et al., 2016). The hypoxia-inducible factors HRE1 and HRE2 are further controlled at the posttranscriptional level through the Arg-Cys/NERP (Gibbs et al., 2011). During hypoxic regulation, HRA1 acts as a feedback repressor of anaerobic gene expression, by interaction with RAP2.12 (Giuntoli et al., 2014). The SINAT pathway, which is an N-end rule pathway-independent proteolysis, is also shown. RAP2.12 can be ubiquitinated by the E3 ligases SINAT1/2 (Papdi et al., 2015). These proteins modulate the autophagy pathway and thereby enhance Arabidopsis tolerance to nutrient starvation (Qi et al., 2017). In fact, autophagy responses are also activated during hypoxia and contribute to plant submergence tolerance (Chen et al., 2015, 2017a and 2017b). This evidence suggests that an additional tier of regulation might connect the ERF-VIIs to submergence responses, through the SINAT factors. Solid lines refer to experimentally established reactions or relationships, dashed lines to hypothetical relationships drawn from observed regulation, and dotted lines depict hypothetical reactions.

stress, and its regulation has not been fully understood. In fact, the flooding response in plants entails the integration of manifold stimuli, represented by hormone signals, reactive oxygen species (ROS) signatures, carbohydrate levels, redox indicators, pH variations, and second messengers. In addition to perceiving changes in oxygen levels, ERF-VIIs are expected to collect part of such vast signaling network. For instance,

the proteasomal turnover of RAP-type AtERF-VIIs has been proposed to be mediated by an additional, oxygen-independent mechanism brought about by the RING finger E3 ligases SINAT1 and SINAT2 (SEVEN IN ABSENTIA OF ARABIDOPSIS 1 and 2; Welsch et al., 2007; Papdi et al., 2015; Fig. 1). Since SINAT1/2 are bridged to starvation and autophagy (Qi et al., 2017), perception of low nutrient conditions, established upon oxygen deprivation, could hypothetically converge on ERF-VII regulation through the SINAT pathway.

An overview of ERF-VII functions has been made by Gibbs et al. (2015). In this update, we highlight the most recent findings regarding N-terminal modifying mechanisms, DNA binding properties, and protein interactions of the Arabidopsis ERF-VII factors. We also highlight the perspective that additional layers of regulation, beyond their oxygen sensitivity, might contribute to expanding the range of their physiological functions.

CONVERGENCE OF REGULATORY MECHANISMS AT THE ERF-VII PROTEIN N TERMINI

Plant Cys Oxidases

Phylogenetic analysis of ERF-VII orthologous proteins in higher plants highlighted the existence of a highly conserved N-terminal NH_2-MCGGAII-COOH sequence (Licausi et al., 2011a). Initial recognition of plant ERF-VII proteins as potential oxygen-sensitive substrates entailed the finding that this consensus contains a redox-sensitive Cys, in such a position as to be amenable to recognition by a specialized proteolytic pathway, known as the Arg-Cys/N-end rule pathway (NERP) for proteasomal degradation (Gibbs et al., 2011; Licausi et al., 2011a). A sequence of orderly reactions involves ERF-VII proteins (Box 1; Graciet et al., 2009; Tasaki et al., 2012; Xiao et al., 2010). Mature ERF-VII proteins expose an N-terminal Cys (Cys2), which functions as a degradation signature ("N-degron") targeting these proteins to the 26S proteasome, upon sequential recruitment of arginyl transferase and E3 ubiquitin ligase enzymes (Bachmair et al., 1986; Varshavsky 2011). In Arabidopsis, the latter enzymatic functions are represented by ATE1/2 (ARGINYLTRANSFERASE 1 and 2) and PRT6 (PROTEOLYSIS-6) proteins, respectively (Fig. 1).

The existence of a conserved oxygen-dependent N-degron on the ERF-VII factors represents a promising link between cellular oxygen levels and those coordinated transcriptional adjustments that constitute the hallmark of plant hypoxic responses. Despite this, only recently has light been shed on the mechanism by which the Arg-Cys/N-end rule is initiated in response to oxygen. A family of plant-specific metalloproteins, named plant Cys oxidases (PCOs), has been found to be related to Cys2 oxidation in ERF-VII proteins. *PCOs* encompass five members in Arabidopsis, two of which (*PCO1/2*) are part of the core response to low oxygen (Mustroph et al., 2009 and 2010). Genetic dissection of the Arg-Cys/N-end rule pathway has demonstrated that *PCO1/2* act upstream of *ATE1/2* and *PRT6* to redundantly repress anaerobic gene induction under hypoxia. In line with this, individual overexpression of either gene determines lower tolerance to submergence (Weits et al., 2014). In the plant, PCO enzymes impact ERF-VII protein levels, in that the stability of AtRAP2.12 correlates negatively with *PCO1/2* expression (Weits et al., 2014). In addition, in vitro evidence suggests that PCOs influence ERF-VII in vivo turnover by direct Cys2 oxidation. Purified recombinant PCO enzymes consume molecular oxygen in the presence of either L-Cys or synthetic peptides corresponding to AtRAP2.12 N terminus (Weits et al., 2014) and catalyze the reaction of N-terminal Cys to Cys-sulfinic acid ($CysO_2$; White et al., 2017). A recombinant plant ATE1 enzyme can also conjugate Arg to a synthetic NH_2-CGGAIISDFI-COOH peptide, derived from the AtRAP2.12 N terminus, only in the presence of both PCO and oxygen. This thus provides proof for the generation of an active oxygen-sensitive N-degron on such substrates (White et al., 2017).

These milestone studies lay the foundation for a model of plant O_2-sensor switch in vivo. Plant PCOs qualify as the first cysteinyl dioxygenase enzymes discovered, since before that date only bacterial and mammalian Cys dioxygenases had been known, which promote free L-Cys conversion, contributing to its homeostasis to prevent cytotoxicity (Dominy et al., 2006; Ye et al., 2007). In animals, the existence of enzymatic activities mediating N-terminal Cys oxidation is debated (Kwon et al., 2002; Hu et al., 2005). Therefore, the recent findings regarding PCOs have opened a fascinating perspective on the diversification of the Arg/N-end rule pathway among kingdoms. Despite the connection established between Cys oxidation and N-terminal protein arginylation (Hu et al., 2005), knowledge regarding the targets of the specialized Arg-Cys/NERP branch in mammals is limited to the RGS4/5 proteins (REGULATOR OF G-PROTEIN SIGNALING; Lee et al., 2005), activators of G-protein α subunits in cardiomycetes (Lee et al., 2012), and to the proapoptotic protein BRCA1 (Piatkov et al., 2012). Unlike plants, no target of this pathway has been associated with hypoxic responses, which instead rely on an unrelated, albeit functionally parallel, mechanism regulating the oxygen-sensitive TF HIFα (HYPOXIA-INDUCIBLE FACTOR 1α; Jaakkola et al., 2001). It is tempting to speculate that the specific evolution of an enzymatic control point for Cys oxidation in the plant kingdom, represented by PCOs, enabled plants to couple the N-end rule with oxygen sensing.

Cys oxidases are nonheme iron-dependent oxygenases, which make use of a coordinated iron ion to activate oxygen during catalysis, forming a putative Fe(III)-superoxo intermediate (White and Flashman, 2016). Given this enzymatic requirement, PCOs qualify as a potential convergence point between iron and low

BOX 1. ERF-VII Factors as N-end Rule Substrates

Group-VII ERFs were the first plant substrates of the Arg/N-end rule pathway (NERP) to be discovered. The NERP is a proteolytic system that promotes the turnover of proteins containing N-terminal sequences (N-degrons) that work as degradation signals (Bachmair et al., 1986). Substrate proteins are degraded through the proteasome, following polyubiquitination by specific N-degron-recognizing E3 ubiquitin ligases (N-recognins). Primary destabilizing residues are directly targeted by E3 ligases. They consist in basic or bulky hydrophobic residues (Arg/NERP; Varshavski, 2011), acetylated residues (Ac/NERP; Hwang et al., 2010), and proline (Pro/NERP; Chen et al., 2017). Secondary and tertiary destabilizing residues, instead, can be converted into primary ones upon enzymatic modifications, such as N-terminal deamidation (Gln, Asn), arginylation (Glu, Asp, *Cys), acetylation (Gly, Ala, Ser, Thr, Val, Cys), or oxidation (Cys). Therefore, the NERP assumes a hierarchical architecture, which is conserved across eukaryotes (Tasaki et al., 2012).

ERF-VII proteins enter the Arg/NERP upon co-translational Met cleavage, enabled by the small side chain of the neighboring Cys2, which matches the substrate specificity of methionine aminopeptidases (MAPs; Xiao et al., 2010). Exposed Cys behave as tertiary destabilizing residues, being converted into oxidized cysteine (*Cys, secondary destabilizing residue) in the presence of oxidizing agents, such as O2, NO, and hypothetically, ROS (Fig. 1). Chemical similarity between acidic residues and *Cys underlies its recognition by plant arginyl transferases (ATE1/2, in Arabidopsis; Graciet et al., 2009), triggering Arg conjugation and subsequent targeting by the Arg-specific E3 ligase PRT6 (Proteolysis 6; Garzón et al., 2007). Therefore, Cys2 oxidation provides a link between oxygen and NERP regulation, in plants as well as in animals (Hu et al., 2005).

Direct Cys-dependent ERF-VII regulation by the proteasome was first shown in heterologous rabbit reticulocyte lysate assays (Gibbs et al., 2011). In vivo, the amenability of ERF-VII proteins to Arg-Cys/NERP regulation has been mainly investigated by the expression of reporter substrates or HA-tagged ERF-VIIs. MC-ERF-VII-HA over-expressors have been used for immunological detection, to display protein dynamics (1) under hypoxia and post-stress re-oxygenation, (2) in the prt6 mutant (Gibbs et al., 2011), and (3) after NO manipulation by chemical treatments or genetic impairment of NO biosynthesis (Gibbs et al., 2014). Alternatively, the impact of these conditions on ERF-VII stability has been shown by means of MC-GUS and UBI-C-GUS histochemical reporters (Vincente et al., 2017; Gibbs et al., 2014). MC-GUS consists in a translational fusion between an ERF-VII-derived Cys-N-degron and the β-glucuronidase reporter, UBI-C-GUS in a ubiquitin-Cys reporter fusion, from which an N-terminal Cys-reporter is generated by ubiquitin-specific endoproteases (Garzón et al., 2007).

oxygen signaling. There is a partial overlap in the transcriptional adjustments between low Fe and hypoxia, possibly as a consequence of a higher energy demand, due to a compensatory increase in iron uptake, which enhances the mitochondrial activity (López-Millán et al., 2000; Vigani, 2012). Hypoxia also stimulates the expression of Fe deficiency genes, with the involvement of the ethylene-responsive TFs EIN3/EIL1 (ETHYLENE-INSENSITIVE 2/EIN3-LIKE 1; García et al., 2014; Lucena et al., 2015). In turn, it has further been shown that ethylene-mediated iron homeostasis involves EIN3/EIL1 interaction with the subunit Med25 of Mediator (a large multiprotein complex that bridges cis-element-bound TFs and the basal transcriptional machinery; Dolan and Chapple, 2017; Yang et al., 2014). In the human body, the oxygen-sensing pathway contributes to iron homeostasis and deficiency responses with the action of HIF prolyl hydroxylase (P4H) enzymes (Salahudeen and Bruick, 2009; Anderson et al., 2013; Siegert et al., 2015). P4Hs are nonheme Fe- and 2-OG-dependent oxygenases that work as sensors for oxygen in metazoans, in the same way as the phylogenetically unrelated PCOs do in plants. While a role in iron responses has been proposed for plant P4H homologs (Vigani et al., 2013), no connection has yet been proposed between these processes and PCOs. Given the impaired metal uptake capability by waterlogged root systems (Martínez-Cuenca et al., 2015), a mechanism might have been evolved in plants to integrate the perception of hypoxia with intracellular metal ion homeostasis. Hypothetically, PCO activity could be regulated through metal ion switching at its active site, thereby providing a proxy for the intracellular Fe status, which has been proposed as being estimated from its balance with other transition metal ions (Kobayashi and Nishizawa, 2014).

PCOs also represent a potential node in the interplay between O_2 signaling and ROS homeostasis. In mammalian cells, ROS, produced during hypoxia by the malfunctioning of Complex III at the mitochondrial electron transport chain indirectly impact on HIFα stability by inhibiting the activity of P4Hs (Chandel et al., 2000; Bell et al., 2007). It would therefore be interesting to investigate whether a similar mechanism acted on the functionally equivalent PCO enzymes.

N-Terminal Cys Modifications

Cys2 in ERF-VII TFs is a regulatory Cys (Formenko et al., 2010; Couturier et al., 2013). Its thiol group is highly susceptible to oxidation (Reddie and Carroll, 2008) by oxygen, ROS, and reactive nitrogen species, and it can be used in a variety of redox reactions (Giles

et al., 2003) and can undergo additional enzymatic modifications, such as S-acylation and N-acetylation (Polevoda and Sherman, 2003; Hwang et al., 2010). Therefore, Cys2 has the potential to accept diversified signaling pathways, promoting the integration of manifold stimuli, and might have been selected accordingly (Marino and Gladyshev, 2010).

Low H_2O_2 concentrations, compatible with the signaling range, promote Cys oxidation to Cys-sulfenic acid (CysO) (Schieber and Chandel, 2014). CysO can be reverted to thiolate by disulfide reductases, thioredoxin and glutaredoxin, or act as an intermediate for disulfide bond formation (Poole, 2015). Instead, higher H_2O_2 levels, generated under ROS stress, can push oxidation to Cys-sulfinic ($CysO_2$) or irreversibly further to Cys-sulfonic forms ($CysO_3$; Schieber and Chandel, 2014; Fig. 1). Once viewed as a transient species in disulfide bond production, CysO has been found to be significant for catalysis and protein functionality (Gupta and Carroll, 2014). Thiol-disulfide transitions, on the other hand, are frequently associated with redox regulation in plants. For instance, intra- or intermolecular Cys-Cys formation is believed to determine the nuclear localization of the redox TF HSFA8 in response to H_2O_2 (Giesguth et al., 2015). Despite the fact that redox-sensitive nonclustered cysteines have been identified and examined in plant transcription factors before (Schmidt and Schippers, 2015), they have never been found to belong to the set of Met-Cys initiating ("MC") proteins. The fact that the conversion of a synthetic RAP2.12-derived peptide released $CysO_2$ as the sole reaction product in vitro (White et al., 2017) does not rule out that additional, biologically significant, oxo-species may be formed by the ERF-VII N termini in the cellular environment and contribute to their half-life, localization, or function.

In the case of the mammalian GTPase-activating proteins RGS4/5, a nonenzymatic S-nitrosylation reaction has been proposed to precede Cys2 oxidation. The biological chemistry of *S*-nitroso-Cys has not yet been fully clarified (Gould et al., 2013). Although in vitro PCO can process Cys2 in the absence of NO, i.e. without previous S-nitrosylation (White et al., 2017), a role for NO in the degradation of the ERF-VIIs has been observed in vivo. In fact, NO has been shown to promote an N-end rule pathway-dependent proteolysis of full-length ERF-VII factors, as well as artificial ERF-VII-derived Arg-Cys/NERP substrates (MC-GUS, UBI-C-GUS) in Arabidopsis and barley (Gibbs et al., 2014; Vicente et al., 2017). However, the mechanism connecting NO to ERF-VII stability still needs to be revealed (Gibbs et al., 2015; Fig. 1).

Exposure of an amino-terminal Cys is a requisite for implementing N-end rule reactions. Thus, even prior to Cys2 modification, regulation of the N-terminal Met cleavage could represent a general mechanism affecting MC-protein stability. Human MetAP2 activity responds to the cytosolic redox state through the thioredoxin-dependent conversion of a Cys_{228}-Cys_{448} disulfide bond (Chiu et al., 2014). Such evidence provides a potential link between cellular ROS content and, ultimately, the availability of MC proteins for N-end rule pathway-mediated degradation.

The functions of ERF-VII Cys2 might be extended by the covalent attachment of lipid moieties (lipidation). This process has emerged as a major regulatory mechanism in a variety of subcellular responses in animals, yeast, and, more recently, plants. In fact, the conjugation of a hydrophobic moiety can have a strong impact on the structure, interaction, and, primarily, membrane targeting of soluble proteins (Aicart-Ramos et al., 2011; Hemsley, 2015). Cysteines can establish dynamic thioesther bonds with fatty acids (S-acylation), by the action of endomembrane-associated protein S-acyltransferase and palmitoyl thioesterase enzymes (Hang and Linder, 2011).

Cys2 palmitoylation has been demonstrated for human RGS proteins (De Vries et al., 1996). Cys2 mutation does not affect RGS4 direct association with phospholipid bilayers (Srinivasa et al., 1998), which is in fact due to the folding of its N terminus in an alpha-helical structure (Bernstein et al., 2000). However, Cys2 palmitoylation is crucial for RGS4 and RGS16 activity, possibly by affecting their affinity for Gα target subunits (Druey et al., 1999; Tu et al., 1999).

AtRAP2.12 has been found to reside at the plasma membrane in aerobic leaf cells (Fig. 1), unless its N-terminal domain is ablated (Licausi et al., 2011a; Giuntoli et al., 2017). In turn, ERF-VII association with the plasma membrane is believed to depend on acyl-CoA binding proteins, according to the observed interaction between members of the two families (Li and Chye, 2004; Li et al., 2008; Licausi et al., 2011a). During hypoxia, AtRAP2.12 is quickly displaced from the plasma membrane toward the nucleus (Kosmacz et al., 2015). Given that the regulation of fatty acid profiles has been associated with hypoxic stress (Klinkenberg et al., 2014; Xie et al., 2015a; Xie et al., 2015b), the evidence available can be the basis of a speculative model of hypoxia sensing, in which the dynamic acylation state of the ERF-VII TFs collects indirect low oxygen signals to regulate their intracellular trafficking and activity.

LATEST INSIGHTS INTO THE TRANSCRIPTION FACTOR PROPERTIES OF THE ERF-VIIS

Longstanding efforts have focused the quest for DNA regulatory elements that enable plants to coordinate the activation of low-oxygen responsive promoters, under the assumption that coexpressed promoters should share common features, thus mediating their recognition by the transcriptional machinery (Rombauts et al., 2003).

In vitro DNA-binding assays associate ERF family members with GCC-box motifs (5′-AGCCGCC-3′; Ohme-Takagi and Shinshi, 1995; Hao et al., 1998), which have been traced out in many genes induced by ethylene, pathogenesis, wounding, or jasmonate (Brown et al., 2003; Zarei et al., 2011). However, the fact

that GCC-boxes have not been recognized as part of the anaerobic response promoter element (known as ARE; Olive et al., 1990) raised the question as to whether group VII ERFs had a different DNA binding affinity. AtRAP2.2 was isolated as an interactor of the unrelated 5′-ATCTA-3′ motif, claimed to contain the minimum determinant for ERF-VII DNA binding (Welsch et al., 2007). Although present in the 5′-upstream sequence of many anaerobic genes (Licausi et al., 2011b), evidence presented by Gasch et al. (2016) indicates that this motif is not likely to be responsible for their activation by the anaerobic TFs.

A promising candidate as a functional anaerobic promoter element in plants was recently revealed. Phylogenetically related core anaerobic genes from 25 species were compared, and clustering of the detected DNA motif patterns resulted in nine conserved consensus sequences (Gasch et al., 2016). Of these, a 12-bp-long bipartite motif composed of GC- and GT-rich halves was validated as a regulator of the anaerobic targets *LBD41* and *PCO1* from Arabidopsis and named hypoxia-responsive promoter element (HRPE; Fig. 1). The resemblance of HRPE to the previously annotated ARE sequence from maize (*Zea mays*), and the demonstration that RAP-type ERF-VIIs were able to bind a synthetic ARE promoter, suggest that an actual connection point has been found between the long-sought after plant hypoxia response element and the ethylene responsive factors.

Two of the other conserved motif clusters matched known regulatory elements, namely the ABA-responsive element and the GCC-box. The detection of the ABA-responsive element, which harbors a G-box element, complies with previous reports on the involvement of G-box-binding bZIP factors in the regulation of the *ADH* promoter (McKendree and Ferl, 1992; Meier and Gruissem, 1994; de Bruxelles et al., 1996). The enrichment of core anaerobic promoters with GCC-boxes also suggests that the ERF-VII factors retained the ability to bind this canonical motif. The experimental reports on the relationship between ERF-VIIs and GCC-boxes are not all in agreement. A direct interaction was identified between AtRAP2.3 and a GCC-box-containing promoter region of the *ABI5* gene (Gibbs et al., 2014), and RAP2.3 was able to transactivate a synthetic promoter containing tandem GCC-box copies from *HOOKLESS1* (Marín-de la Rosa et al., 2014). However, a yeast one-hybrid experiment failed to detect an interaction between AtRAP2.2 and a prey construct composed of tandem copies of a GCC-box when flanking nucleotides from the Arabidopsis *PDF1.2* promoter were included (Ou et al., 2011). Finally, the binding of AtHRE2 to a synthetic GCC-box probe was shown to occur in vitro by Lee et al. (2015).

The mechanism of ERF-VII interaction with HRPE still awaits an experimental description. Unlike RAP-type proteins, HRE1 and HRE2 have not proven capable of activating natural anaerobic promoters (Bui et al., 2015). Their inability to associate with HRPE (Gasch et al., 2016) points to a deficiency in DNA binding by either HRE1 or HRE2, despite the presence of a fully conserved AP2/ERF domain (Nakano et al., 2006). This would imply more specific needs for HRPE recognition than those provided by this well-characterized DNA-binding domain. In other words, additional protein domains, exclusively present on RAP-type ERF-VIIs, might be necessary for the interaction with HRPE. One hypothesis is that the AP2/ERF domain might contact the GC-rich region of HRPE (Yang et al., 2009), whereas neighboring amino acids would mediate the interaction with the GT portion.

Specific DNA-binding properties might underlie the functional diversification reported for the Arabidopsis ERF-VII factors under hypoxia. Here, HREs are needed to sustain anaerobic gene expression but are not essential for the initiation of transcriptional responses (Licausi et al., 2010). Rather than associating with target promoters, HREs may contribute a transcriptional activation function to—still unresolved—protein complexes that form after a primary, RAP-type-dependent, response to low oxygen. In fact, HREs share a conserved C-terminal hydrophobic motif with the other ERF-VII factors, called CMVII-8 (van Veen et al., 2014). CMVII-8 is sufficient to confer transactivation properties, when fused to a GAL4 DNA-binding domain (Bui et al., 2015) or evaluated inside native AtRAP2.2/12 proteins (Licausi et al., 2011a). Thus, ERF-VIIs generally qualify as activators.

The isolation of RAP2.12 as a partner of the Med25 subunit of the Mediator complex suggests that the recruitment of RNA polymerase II by the ERF-VIIs occurs through their interaction with particular Mediator (Med) proteins (Ou et al., 2011). The degree of specialization of Med proteins, in terms of TF interaction preferences, is assumed to be low, because of the limited number of Med subunits encoded by the proteome (the approximate proportion in Arabidopsis is 30 as against more than 1,500 TFs). However, the observation that individual mutated subunits produce particular—although pleiotropic—phenotypes implies the existence of specific functions (Samanta and Thakur, 2015). Med25 has emerged as a master regulator in plants (Kazan, 2017), involved in hormone signaling, iron homeostasis, flowering regulation, and abiotic stress responses. Whether it plays a role in hypoxic responses still needs to be investigated.

EXPANDING ROLES OF ERF-VII FAMILY FACTORS

Growing evidence supports ERF-VII involvement in transcriptional adjustments that go beyond the activation of a set of conserved hypoxia-responsive genes (Mustroph et al., 2010). The recent literature links these proteins to developmental processes controlled by ABA, ethylene, and gibberellin, to abiotic stress tolerance and resistance to fungal attack. Participation of the ERF-VII in some of these physiological phenomena might in principle be enabled by the existence of hypoxic microenvironments in plant tissues. On the other

hand, ERF-VII functionality seems to be expanded through additional, low-oxygen-independent mechanisms that subtract these TFs from aerobic degradation and confer them condition-, tissue-, or cell-specific stability, expression, and activity.

ERF-VII Involvement in ROS- and NO-Dependent Responses

ERF-VIIs can impact on plant responses under various abiotic and biotic stress conditions with oxidative stress components. In Arabidopsis, RAP-type ERF-VIIs participate in oxidative and osmotic stress tolerance (Papdi et al., 2015), *AtRAP2.2* expression is positively correlated with plant resistance to the necrotrophic fungus *Botrytis cinerea* (Zhao et al., 2012), and AtHRE2 contributes to salinity and osmotic stress tolerance (Park et al., 2011). Ectopic expression of ERF-VII homologous sequences in several plant species triggers protective responses against dehydration, salt, mannitol, heavy metals, heat, as well as against a wide range of tested pathogens (for review, see Gibbs et al., 2015). Constitutive *ERF-VII* gene expression relates to more sustained activation of ROS scavenging reactions, and, conversely, mutations inside the gene family lead to elevated ROS under stress. Therefore, prompt and sustained ROS scavenging is believed to account for the positive impact of ERF-VII overexpression in those conditions entailing oxidative stress (Ogawa et al., 2005; Tang et al., 2005; Park et al., 2011; Yao et al., 2017; Vicente et al., 2017).

ROS production is enhanced under low-oxygen conditions (Steffens et al., 2013). Thus, the same protective mechanisms can act as an integral part of the ERF-VII dependent response strategy to hypoxia. Improved ROS management has been associated with superior submergence tolerance in maize and *Brachypodion distachyon* (Campbell et al., 2015; Rivera-Contreras et al., 2016), while the activation of antioxidant responses in the posthypoxic phase is crucial for survival in Arabidopsis (Paradiso et al., 2016; Yuan et al., 2017). Interestingly, a target of RAP-type ERF-VIIs, namely *HYPOXIA RESPONSIVE UNIVERSAL STRESS PROTEIN1*, has been shown to coordinate oxygen sensing by PCO/RAP2.12 with H_2O_2 production by NADPH oxidases, indicating that there is a network connecting diverse signaling pathways downstream of ERF-VII targets (Gonzali et al., 2015). At the same time, ERF-VII involvement in the regulation of stress responses occurring under oxygen-replete conditions implies that the posttranslational system controlling their stability depending on oxygen is intertwined with additional regulatory mechanisms. The Arg-Cys/NERP has recently been proven to promote Arabidopsis and barley tolerance to salinity, drought, and heat (multiple abiotic stresses sharing an oxidative stress component) through ERF-VIIs. It has been proposed that, during salinity, ERF-VII proteins become stabilized following a decline in nitrate reductase activity and subsequent decrease in NO levels (Vicente et al., 2017).

NO is an elusive gaseous signal involved in a range of plant stress and developmental responses, including hypoxia (Pucciariello and Perata, 2017). Reports regarding the influence of nitric oxide on a plant's ability to cope with hypoxia are controversial (Perazzolli et al., 2004; Gupta and Igamberdiev, 2016; Mira et al., 2016; Peng et al., 2016), making it hard to draw conclusions regarding the impact of the described regulation under hypoxia. However, ERF-VII behavior as novel NO sensors has made it possible to connect them to the physiology of this gaseous signal. NO levels have been manipulated in *prt6* and in combinatorial *prt6erfvii* mutants, in order to reveal the ability of stabilized ERF-VII factors to mediate specific responses. It has been found that germination, inhibited hypocotyl elongation in the dark, and stomatal closure responses are promoted by NO as a result of the degradation of the constitutively expressed ERF-VIIs (Gibbs et al., 2014). Detailed examination of the NO-dependent release of seed dormancy has shown that a repressor of germination, *ABI5*, is directly targeted by the ERF-VII (Gibbs et al., 2014). This further qualifies ERF-VIIs as novel players in the antagonistic interplay between ABA and NO during germination, which has already been found to converge on ABI5 through S-nitrosylation of a regulatory moiety that facilitates its proteasomal degradation (Albertos et al., 2015). Another investigated process prone to NO regulation is the repression of the apical hook opening during seedling skotomorphogenesis. In this case, all subfamily members have been found to be active (Abbas et al., 2015), suggesting that the involvement of HRE-type factors in the control of other physiological responses might be restrained by specific regulatory mechanisms.

ERF-VII in Plant-Pathogen Interactions

Genetic approaches have recently highlighted the participation of group VII ERFs in responding to biotrophic pathogens and to the necrotroph *B. cinerea*. The mechanisms enabling ERF-VII stabilization and operation in both phenomena have not been revealed yet. In the case of the tumorigenic pathogens *Plasmodiophora brassicae*, *Meloydogyne japonica*, and *Agrobacterium tumefaciens*, infection has been observed to up-regulate fermentative genes, along with a significant proportion of ERF-VII and Arg-Cys/NERP targets (Gravot et al., 2016). In addition, root gall formation, caused by the protist *P. brassicae* in the secondary infection phase, is enhanced by ERF-VII stabilization (Gravot et al., 2016). Switching on the hypoxic metabolism, downstream of the ERF-VIIs, may benefit gall-forming pathogens (Gravot et al., 2016).

During Arabidopsis interaction with *B. cinerea*, instead, RAP2.2 participates in plant defense downstream of ethylene signaling (Zhao et al., 2012), along with its partner Med25 (Ou et al., 2011; Fig. 2A). Since the occurrence of hypoxia during such a pathogen attack has been ruled out (Zhao et al., 2012), a few

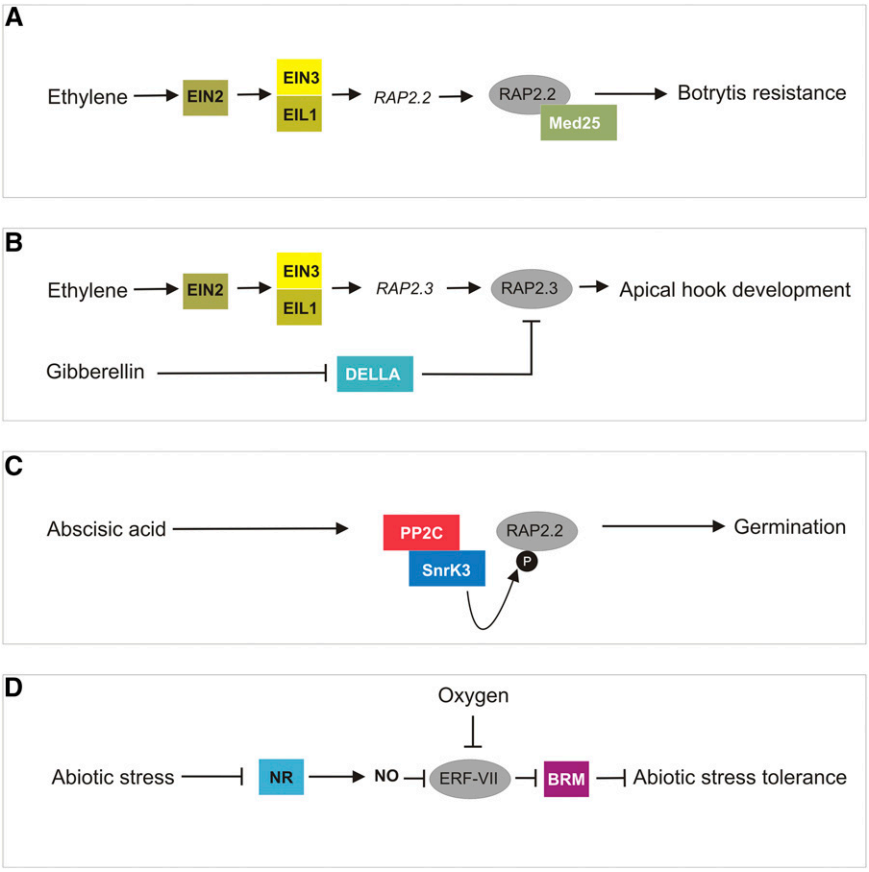

Figure 2. Additional roles of ERF-VIIs through interaction with distinct protein partners. A, Resistance to necrotrophic fungi (Zhao et al., 2012). After *B. cinerea* infection, ethylene accumulation leads to *RAP2.2* gene induction downstream of the EIN2-EIN3/EIL ethylene signaling cascade. RAP2.2 activates the resistance genes *PDF1.2* and *ChiB* by interaction with its partner Med25 (Ou et al., 2011) and contributes positively to Arabidopsis resistance to fungal attack. B, RAP2.3 is a positive regulator of apical hook development in Arabidopsis seedlings, and its action is counteracted by interacting DELLA proteins (Marín-de la Rosa et al., 2014). In etiolated seedlings, *RAP2.3* gene expression is promoted by dark-induced ethylene production, while low levels of DELLA proteins prevent RAP2.3 functional restriction. Therefore, RAP2.3 participates in the interplay between ethylene and GA, which regulates apical hook formation (Abbas et al., 2013), by hindering premature hook opening under darkness. C, During germination, RAP2.2 is as a negative regulator of ABA responses (Lumba et al., 2014). This function has been associated with RAP2.2 phosphorylation, following its interaction with an SNRK3 kinase complex that mediates ABA insensitivity. D, ERF-VII stabilization enhances plant tolerance to multiple abiotic stresses (Vicente et al., 2017). During salinity, decreased NO biosynthesis due to NR enzyme impairment has been proposed to lead to ERF-VII protein stabilization in the presence of oxygen. The beneficial effects of the ERF-VIIs on plant tolerance to salinity is antagonized by its interacting partner BRM, possibly due to competition for the same cis-element on the target gene promoters (Vicente et al., 2017). EIN2, Ethylene-insensitive2; EIN3, Ethylene-insensitive2; EIL, EIN3-like; Med25, Mediator subunit25; DELLA, GRAS-domain family proteins (GAI, RGA, RGLs); PP2C, Protein phosphatase2C; SnRK3, SNF1-related protein kinase3; NR, nitrate reductases; NO, nitric oxide; BRM, BRAHMA ATPase.

scenarios can be put forward to explain the postulated impairment of RAP2.2 degradation. In principle, factors affecting the activity of Arg-Cys/NERP components can modify the stability of the ERF-VII proteins independently of oxygen. During infection, PCO enzymes may be sensitive to different signals, such as the redox status, ROS and NO generation, and the availability of micronutrients, as discussed previously. In such case, lower PCO activity under *B. cinerea* attack could explain why *RAP2.2* overexpression was not sufficient to up-regulate pathogenic markers in the absence of fungal infection (Zhao et al., 2012). PRT6 activity might also change in specific conditions; the synthetic R-GUS substrate could be used to visualize the PRT6 activity pattern in vivo during pathogen attack (Garzón et al., 2007).

Several ERF transcription factors promote the integration of intracellular stimuli (Müller and Munné-Bosch, 2015). RAP2.2 involvement in pathogen responses downstream of ethylene suggests that ERF-VIIs might have emerged as bridging elements of low oxygen- and immune responses (Zhao et al., 2012). In

fact, the setup of defense mechanisms is particularly appropriate in flooded plants, in which infection can be facilitated by the extent of submergence and post-submergence injury events. Submergence-triggered immunity has been observed in Arabidopsis and ascribed to one of the submergence-inducible WRKY TFs, *WRKY22* (Hsu et al., 2013). Interestingly, the observed transcriptional responses caused by constitutive WRKY22 expression are very similar to the transcriptome-level changes triggered by AtRAP2.12 stabilization under fully aerated conditions (Giuntoli et al., 2017). In this case, the removal of the oxygen-sensitive N-terminal domain and overexpression of the resulting RAP2.12 protein leads to the activation of defense markers (*WRKY* and *pathogenesis-related* genes), components of salicylic acid and ABA metabolism and ROS-responsive genes, supporting the hypothesis of pathway convergence. A defense network involving ERF-VII proteins might also include mitogen-activated kinases (MPKs) and the TF WRKY33. MPK3/6 act in retrograde signaling following mitochondrial ROS stress during hypoxia (Chang et al., 2012) and activate ERF6 (a hub of immunity, ROS, and hormone responses; Huang et al., 2016) upon *B. cinerea* attack or ROS treatment (Meng et al., 2013). WRKY33 contributes to Arabidopsis resistance to *B. cinerea* (Liu et al., 2015) and submergence tolerance (Hwang et al., 2011).

Novel Protein Interactions of the ERF-VII Factors

The previous examples indicate that, when subtracted from proteolytic degradation, the ERF-VII transcription factors can promote the activation of specific responses (e.g. to hypoxia, oxidative stresses, darkness, or pathogens). It would seem that specificity is achieved by the regulation of particular, only partially overlapping, subsets of target genes. Selection of different protein partners and recruitment in distinct multiprotein complexes could modulate ERF-VII activity. This has been illustrated by a few recent studies.

AtRAP2.3 and AtRAP2.12 associate with DELLA proteins (Marín-de la Rosa et al., 2014). DELLAs were known to prevent ethylene-induced gene expression by sequestering the transcriptional activator EIN3 (An et al., 2012). In this case, the interplay between GA signaling and ethylene is enriched by sequestration of RAP-type ERFs, shown to be downstream targets of the EIN3/EIL TFs (Hinz et al., 2010; Zhao et al., 2012). This interaction helps prevent premature apical hook opening in etiolated seedlings (Fig. 2B). Since association with the DELLA protein GAI involves the N-terminal half of RAP2.3, including its AP2/ERF DNA binding domain, it has been suggested that DELLAs regulate ERF-VII activity by hindering DNA binding, specifically to GCC-box containing ethylene- and GA-target promoters (Marín-de la Rosa et al., 2014). DELLAs may also mask the oxygen-sensitive ERF-VII domain, in such a way that the TFs would be exposed to the N-end rule pathway only in the presence of GA. Whether the protected ERF-VIIs are then available for association with other DNA motifs, i.e. not the GCC-box, requires further experimentation. This example suggests that ERF-VII abundance and promoter preferences might be reshaped in different physiological pathways, upon specific partner selection.

RAP2.2 has been revealed to be part of a PP2C-SNRK3 complex that promotes ABA insensitivity (Lumba et al., 2014; Fig. 2C). This notable study demonstrates that ERF-VII functions can be tuned by posttranslational modifications (e.g. RAP2.2 phosphorylation by SNRK3) and partner selection. Specifically, the interaction of RAP2.2 with the SNRK3.15/22 isoforms enables it to mediate negative ABA responses, acquiring an opposite function to that observed by Gibbs et al. (2015).

Finally, specificity in promoter targeting can be achieved by the constitution of different transcriptional complexes. RAP2.12 and RAP2.3 interact with BRAHMA (BRM; Vicente et al., 2017), a SWI/SNF chromatin-remodeling ATPase that, among other functions, represses ABA responses. BRM and RAP2.3 share a GCC-box binding site on the *ABI5* promoter. It has been proposed that, during salinity, the interplay between BRM and the stress-stabilized ERF-VIIs balances ABA-responsive gene expression through opposite functionalities (Fig. 2D).

CONCLUSION

Knowledge of the role of ERF-VIIs has increased significantly over the last decade. A perspective is

OUTSTANDING QUESTIONS

- Which signals and mechanisms (e.g. metal ion switching and ROS) regulate PCOs activity beyond intracellular oxygen tension?
- Can ROS and RNS impact on ERF-VII stability through the Arg/Cys-N-end rule pathway?
- Regarding the Arg-Cys/N-end rule pathway, is the development of an enzymatic control point for Cys2 oxidation a particular feature of plant evolution?
- Which mechanism shuttles AtRAP2.12 to the plasma membrane when cells are normally aerated and what mediates its release to the nucleus upon hypoxia?
- Do different protein-protein interactions determine ERF-VII specificity in stress and hormone response regulatory modules?
- What is the pattern of promoter occupancy by the ERF-VIIs at the genome-wide level?
- How is ERF-VII activity regulated by posttranslational modifications (e.g. phosphorylation, acylation, glycosylation and sumoylation) different from Cys2 oxidation?

emerging in which a diversified set of mechanisms can influence ERF-VII expression, availability, and activity in order to specify their functions in a wider network of physiological pathways activated by stress and hormones. However, important questions need to be addressed, before a more detailed picture of ERF-VII regulation is gained (see Outstanding Questions Box). The most recent observations suggest novel mechanisms enriching the role of ERF-VIIs in the hypoxic response and connecting it to additional physiological, developmental, and stress-related processes.

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