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The low oxygen, oxidative and osmotic stress responses synergistically act through the ethylene response factor VII genes *RAP2.12*, *RAP2.2* and *RAP2.3*

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SUMMARY

The ethylene response factor VII (ERF-VII) transcription factor RELATED TO APETALA2.12 (RAP2.12) was previously identified as an activator of the *ALCOHOL DEHYDROGENASE1 promoter::luciferase* (*ADH1-LUC*) reporter gene. Here we show that overexpression of RAP2.12 and its homologues RAP2.2 and RAP2.3 sustains ABA-mediated activation of *ADH1* and activates hypoxia marker genes under both anoxic and normoxic conditions. Inducible expression of all three RAP2s conferred tolerance to anoxia, oxidative and osmotic stresses, and enhanced the sensitivity to abscisic acid (ABA). Consistently, the *rap2.12-2 rap2.3-1* double mutant showed hypersensitivity to both submergence and osmotic stress. These findings suggest that the three ERF-VII-type transcription factors play roles in tolerance to multiple stresses that sequentially occur during and after submergence in Arabidopsis. Oxygen-dependent degradation of RAP2.12 was previously shown to be mediated by the N-end rule pathway. During submergence the RAP2.12, RAP2.2 and RAP2.3 are stabilized and accumulates in the nucleus affecting the transcription of stress response genes. We conclude that the stabilized RAP2 transcription factors can prolong the ABA-mediated activation of a subset of osmotic responsive genes (e.g. *ADH1*). We also show that RAP2.12 protein level is affected by the REALLY INTERESTING GENE (RING) domain containing SEVEN IN ABSENTIA of *Arabidopsis thaliana* 2 (SINAT2). Silencing of SINAT1/2 genes leads to enhanced RAP2.12 abundance independently of the presence or absence of its N-terminal degron. Taken together, our results suggest that RAP2.12 and its homologues RAP2.2 and RAP2.3 act redundantly in multiple stress responses. Alternative protein degradation pathways may provide inputs to the RAP2 transcription factors for the distinct stresses.

Keywords: ethylene response factor, RAP2, anoxia, osmotic and oxidative stress, luciferase reporter, *Arabidopsis thaliana*, SINAT.

INTRODUCTION

To survive in adverse environmental conditions, plants have evolved sophisticated regulatory systems that mount adaptation to various forms of abiotic stresses (Nakashima *et al.*, 2009; Ahuja *et al.*, 2010; Hirayama and Shinozaki, 2010; Golldack *et al.*, 2014). Recent genetic and molecular studies have led to significant advances in deciphering details of stress signalling pathways, although understanding these regulatory networks remains a challenging task. Screening for altered expression of stress-regulated reporter genes in forward genetic screens has facilitated the identification of components of abscisic acid (ABA), salt and osmotic stress signalling pathways (Chinnusamy *et al.*, 2002; Gong *et al.*, 2002; Zhu *et al.*, 2005; Aboul-Soud *et al.*, 2009). By screening an estradiol-inducible cDNA library to transactivate the *ADH1 promoter::luciferase* (*ADH1-LUC*) reporter in Arabidopsis plants, we identified RELATED TO APETALA2.12 (RAP2.12), an ethylene response factor VII (ERF-VII) family protein. We showed that *ADH1-LUC* and endogenous *ADH1* mRNA were transiently induced by ABA, which became sustained upon RAP2.12 overexpression (Papdi *et al.*, 2008).

The ERF-VII family of Arabidopsis transcription factors (TFs) consists of five members: RAP2.12 (At1g53910), RAP2.2 (At3g14230), RAP2.3 (At3g16770), Hypoxia Responsive ERF1 (HRE1; At1g72360) and HRE2 (At2g47520). These ERF-VII TFs and their orthologs in other plant species share a conserved APETALA2 (AP2) domain that is necessary for protein–DNA interactions, and an N-terminal degron motive MCGGAI/V (N-degron) that plays a regulatory role in protein turnover. The stability of ERF-VII proteins is modulated by the presence of molecular oxygen, implying that they function in an oxygen sensing mechanism via the N-end rule protein degradation pathway (NERP, Gibbs *et al.*, 2011; Licausi *et al.*, 2011). ERF-VIIs are post-translationally modified by aminopeptidases, which remove the start Met from their N-terminal ends thereby placing a Cys residue at the N-terminal position. As a consequence, in the presence of oxygen Cys is oxidised to Cys-sulfonic or Cys-sulfinic acid. Thereby, ERF-VIIs become substrates for Arg-tRNA protein transferases (ATEs) catalysing the formation of arginyl-protein conjugates. Thiol oxidase-related cysteine oxidases (PCOs) were shown to catalyse the oxidation of N-terminus cysteine residues in the presence of oxygen (Weits *et al.*, 2014). Subsequent addition of an Arg residue to the N-terminus by ATEs provides a signal for polyubiquitination by the proteolysis 6 (PRT6) ubiquitin ligase, which targets ERF-VII TFs for degradation by the 26S proteasome. This molecular oxygen sensing mechanisms via NERP are widely conserved among plant species (Bailey-Serres *et al.*, 2012).

Although plants are able to produce oxygen in their photosynthetic tissues, adverse environmental conditions, such as submergence can cause oxygen deprivation. Inefficient oxygen supply negatively affects the energy production and therefore developmental processes (Voesenek and Bailey-Serres, 2013). Molecular responses to hypoxia promote a switch from aerobic to anaerobic respiration that decreases the cellular ATP demand and initiates alternative metabolic processes, such as fermentation and utilization of pyrophosphate as alternate energy source (Stitt, 1998). In parallel, transcriptional regulation also contributes alleviating energy depletion. As activators of hypoxia-induced genes, the ERF-VII TFs emerged as pivotal regulators of oxygen deprivation (van Dongen and Licausi, 2015). In rice cultivars, variations between the *SUBMERGENCE 1* (*SUB1*) ERF-VII orthologs underlie important quantitative traits determining flooding tolerance. Although most rice cultivars carry *SUB1B* and *SUB1C,* apparently the *SUB1A-1* allele represents a key determinant of submergence tolerance, *SUB1C* can also promote quiescence-mediated tolerance in wild rice varieties lacking *SUB1A* (Pucciariello and Perata, 2013). Interestingly, turnover of both SUB1A and SUB1C proteins is unaffected by the oxygen-dependent NERP (Gibbs *et al.*, 2011), suggesting that this pathway became divergent in rice.

Overexpression and knockout mutations of Arabidopsis ERF-VII TFs influence plant survival rates under low oxygen conditions. Overexpression of HRE1 but not HRE2 was reported to confer tolerance to anoxia, while the double *hre1hre2* mutant was more sensitive to anaerobic treatment compared to wild type and single *hre* mutants (Licausi *et al.*, 2010). Analogously, RAP2.2 and RAP2.12 overexpressing plants show higher survival rates after hypoxia (Hinz *et al.*, 2010; Licausi *et al.*, 2011), whereas inactivation of RAP2.2 confers enhanced sensitivity to oxygen limitation (Hinz *et al.*, 2010). The role of RAP2.3 in low oxygen responses is so far unexplored.

Besides hypoxia, the ERF-VII TFs were implicated in a range of other stress responses. Elevated expression of rice SUB1A was found to increase osmotic and oxidative stress tolerances (Fukao *et al.*, 2011), whereas overexpression of the barley ERF-VII HvRaf in Arabidopsis conferred salt tolerant root growth and enhanced resistance to a pathogenic fungus (Jung *et al.*, 2007). In addition, Arabidopsis HRE2 was shown to contribute to salt and osmotic stress tolerance (Park *et al.*, 2011), whereas overexpression and inactivation of RAP2.2 yielded enhanced resistance and sensitivity to necrotrophic *Botrytis* species, respectively (Zhao *et al.*, 2012). NERP-related destabilization of ERF-VII TFs has also been linked to the perception of nitric oxide (NO) and ABA signalling by their ability to activate *ABSCISIC ACID INSENSITIVE5* (*ABI5,* Gibbs *et al.*, 2014).

Consequent to flood, plants experience first oxidative damage followed by dehydration (Fukao *et al.*, 2011; Shingaki-Wells *et al.*, 2014). In this study we report that, in addition to their roles in response to low oxygen, RAP2.2, RAP2.3 and RAP2.12 also play a role in modulating oxidative and osmotic stress tolerance during submergence acclimation. Furthermore, we show that the NERP is not the sole modus for destruction of ERF-VIIs by demonstrating that abundance of the N-terminal epitope tagged RAP2.12 is degraded by the proteasome. We also show that RAP2.12 protein level is affected by the putative E3 ubiquitin ligases SINAT1 and 2.

RESULTS

ERF-VII family mRNAs show differential responses to low oxygen and ABA

We have shown that estradiol-induced expression of RAP2.12 leads to prolonged transcriptional activation of *ALCOHOL DEHYDOGENASE 1* (*ADH1*) by ABA (Papdi *et al.*, 2008). This prompted us to further explore the connection between RAP2.12 and ABA signalling. First, we compared the low oxygen response of ERF-VII family TFs with that of ABA regulation by quantitative RT-PCR (qPCR) measurement of transcript levels in wild type (Col-0) Arabidopsis seedlings. Anoxia treatment led to characteristic induction of both *HRE1* and *HRE2* genes, in contrast to a gradual

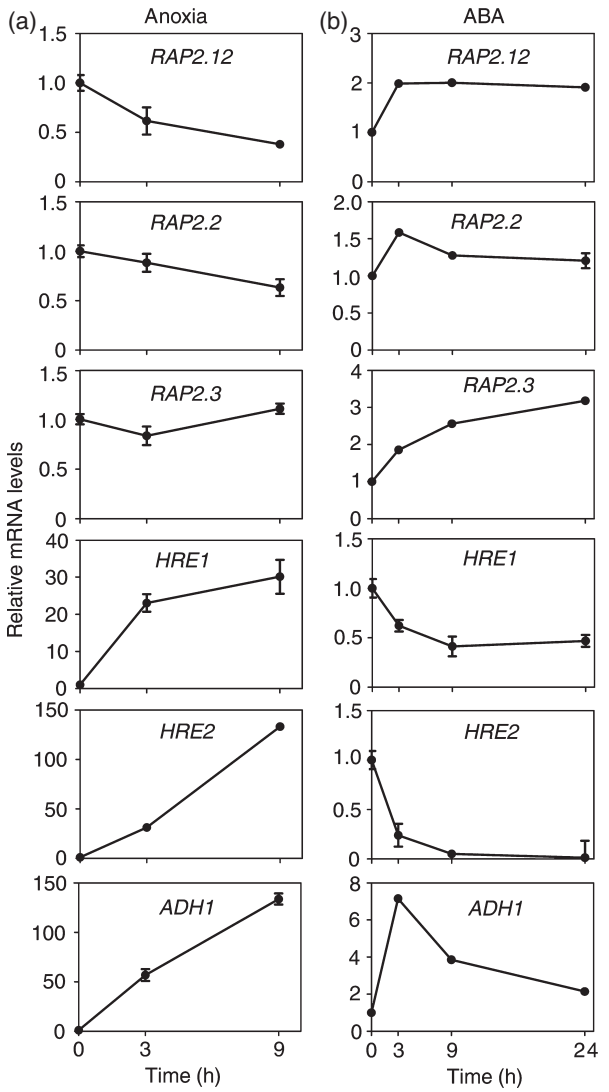

Figure 1. Comparative analysis of ERF-VII gene expression.
(a) Gene expression profiles of ERF-VII transcription factors in Col-0 wild type plants during anoxia. Arabidopsis seedlings were subjected to 3 or 9 h anoxia treatments.
(b) Effect of abscisic acid (ABA) treatment on ERF-VII gene regulation. One-week-old wild type plants were treated with 30 μM ABA and samples were taken 0, 3, 9 or 24-h timepoints.

decrease of *RAP2.12* and *RAP2.2* mRNA levels (Figure 1a) confirming the previous results of Licausi *et al.* (2010) obtained with hypoxia (1% oxygen) exposed plants. Contrary to anoxia, *RAP2.12, RAP2.3,* and to a lesser extent *RAP2.2* were induced, whereas *HRE1* and *HRE2* were downregulated by ABA (30 μM) treatment (Figure 1b). The ERF-VII target, *ADH1* showed a steady increase of mRNA by anoxia while it was transiently induced by ABA (Figure 1a,b). Based on their different transcriptional responses to hypoxia and ABA, the ERF-VII TF genes could thus be classified into two subgroups. *RAP2.12, RAP2.2* and *RAP2.3* were activated by ABA but repressed or unaffected by low oxygen, whereas *HRE1* and *HRE2* were induced by anoxia and clearly repressed by ABA. This split into two groups of ERF-VII genes also hold for the expression levels in different organs based on data in the Genevestigator (Hruz *et al.*, 2008); the *RAP2.12, RAP2.2* and *RAP2.3* show high, while *HRE1* and *HRE2* low basal expressions (Figure S1). The ERF-VIIs are also distinct in their abiotic stress regulation (Figure S2). *RAP2.12, RAP2.2* and *RAP2.3* were increased by osmotic stress, while *RAP2.2* was also upregulated by cold, heat and salinity. *HRE1* and *HRE2* were decreased upon cold and heat, while *HRE2* was induced by salt and osmotic stresses.

RAP2.12, RAP2.2 and RAP2.3 activate *ADH1* expression under normoxic condition and sustained its ABA induction

Next, we compared transcriptional activation of *ADH1-LUC* by estradiol-inducible overexpression of the *RAP2.12, RAP2.2* or *RAP2.3* genes in Arabidopsis (AE-RAP2 lines). Induced overexpression of any of these RAP2s resulted in trans-activation of *ADH1-LUC* showing a steady increase over a 24-h period but not in controls including ADH-LUC without RAP2 overexpression or mock treatments (Figures 2a,b and S3). ABA treatment (30 μM) led to transient activation of *ADH1-LUC* peaking at about 3 h in the control ADH-LUC and the three AE-RAP2 lines (Figure 2b). The estradiol-induced overexpression of RAP2 genes modified the ABA response of *ADH1-LUC;* the initial rapid increase was unchanged but it remained elevated throughout the time course (Figure 2b). These results showed that all three RAP2 factors, when overexpressed, can transactivate *ADH1-LUC* and their overexpression sustains the transient ABA response.

RAP2.12, RAP2.2 and RAP2.3 overexpression upregulate hypoxia genes at normal conditions while it potentiates the anaerobic stress response

Not only *ADH1,* but other hypoxia-induced genes such as *PYRUVATE DECARBOXYLASE1 (PDC1*), *SUCROSE SYNTHASE1 (SUS1)* and *SUS4* were all markedly increased in normoxic conditions in the estradiol-treated AE-RAP2 plants, but not in the control ADH-LUC line. Anoxia induced the expression of these four hypoxia genes but this was greatly enhanced by the estradiol-dependent overexpression of the RAP2 genes (Figure 3). These data indicate that inducible overexpression of RAP2 genes can surpass the requirement for hypoxia to activate genes under normoxic condition, while constitutive overexpression of RAP2.2 and RAP2.12 were found insufficient for this process (Hinz *et al.*, 2010; Licausi *et al.*, 2011).

Trans-activation of *ADH1* by RAP2.12 is independent of ABA

To examine whether ABA is required for RAP2-mediated trans-activation of *ADH1,* we introduced by crossing the

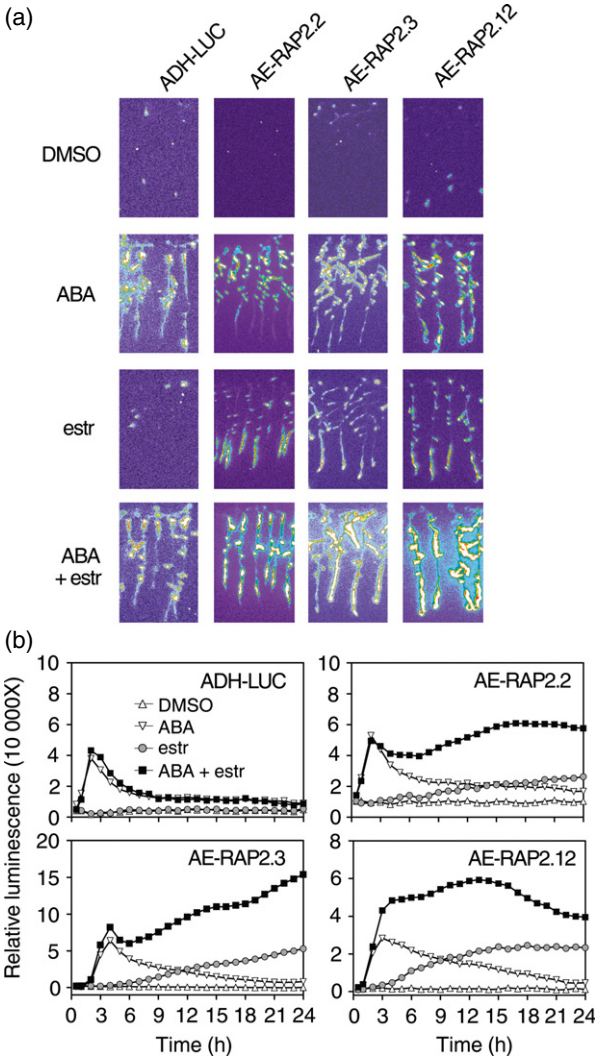

Figure 2. Bioluminescence detection of *ADH1-LUC* activity in RAP2.2, RAP2.3 and RAP2.12 overexpressing lines.
(a) Images of ADH-LUC, AE-RAP2.2, AE-RAP2.3 and AE-RAP2.12 plants, showing LUC activities after 9 h of spraying with 0.04% DMSO solvent as control, 30 μM ABA and/or 5 μM estradiol (estr).
(b) Kinetics of bioluminescence of the *ADH1-LUC* reporter for 24 h, measured in different genetic backgrounds, treated as above.

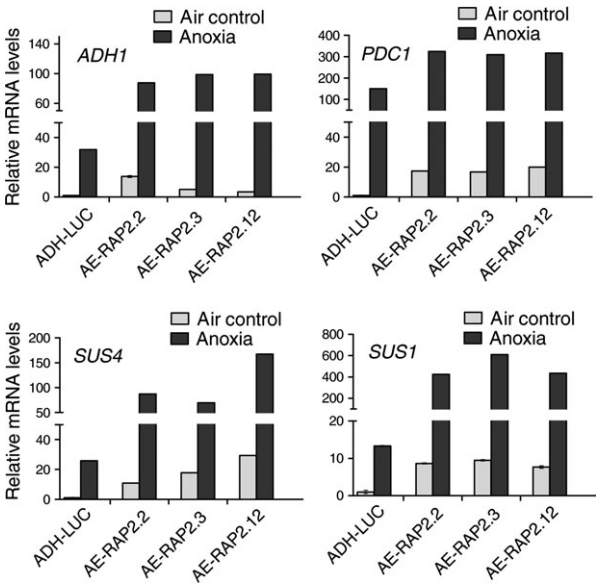

Figure 3. RAP2.2, RAP2.3 and RAP2.12 upregulate hypoxia marker genes.
Seven-day-old plants of ADH-LUC, AE-RAP2.2, AE-RAP2.3 and AE-RAP2.12 lines were treated with 5 μM estradiol, then half of the plants were subjected to anoxia for 3 h, the rest were kept in normoxic conditions in the dark (air control). Three hours later, samples were taken and used for qPCR analysis to test expression of hypoxia marker genes: *ALCOHOL DEHYDROGENASE 1* (*ADH1, AT1G77120), PYRUVATE DECARBOXYLASE 1* (*PDC1,* AT4G33070), *SUCROSE SYNTHASE 4* (*SUS4,* AT3G43190) and *SUCROSE SYNTHASE 1* (*SUS1,* AT5G20830).

ADH1-LUC and estradiol-inducible AE-RAP2.12 constructs into the *aba2-3* mutant impaired in ABA biosynthesis. Treatment of AE-RAP2.12 and *aba2-3*× AE-RAP2.12 seedlings for 3 h with either anoxia, estradiol or the combination of both resulted in comparable trans-activation of the *ADH1-LUC* reporter in both lines (Figure 4a). Thus, the trans-activation of *ADH1-LUC* by anoxia does not involve ABA biosynthesis. Osmotic stress responses are known to be mediated by ABA signalling (Seki *et al.*, 2007). Next we examined how the *ADH1-LUC* reporter responds to osmotic stress in the AE-RAP2.12 and *aba2-3*× AE-RAP2.12 seedlings by treatment with 300 mM mannitol. In wild type background, the transient induction of *ADH1-LUC* peaking at around 3 h upon osmotic stress closely paralleled what we observed with ABA. In the *aba2-3* background mannitol treatment alone cannot induce *ADH1-LUC*. Thus osmotic stress induces *ADH1-LUC* through ABA. As was the case for ABA induction of *ADH1-LUC,* the RAP2.12 overexpression also modified the transient osmotic response to become sustained and even further increased in the wild type, but not in the *aba2-3* background (Figure 4b). We conclude that RAP2.12 transactivates the *ADH1-LUC* reporter independently of ABA.

ERF-VII RAP2s modulate ABA sensitivity and tolerance to anoxic, oxidative and osmotic stress

The role of RAP2.3 is largely unexplored, but other members of the ERF-VII group appear to have similar roles in low oxygen tolerance (Hinz *et al.*, 2010; Licausi *et al.*, 2010, 2011), but other abiotic stress functions remained unclear. We studied the physiological responses to osmotic and oxidative stresses as well as to low oxygen in the AE-RAP2.12, AE-RAP2.2 and AE-RAP2.3 lines compared to ADH-LUC parental control. Whereas after 8 h of anoxia treatment there was only a minor difference among the control and AE-RAP2 seedlings; all showed around 20% reduction in survival rate. However, after 14 h of anoxia the difference became apparent; only about 10% of control

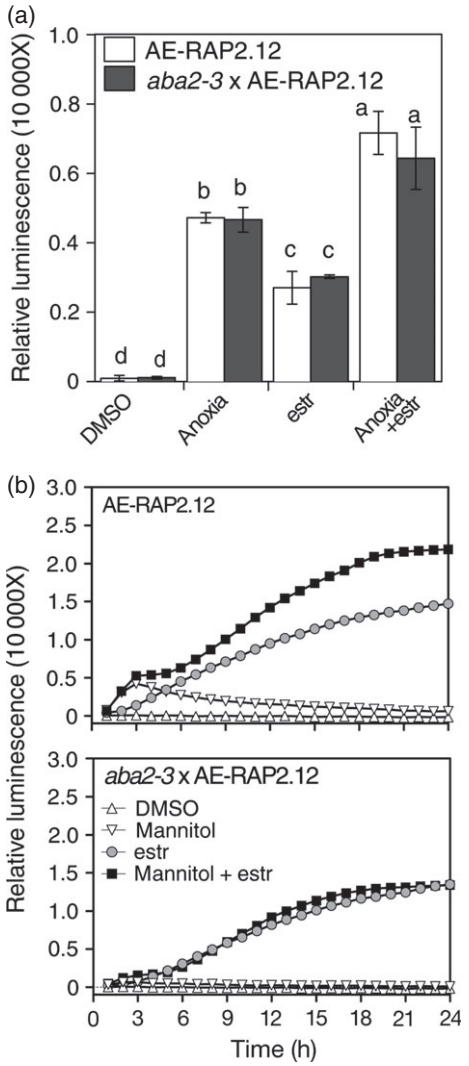

Figure 4. Bioluminescence detection of *ADH1-LUC* in AE-RAP2.12 and *aba2-3*× AE-RAP2.12 lines.
(a) *ADH1-LUC* activity in AE-RAP2.12 and *aba2-3*× AE-RAP2.12 lines, subjected to anoxia. Bioluminescence imaging was performed 3 h after anoxia and/or 5 μM estradiol (estr) treatments. Relative values of luciferase activities are shown. Statistical differences were calculated with one-way ANOVA Tukey's test ($P < 0.05$).
(b) Bioluminescence imaging of AE-RAP2.12 line (up) and *aba2-3*× AE-RAP2.12 (down), monitored for 24 h. Mannitol treatment enhanced the effect of RAP2.12 overexpression on *ADH1-LUC* in wild type but not in *aba2-3* mutant background. Plants were treated with 0.04% DMSO, 400 mM mannitol and/or 5 μM estradiol.

plants remained viable compared with a 17–22% survival rate of AE-RAP2 lines (Figure 5a,b). To assess tolerance to osmotic and oxidative stresses, leaf area of seedlings was measured at different time points after transferring seedlings on media containing estradiol and either 300 mM mannitol or 2 mM hydrogen peroxide (H_2O_2). Rosette growth of ADH-LUC control and AE-RAP2 plants were indistinguishable on medium containing estradiol without any stress. Compared with estradiol-treated plants, the leaf area of the parental ADH-LUC control was reduced by 80% in contrast with 55–60% of AE-RAP2 plants following exposure to estradiol and H_2O_2 for 15 days (Figure 5c,d). Compared to the control, the leaf area of AE-RAP2 lines remained about twice as large following mannitol treatment for 12 days, indicating that estradiol-induction of RAP2.2, RAP2.3 and RAP2.12 conferred similar enhancement of oxidative and osmotic stress tolerance of leaf growth (Figure 5e,f). To determine whether enhanced stress tolerance reflected a change in ABA sensitivity of AE-RAP2 lines, their root elongation was tested after transferring 4-day-old seedlings for 7 days on estradiol-containing media with or without 10 μM ABA. Compared to seedlings growing in ABA-free medium, the average root length of control was reduced by 50%, whereas the AE-RAP2 seedlings showed a reduction of 38–40% indicating a slight increase of ABA sensitivity (Figure 5g,h).

To investigate the molecular basis of stress tolerance conferred by RAP2 TFs, we analysed gene expression changes of selected osmotic stress and ABA-inducible genes (Hirayama and Shinozaki, 2010) upon RAP2.12 overexpression. After 9 h ABA treatment the expression of *RESPONSE TO DROUGHT 29A* (*RD29A), RESPONSE TO DROUGHT 22* (*RD22)* and *DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1* (*P5CS1*) genes were 23–40% higher in the RAP2.12 overexpressing plants than in the control line (Figure 5i). Expression of other selected genes was unaffected by RAP2.12 overexpression (Figure S4).

Mutants of *rap2.2, rap2.3* and *rap2.12* show distinct sensitivities to various abiotic stresses

To identify whether *RAP2.2, RAP2.3* and/or *RAP2.12* are required for abiotic stress functions, we characterized mutant lines carrying T-DNA insertions in their coding regions (Figures 6a and S5). We compared stress tolerance traits of the *rap2.2-4, rap2.3-1, rap2.12-2* single and *rap2.12-2 rap2.3-1* double mutant lines to wild type. Submergence in the dark causes hypoxia with energy deprivation generating stress, which affects plant growth and survival (Bailey-Serres and Voesenek, 2008). Whereas about 80% of wild type plants survived 44-h submergence in the dark, 50% of *rap2.2-4, rap2.3-1* and *rap2.12-2* mutants and only 36% of *rap2.12-2 rap2.3-1* double mutant plants remained viable (Figure 6b). Although the *rap2* single mutants were hypersensitive to submergence, the growth rate of single *rap2* mutants was comparable to wild type on 300 mM mannitol-containing medium, while the *rap2.12-2 rap2.3-1* double mutant displayed about 50% reduction of rosette sizes compared to wild type, indicating enhanced osmotic stress sensitivity (Figures 6c,d and S6). By contrast, 2 mM H_2O_2 made no difference in growth of single and double *rap2* mutants and wild type (Figure 6c).

To determine whether the enhanced osmotic sensitivity of *rap2* mutants correlated with changes in the activities of

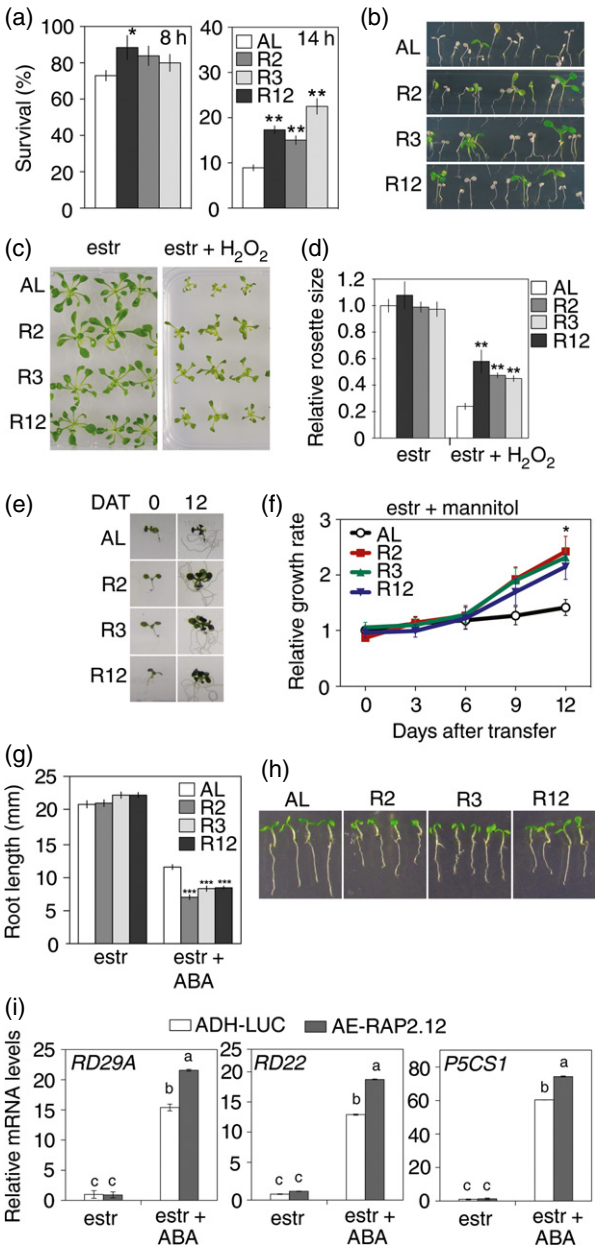

Figure 5. Overexpression of RAP2 transcription factors confers tolerance to various abiotic stresses.
(a) Survival rates of ADH-LUC and the AE-RAP2 plants treated with 5 μM estradiol and subjected to 8 or 14 h anoxia. Survival was scored after 7 days' recovery.
(b) Images of plants 1 week after the 14 h anoxia treatment.
(c) Overexpression of RAP2 TFs confers oxidative stress tolerance. Four-day-old seedlings of ADH-LUC or AE-RAP2s were transferred to ½MS medium supplemented by 5 μM estradiol (estr) with or without 2 mM hydrogen peroxide (H_2O_2) and grown for 15 days.
(d) Relative rosette sizes, averages of 15 plants.
(e) ADH-LUC and RAP2s overexpressing plantlets grown on agar plates containing 300 mM mannitol and 5 μM estradiol for 0 or 12 days. DAT, days after transfer.
(f) Rosettes sizes of 15 plantlets grown on high osmotic medium.
(g) ABA sensitivity of root elongation. Four-day-old seedlings were transferred to media supplemented by 5 μM estradiol with or without 10 μM ABA. Graphs show average root lengths of 14 seedlings grown for 7 days.
(h) Images of typical AE-RAP2 plants showing differences in root growth. Abbreviations used for the lines in physiological studies: AL: ADH-LUC; R2: AE-RAP2.2; R3: AE-RAP2.3; R12: AE-RAP2.12. If otherwise not stated values represent mean of three technical replicates, each contained at least 25 plantlets. Error bars show standard error (SE), asterisks indicate statistically significant differences compared with ADH-LUC parental line, one-way ANOVA multiple comparison test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
(i) qPCR analysis of selected ABA and osmotic stress induced genes in ADH-LUC and AE-RAP2.12 lines after 9 h 5 μM estradiol with or without 30 μM ABA treatment: *RD29A* (AT5G52310), *RD22* (AT5G25610) and *P5CS1* (AT2G39800). Statistical analysis was performed using one-way ANOVA Tukey's test ($P < 0.05$).

RAP2-responsive marker genes, 10-day-old wild type and *rap2* mutant seedlings were transferred to medium supplemented with 300 mM mannitol. *ADH1* and *SUS1* transcript levels were tested after 0, 3 and 9 h of osmotic stress (Figure 6e; Dejardin *et al.*, 1999; Papdi *et al.*, 2008). Whereas the wild type and single *rap2* mutants showed comparable *ADH1* expression, it was 40% lower in the *rap2.12-2 rap2.3-1* double mutant after 9 h of mannitol treatment. At the same time, *SUS1* transcript levels were decreased by 20–35% in the single *rap2* mutants and by 80% in the *rap2.12-2 rap2.3-1* double mutant compared with wild type plants (Figure 6e). However, expression level of *RD29A, RD22* and *P5CS1,* which was enhanced by ABA treatment in RAP2.12 overexpressing lines, was unaffected by 9 h mannitol treatment in the *rap2.12-2 rap2.3-1* mutant (Figure S7).

SINAT2 affects RAP2.12 protein abundance

Overexpression of Arabidopsis RAP2.12 has been reported to confer submergence tolerance, which is abolished by fusing RAP2.12 to an N-terminal haemagglutinin tag (HA:RAP2.12) that interferes with NERP-mediated proteolysis (Licausi *et al.*, 2011). We generated an Arabidopsis line that expressed HA:RAP2.12 estradiol-inducibly; (Figures 7a and S8). Surprisingly, when HA:RAP2.12 expressing plants were treated with the proteasome inhibitor MG132, an unambiguously higher abundance of HA:RAP2.12 was detected, indicating that despite its N-terminal modification HA:RAP2.12 remained a substrate for proteasomal degradation (Figure 7b). To confirm that MG132 stabilizes HA:RAP2.12, we also tested its effect in a cytoplasmic extract *in vitro* that rules out transcriptional regulation. Protein extract of XVE-HA:RAP2.12 kept on ice for 2 h resulted in a time-dependent degradation of HA:RAP2.12 protein, but this was considerably reduced by MG132 (Figure S9). As the N-terminal HA-tag on RAP2.12 is expected to interfere with the N-degron (Figure S8), we concluded that HA:RAP2.12 is likely to be targeted to proteosome-dependent proteolysis that is independent of NERP.

RAP2.2 was previously reported to interact with the putative E3 ubiquitin ligase SEVEN IN ABSENTIA (SINA) of

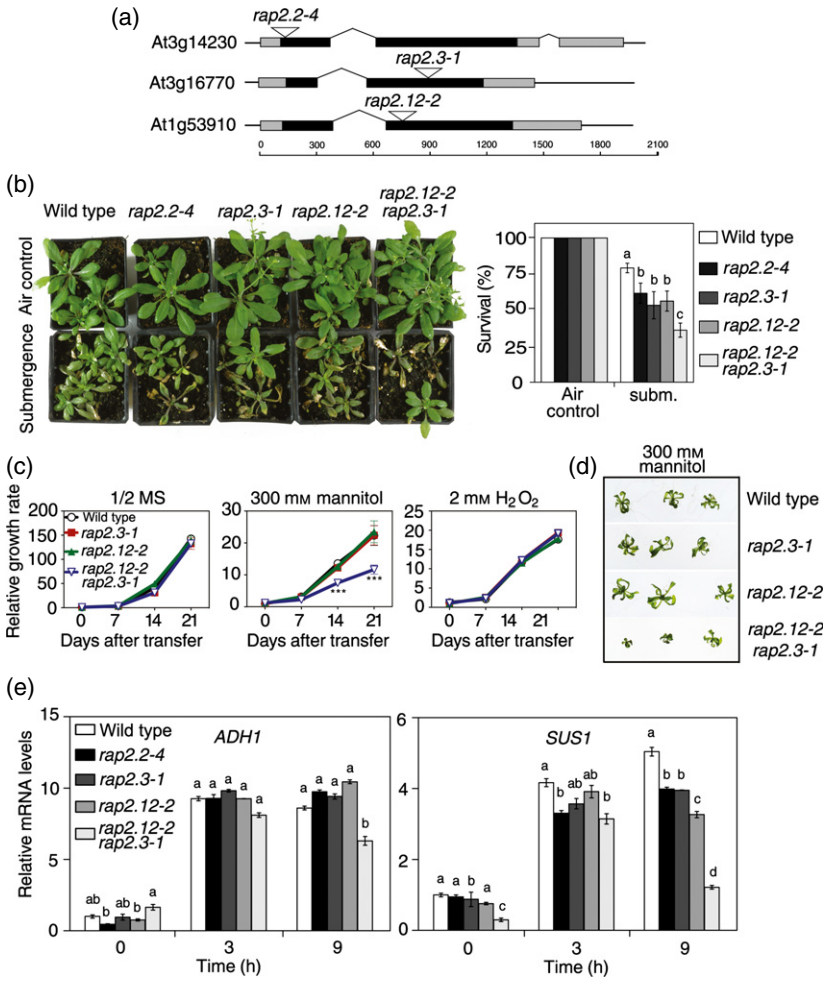

Figure 6. Knockout mutation of *RAP2.2, RAP2.3* and *RAP2.12* genes causes stress hypersensitivity.
(a) T-DNA insertions are localized in the exons of At3g14230 (*RAP2.2),* At3g16770 (*RAP2.3*) and At1g53910 (*RAP2.12).*
(b) Mutant lines have enhanced sensitivity to submergence compared with wild type. Left panel shows images of plants after 1 week recovery of 44 h submergence. Control plants were kept in the air but in dark for 44 h. Right panel shows survival rates of plants (%) after submergence. Statistical differences were calculated with one-way ANOVA Tukey's test ($P < 0.05$).
(c) Rosette growth of wild type, *rap2.3*-1 and *rap2.12-2* mutants and *rap2.12-2 rap2.3-1* double mutants on standard medium (½MS) supplemented with 300 mM mannitol or 2 mM H_2O_2. Rosette sizes of 15 plants were measured from each line. Asterisks indicate significant differences after one-way ANOVA Dunnett's test ($P < 0.05$).
(d) Images of wild type and mutant plants after 21 days growth on medium containing 300 mM mannitol.
(e) Transcript levels of *ADH1* (*AT1G77120*) and *SUS1* (*AT5G20830*) determined by qRT-PCR analysis. Ten-day-old plants of wild type, *rap2.2-4, rap2.3-1, rap2.12-2* and double *rap2.12-2 rap.3-1* mutant lines were stressed by 300 mM mannitol for 0, 3, or 9 h. Statistical analysis one-way ANOVA Tukey's test was performed independently at each time point ($P < 0.05$).

Arabidopsis thaliana 2 (SINAT2, Welsch *et al.*, 2007). The conserved SINA proteins possess two characteristic domains, an N-terminal RING-type zinc finger domain and a C-terminal TRAF-like substrate-binding domain (Hu and Fearon, 1999; Polekhina *et al.*, 2002). Plant SINA-related proteins were reported to have E3 ubiquitin ligase activity (Xie *et al.*, 2002; Ning *et al.*, 2011; Peralta *et al.*, 2013) and are involved in the regulation of stress responses (Kim *et al.*, 2006; Ning *et al.*, 2011; Bao *et al.*, 2014). Because of the high sequence similarity between RAP2.12 and the SINAT2-interacting RAP2.2 protein, we decided to test whether SINAT2 may influence RAP2.12 protein levels.

The Arabidopsis genome encodes 18 SINA-related proteins (Figure S10). Comparison of SINA-related proteins indicates that SINAT2 shares 95% of amino acid identity with SINAT1. Additionally, *SINAT1* and *SINAT2* show similar transcriptional profiles in Arabidopsis suggesting that they probably perform overlapping functions (Figure S11). To investigate, whether these E3 ubiquitin ligases control RAP2.12 abundance in Arabidopsis, a specific artificial microRNA (amiRNA)-expressing construct was created to generate transgenic plants that simultaneously silence both *SINAT1* and *SINAT2* (Figure S12). The XVE-amiSINAT1/2 lines were designed to express the SINAT-targeted

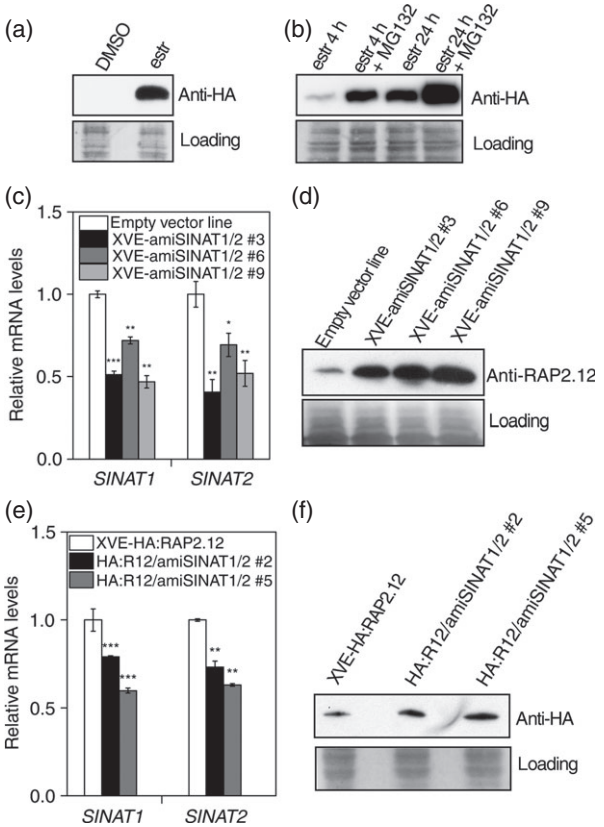

Figure 7. Abundance of RAP2.12 is affected by SINAT proteins.
(a) Western detection of HA:RAP2.12 in XVE-HA:RAP2.12 line. HA:RAP2.12 was detected after 5 μM estradiol, but not after solvent (0.04% DMSO) treatment.
(b) HA:RAP2.12 is stabilized by the proteasome inhibitor MG132. XVE-HA:RAP2.12 plants treated with 5 μM estradiol for 4 or 24 h. To inhibit proteasomes, plants were treated with 100 μM MG132.
(c) Transcript levels of *SINAT1* and *SINAT2* in three independent XVE-amiSINAT1/2 lines, in 10-day-old plants, treated with 10 μM estradiol for 48 h. Statistical analysis was performed using one-way ANOVA Dunnett's test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
(d) RAP2.12 protein detected in independent SINAT1/2 silenced lines using anti-RAP2.12 antibody.
(e) Transcript levels of *SINAT1* and *SINAT2* in two independent, constitutively expressing HA:R12/amiSINAT1/2 lines, carrying the XVE-HA:RAP2.12 construct. Statistical analysis was performed using one-way ANOVA Dunnett's test (**$P < 0.01$, ***$P < 0.001$). Note similarly reduced expression of both *SINAT1* and *SINAT2*.
(f) Detection of HA:RAP2.12 protein in XVE-HA:RAP2.12 line and two HA:R12/amiSINAT1/2 lines after 5 μM estradiol treatment. Note western signal obtained with anti-HA antibody.

amiRNA in an estradiol-dependent manner. Three lines were selected in which the *SINAT1* and *SINAT2* mRNA levels were reduced to 40–70% after estradiol treatment compared to control carrying the empty pER8 vector (Figure 7c). The abundance of endogenous RAP2.12 detected by western blotting with an anti-RAP2.12 antibody was remarkable higher in these lines compared to the control suggesting that SINAT1/2 influences RAP2.12 protein levels (Figure 7d).

Next, we investigated whether *SINAT1* and *SINAT2* co-silencing can also affect HA:RAP2.12 that cannot be targeted through NERP. Using the estradiol-inducible XVE-HA:RAP2.12 construct in plants into which we introduced a CaMV 35S promoter-driven *SINAT1*/2 silencing amiRNA construct resulted in around 20–40% reduction of *SINAT1* and *SINAT2* (Figure 7e). The HA:RAP2.12 protein was slightly elevated in two independent HA:R12/amiSINAT1/2 lines (Figure 7f), indicating that SINAT-silencing can also influence the HA-tagged RAP2.12 independent of the NERP.

DISCUSSION

ERF-VII RAP2s modulate responses to different stress conditions

Originally, we identified RAP2.12 as a transcriptional activator of *ADH1,* which is induced by ABA and abiotic stresses, including hypoxia, high osmotics and salinity (Papdi *et al.*, 2008). Subsequently, RAP2.12 and other members of the ERF-VII TF family were described as principal components of oxygen and NO perception, which are targeted for proteasomal degradation through the NERP (Gibbs *et al.*, 2011, 2014; Licausi *et al.*, 2011). Although all members of the ERF-VII family play role in low oxygen signalling, only RAP2.12, RAP2.2 and RAP2.3 are thought to function as primary actors in oxygen sensing, whereas HRE1 and HRE2 appear to be required only for maintenance of anaerobic responses (Sasidharan and Mustroph, 2011; Licausi *et al.*, 2013). Our data show that mRNA levels of RAP2s and HREs are differently regulated by ABA, which stimulates the expression of *RAP2.12* and *RAP2.3* but represses *HRE1* and *HRE2* (Figure 1b).

We reported earlier that estradiol-inducible overexpression of RAP2.12 transactivates the *ADH1-LUC* reporter and endogenous *ADH1* gene (Papdi *et al.*, 2008). By extending this observation, our current study indicates that activation of *ADH1-LUC* occurs with a comparable kinetics in response to induction of RAP2.2, RAP2.3 and RAP2.12 (Figure 2). Overexpression of RAP2.2 and RAP2.12 was reported to enhance, while their silencing by artificial miRNAs to reduce anaerobic induction of a set of hypoxia responsive genes (Hinz *et al.*, 2010; Licausi *et al.*, 2011). Our data show that estradiol-induction of the three ERF-VIIs activates the hypoxia-induced *ADH1, PDC1, SUS1* and *SUS4* marker genes also in the absence of low oxygen stress (Figure 3), most likely because the induced overexpression can surpass NERP degradation.

Overexpression of the Arabidopsis RAP2.2 and RAP2.12, and the related rice SUB1A-1 TF was reported to enhance survival under low oxygen conditions (Xu *et al.*, 2006; Hinz *et al.*, 2010; Licausi *et al.*, 2011). Our data indicate that overexpression of any of RAP2.2, RAP2.3 and RAP2.12 results in enhanced tolerance also to osmotic and oxidative stresses in addition to anoxia (Figure 5). However, the

threshold of stress tolerance conferred by RAPs appears to be different in the case of different stresses. In contrast to hypoxia genes, RAP2.12 overexpression did not affect the basal expression of ABA and osmotic stress induced genes; *RD22, RD29A* and *P5CS1,* but only when the plants were concomitantly subjected to ABA treatment (Figure 5i). Molecular mechanisms by which these TFs contribute to osmotic and oxidative stress responses remains unexplored.

Knockout mutations of the RAP2 genes compromised low oxygen stress tolerance but did not alter growth responses to mannitol and H_2O_2. Nevertheless, combination of the *rap2.3-1* and *rap2.12-2* mutations conferred increased sensitivity to submergence and osmotic stress (Figure 6b–d). Consequently, *ADH1* and *SUS1* showed similar activation by mannitol treatment in the wild type and single *rap2* mutants, but their transcript levels were markedly reduced in the *rap2.12-2 rap2.3-1* double mutant upon extended osmotic stress (Figure 6e). Interestingly, the expression levels of genes implicated in osmotic protection, such as *RD29A, RD22* and *P5CS1* remained comparable with wild type during osmotic stress in *rap2.12-2 rap2.3-1* line (Figure S7). These observations suggest that ERF-VII factors only contribute to certain aspects of stress tolerance mechanisms related to growth but not osmoprotection. In fact, the growth-modulation is also linked to survive submergence (Voesenek and Bailey-Serres, 2013). Molecular processes that regulate plant growth during abiotic stress conditions are coordinated by the gibberellin-DELLA signalling pathway (Achard *et al.*, 2006; Skirycz and Inze, 2010). Recently uncovered physical interaction of GIBBERELLIN INSENSITIVE (GAI) DELLA protein with RAP2.3 and RAP2.12 (Marin-de la Rosa *et al.*, 2014) thus suggests that the functions of ERF-VII might be modulated in a DELLA-dependent fashion.

ERF-VII TFs induce ADH1 independent of and synergistic with ABA

ABA transiently induced the *ADH1-LUC* reporter, reaching maximal level by approximately 3 h after the treatment. We observed that the transient induction of *ADH1-LUC* was sustained by overexpressing RAP2.2, RAP2.3 or RAP2.12, suggesting that these TFs might negatively affect the responses that mediate the decline in *ADH1* activity. Recently, it was reported that after hypoxia treatment RAP2.12 remained stable for at least 2 h in the nucleus (Kosmacz *et al.*, 2015). Based on these observations, it can be hypothesized that higher abundance of RAP2s at the end of the submergence can further sustain the ABA induction of *ADH1* and other ABA-inducible target genes in the subsequent osmotic stress. Following submergence plants are subjected to oxidative stress during reoxygenation and dehydration when floods subside (Fukao *et al.*, 2011; Shingaki-Wells *et al.*, 2014; Tsai *et al.*, 2014). The synergistic effect of RAP2s and ABA on stress genes thus could provide a sustained response to these different forms of stresses (Figure S13).

In analogy to enhanced ABA sensitivity of root elongation observed in the RAP2 overexpressing Arabidopsis AE-RAP2 lines (Figure 5g,h), overexpression of rice SUB1A was found to result in pronounced inhibition of shoot growth by ABA (Fukao *et al.*, 2011). Recently, the *ABSCISIC ACID INSENSITIVE5* (*ABI5*) gene was found to be upregulated by RAP2.2, RAP2.3 and RAP2.12 in mesophyll-derived leaf protoplasts of the NERP mutant *prt6* (Gibbs *et al.*, 2014). ABI5 is one of the most important positive regulators of ABA signalling during germination and early seed growth (Finkelstein and Lynch, 2000; Lopez-Molina *et al.*, 2002). Enhanced ABA sensitivity of the RAP2 overexpressing lines might therefore be connected to ABI5 activation.

RAP2.12 was also reported to interact with the ACYL-COA-BINDING PROTEIN (ACBP) 1 and 2 (Licausi *et al.*, 2011), which are thought to enhance ABA sensitivity during germination and seedling development (Du *et al.*, 2013a, b). Our data indicate that RAP2.12 transactivates *ADH1-LUC* similarly in the wild type and ABA-deficient *aba2-3* mutant but stimulation of *ADH1-LUC* by osmotic stress is compromised in the RAP2.12 overexpressing *aba2-3* mutant (Figure 4b). This clearly shows that RAP2.12 functions to enhance the ABA-mediated osmotic response, but it activates *ADH1* gene independently of ABA.

Stability of RAP2.12 is regulated by multiple pathways

Ubiquitination is a key post-translational mechanism that modulates proteasomal degradation of numerous signalling and TFs involved in the control of plant developmental, hormonal and stress signalling pathways (Lopez-Molina *et al.*, 2003; Dill *et al.*, 2004; Lau and Deng, 2010; Nishizawa-Yokoi *et al.*, 2010; Lyzenga and Stone, 2012; Yang *et al.*, 2012). Members of the ERF-VII TF family are known to function as direct regulators of hypoxia responses through oxygen-dependent modulation in their stability through the NERP (Gibbs *et al.*, 2011; Licausi *et al.*, 2011). The stability of ERF-VII TFs through NERP is also altered in response to NO (Gibbs *et al.*, 2014). In plants, NO signalling influences numerous basic processes, including cell cycle, growth, development, flowering, senescence and responses to environmental stresses (Mur *et al.*, 2013). Changing the N-terminal Cys residue to Ala by site specific mutagenesis led to stabilization of RAP2.12 protein (Gibbs *et al.*, 2011), while N terminal fusion of HA tag to RAP2.12 or deleting the first 13 conserved amino acids abolished submergence tolerance (Licausi *et al.*, 2011) suggesting that the Cys-containing N-degron is essential for N-end rule mediated function of this TF. As our data demonstrated the involvement of ERF-VII TFs in several stress responses other than hypoxia, we examined whether the stability of RAP2.12, as

a representative member ERF-VII family, would also be affected by proteolysis under normal oxygen exposure. In fact, we found that RAP2.12 is still targeted to proteasomal destruction when its N-terminus was modified that compromises the recognition by the NERP (Figure 7b).

RAP2.2 is an interacting partner of the RING finger domain protein SINAT2 (Welsch *et al.*, 2007). The Arabidopsis genome encodes 469 RING domain proteins representing a great diversity and substrate specificity (Stone *et al.*, 2005). A number of RING domain proteins were reported to be responsible for polyubiquitination and subsequent degradation of key transcriptional regulators of stress and ABA responses (Qin *et al.*, 2008; Bu *et al.*, 2009; Cheng *et al.*, 2012; Liu and Stone, 2013). In Arabidopsis, the SINA group of putative E3 ubiquitin ligases includes 18 proteins: SINAT1–5, SINA-like 1–11, SINA and PEX14 (Peralta *et al.*, 2013). These proteins carry conserved RING-finger and TNF RECEPTOR ASSOCIATED FACTOR (TRAF)-like domains, the latter is necessary for substrate binding and dimerization (Hu and Fearon, 1999; Polekhina *et al.*, 2002). In Drosophila, SINA is a key component of R7 photoreceptor cell development (Carthew and Rubin, 1990), whereas the human SINA ortholog SIAH2 is an important regulator of hypoxia-induced gene expression. In humans specific hydroxylases function to destabilize the Hypoxia Inducible Factor α (HIF1α), a key regulator of hypoxia signalling. As a consequence of oxygen deprivation, SIAH2 is activated by phosphorylation, ubiquitinates the hydroxylases, which are then eliminated by the proteasome (Qi *et al.*, 2010). We investigated whether SINAT2 affects RAP2.12 protein level. Silencing of *SINAT2* together with the closely related *SINAT1* gene led to an increase in the abundance of endogenous as well as N-epitope tagged RAP2.12 protein (Figure 7d,f). In accordance to eukaryotic SINA orthologs, it is thus particularly interesting that SINAT1/2 in plants might also be involved in destabilization of ERF-VII TFs involved in hypoxia responses. Our data indicate that abundance of RAP2.12 is modulated by two alternative proteolytic pathways. The NERP connects targeted proteolysis of RAP2.12 to the perception of gaseous (oxygen and NO) signals, whereas SINAT1 and SINAT2 might target RAP2.12 for proteolysis independently of NERP under normoxic stress conditions when the NERP is inactive.

The functional importance of SINAT proteins in the regulation of ERF-VII stability is yet to be investigated. Future research should unravel how SINAT proteins are involved in stress tolerance mechanisms and whether SINATs have any cooperative interactions with the NERP regulation.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 was the background of every line used in this study. The T-DNA insertion mutant *rap2.12-2* (SAIL-1215-H10) was identified from the Syngenta collection, the *rap2.2-4* line (SAIL_18_G09) was received from Ralf Welsch (Welsch *et al.*, 2007), the *rap2.3-1* line was kind gift from Dr Kawai-Yamada (Ogawa *et al.*, 2007). The *aba2-3* line (Laby *et al.*, 2000) was obtained from the European Arabidopsis Stock Centre (http://nasc.nott.ac.uk). Genetic transformation of Arabidopsis Col-0 or ADH-LUC lines (Papdi *et al.*, 2008) were carried out with *Agrobacterium*-mediated floral-dip method (Clough and Bent, 1998). For *in vitro* plant cultures, seeds were surface sterilised, stratified for 2 days and sown on half-strength MS agar-solidified media containing 0.5% sucrose (½MS). Plants in sterile conditions were grown in short-day illumination (8 h light, 16 h dark); in soil 16 h light, 8 h dark.

Stress tolerance assays

For anoxia tolerance assays 7-day-old vertically grown Arabidopsis plants were used. The anoxia treatments were performed in the dark for 10 or 14 h using an anaerobic workstation Anaerobic System model 1025; (Forma Scientific, www.thermoscientific.com) (Licausi *et al.*, 2010) or continuous nitrogen gas flow through a container (Perez-Salamo *et al.*, 2014). After treatments, the plants were returned to standard normoxic condition and survival was scored after 7 days. Six replicate plates with 20 plantlets of each genotype were scored at each time point. For submergence tolerance assay plants were grown in soil for 28 days, then submerged for 44 h or kept in the dark as air control. Following the submergence treatment, plants were allowed to recover for 7 days and then scored for survival. Five pots were used for every genotype, each pot contained four plants.

To analyse osmotic and oxidative stress tolerance, 5-day-old seedlings were transferred onto ½MS agar medium containing 5 μM estradiol and either 2 mM H_2O_2 or 300 mM mannitol. Plant growth was monitored for 3 weeks by taking images every third day of at least 15 plants per treatment. Growth rates were calculated by measuring rosette sizes as described (Ruibal *et al.*, 2012). To measure root growth, 4-day-old seedlings were transferred to media supplemented by 5 μM estradiol and 10 μM ABA. Root lengths were measured after 7 days. The experiments were repeated at least three times. Statistically significant differences were calculated with one-way ANOVA multiple comparison tests using GraphPad Prism software (http://www.graphpad.com/scientific-software/prism/).

qPCR analysis of gene expression

Quantitative RT-PCR (qPCR) was performed as described (Joseph *et al.*, 2014). Total RNA was extracted from plant tissues using RNeasy Mini Kit (Qiagen, https://www.qiagen.com) and DNase-treated with Ambion's TURBO DNA-free DNase kit. Five-hundred ng DNase-treated RNA was added to each RT reaction using the high capacity cDNA kit (Applied Biosystems, http://www.lifetechnologies.com). Quantitative RT-PCR reactions were prepared with Maxima SYBR Green Master Mix (Thermo Scientific, http://www.thermoscientific.com) and performed with ABI PRISM 7900 HT sequence detection system (Applied Biosystems). Reference gene used: *UBIQUITIN 10,* (*AT4G05320*). For gene specific primers, see Table S1. Statistical analyses based on one-way ANOVA Dunnett's or Tukey's test were carried out with GraphPad Prism software.

Measurement of luciferase reporter activity

Transcriptional activation of the *ADH1-LUC* reporter construct was analysed by bioluminescence imaging as described (Papdi *et al.*, 2008). Arabidopsis plantlets were grown in vertical position on

½MS media (1% agar) for 14 days and sprayed with 2 mM D-luciferin solution (Biosynth AG, https://www.biosynth.com). Photon emission was detected by CCD camera system (Visilux Imager, Visitron Systems GmbH, http://www.visitron.de). Images were analysed using the METAVIEW 4.5r6 software (Universal Imaging Corporation, www.universal-imaging.com), luminescence values were normalised to the background and relative luminescence was calculated where 1 equals the normalised value of the parental line at time 0. Sequential detection of bioluminescence was carried out in 1 h intervals for 24 h with 20 min exposure time.

Western blot analysis

Total protein was extracted from 14-day-old plants with protein extraction buffer (Magyar *et al.*, 1997), containing 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM DTT, 1 mM NaF, 0.5 mM Na_3VO_4, 15 mM β-glycerolphosphate, 15 mM *p*-nitrophenylphosphate, 0.1% Tween 20, 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 100 μM MG132. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. Antibodies used in this study: monoclonal anti-HA peroxidase (Roche, http://www.roche.com), 1:500; monoclonal rabbit anti-RAP2.12 (Licausi *et al.*, 2011), 1:1000; and anti-rabbit secondary antibody (Pierce Antibodies, http://www.pierce-antibodies.com), 1:30 000.

Construction of vectors for gene expression

Entry vectors (pDONR201) containing the full length cDNAs of *RAP2.2* and *RAP2.3* were obtained from the TRANSPLANTA collection (Leon *et al.*, 2014), while RAP2.12 was cloned before (Papdi *et al.*, 2008). The three cDNAs were moved into the pER8-GW expression vector (Papdi *et al.*, 2008) by LR clonase reaction (Invitrogen, http://www.lifetechnologies.com). N-terminal haemagglutinin epitope (HA) tagging of RAP2.12 was performed by inserting the full length RAP2.12 cDNA into pMENCHU (Ferrando *et al.*, 2000) using the *Bam*HI/*Eco*RI sites. HA:RAP2.12 coding sequences were cloned into the pENTRY-BS vector (Perez-Salamo *et al.*, 2014) as a *Hin*dIII–*Not*I fragment and subsequently moved by LR clonase reaction into pER8-GW. To construct artificial microRNA for co-silencing the Arabidopsis *SINAT1* and *SINAT2* genes, the recommended protocol was used (http://wmd3.weigelworld.org/downloads/Cloning_of_artificial_microRNAs.doc; Schwab *et al.*, 2006). Briefly, amiRNAs were generated by PCR using RS300. The resulting PCR product was digested by *Xho*I and *Spe*I, inserted into pENTRY-BS and then cloned by LR clonase reaction into the estradiol-inducible pER8-GW and constitutive pB2GW7 (PSB; Ghent University, Belgium) expression vectors.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Expression levels of ERF-VII type transcription factors in public available database.

Figure S2. Stress regulation of ERF-VII type transcription factors.

Figure S3. Trans-activation of ADH1-LUC by estradiol induced RAP2.12, RAP2.2 and RAP2.3 in independent lines.

Figure S4. Selected ABA and osmotic stress induced genes that were unaffected by RAP2.12 overexpression.

Figure S5. Expression of RAP2.12 and RAP2.3 genes in single rap2.12-2, rap2.3-1 and the rap2.12-2 rap2.3-1 double mutant.

Figure S6. Comparison of wild type and rap2.2-4 insertion mutant line in osmotic stress growth assay.

Figure S7. Gene expression changes of osmotic stress induced genes in wild type and rap2.12-2 rap2.3-1 double mutant line.

Figure S8. Amino acid sequence of RAP2.12 and HA:RAP2.12 proteins.

Figure S9. HA:RAP2.12 protein is stabilised by the proteasome inhibitor MG132.

Figure S10. Phylogenetic analysis of TRAF domain-like proteins of Arabidopsis.

Figure S11. Co-expression of the Arabidopsis SINAT1 and SINAT2.

Figure S12. Co-silencing of SINAT1 and SINAT2 genes.

Figure S13. Model of RAP2.12, RAP2.2 and RAP2.3 mediated transcriptional activation of the low oxygen and ABA induced *ADH1* gene.

Table S1. List of primers used in this study.

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