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A homolog of the *Arabidopsis thaliana ERS* gene is actively regulated in *Rumex palustris* upon flooding

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Summary

A cDNA homologous to the ethylene-response sensors (ERS/ETR1) from *Arabidopsis thaliana* was isolated from a *Rumex palustris* cDNA library. This cDNA, RP-ERS1, was 2421 bp long and shared 66% nucleotide homology with ETR1 and ERS in their coding regions. The transcript level of *RP-ERS1* was actively regulated during the leaf-elongation response of *R. palustris* upon flooding. *RP-ERS1* transcript levels increased after submergence, and also after exposure to high concentrations of ethylene and carbon dioxide and low concentrations of oxygen. These results suggest that *R. palustris* plants may respond to flooding stress by increasing the number of their ethylene receptors.

Introduction

The gaseous phytohormone ethylene plays an important role in many aspects of plant growth and development, including seed germination, flowering, senescence, abscission, fruit ripening (Abeles *et al.*, 1992) and the stimulation of shoot elongation under water (Jackson and Pearce, 1991; Osborne, 1984; Voesenek and Blom, 1989). The ethylene biosynthetic pathway has been studied extensively and is now largely understood (Yang and Hoffman, 1984). The conversion of *S*-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase is considered to be the rate-limiting step. ACC is converted to ethylene, CO_2 and cyanide by the enzyme ACC oxidase. ACC oxidase is constitutively present in most tissues, but its mRNA levels and synthesis have been shown to increase during fruit ripening in tomato and in submerged deepwater rice (Kende, 1993; Mekhedov and Kende,1996).

The mechanisms by which plant cells perceive and transduce the ethylene signal are still not understood. However, the recent isolation of an *Arabidopsis thaliana* mutant *etr1-1* that lacks a number of responses to ethylene has been an important step forward in our understanding of these mechanisms. While the ethylene synthesis rate of this mutant is not significantly different, feedback regulation by ethylene does not occur (Bleecker *at al.*, 1988). Chang *et al.* (1993) established that the ethylene-insensitive phenotype of the *etr1-1* mutant is caused by a mutation in the *ETR1* gene (*Ethylene-Response*) that encodes a protein with a predicted carboxyl-terminal sequence that is highly similar to the conserved domains of the prokaryotic two-component system of signal transduction. Schaller and Bleecker (1995) used a yeast expression system to demonstrate that the amino-terminal part of ETR1 binds ethylene. This domain contains the amino acid residues that are mutated in the *etr1* mutants, thereby causing the ethylene-insensitive phenotype. More recently, two genes and one cDNA that share sequence similarities with *ETR1* have been isolated: the *A. thaliana ERS* gene (*Ethylene Response Sensor;* Hua *et al.*, 1995), the *Lycopersicon esculentum NR* gene (*Never-Ripe;* Wilkinson *et al.*, 1995) and the *L. esculentum* eTAE1 cDNA (Zhou *et al.*, 1996). However, so far, only the *A. thaliana* ETR1 has been proven to be an ethylene receptor (Chang *et al.*, 1993; Schaller and Bleecker, 1995). These genes encode two distinct but similar proteins: one with (ETR1-like) and one without (ERS-like) a response-regulator domain. The *ETR1*-like genes appear to be expressed constitutively at the mRNA level in all tissues so far studied (Chang *et al.*, 1993; Zhou *et al.*, 1996), whereas the expression of the *ERS*-like gene, as for the *NR* gene of tomato, increases during fruit ripening and upon ethylene treatment (Bleecker and Schaller, 1996; Wilkinson *et al.*, 1995). *A. thaliana* plants transformed with an altered *ERS* gene are also ethylene-insensitive, and thus ERS might be considered to be a sensor of ethylene, like ETR1 (Hua *et al.*, 1995).

To date, nothing is known about the influence of environmental stresses, such as flooding, on the mechanisms of ethylene perception in plants. Flooding induces major endogenous changes in the concentration of oxygen, carbon dioxide and ethylene gases in the plants. Some aquatic and semi-aquatic plants respond to flooding by increasing the elongation rate of the petioles, leaf blades or stems in order to re-establish contact with the atmosphere (Armstrong *et al.*, 1994; Blom and Voesenek, 1996). Ethylene, the phytohormone that strongly inhibits elongation in most terrestrial plants (Abeles *et al.*, 1992), is largely responsible for promoting fast extension under water (Metraux and Kende, 1983). *Rumex palustris*, a flooding-tolerant species distributed along the river plain, also shows this response (Voesenek and Blom, 1989; Voesenek

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et al., 1993). When this species is submerged, ethylene accumulates in the shoot to a level of approximately 1 μl l^{-1} within 1 h; a 20-fold increase over plants grown in air (Banga *et al.*, 1996). In addition to ethylene, gibberellin and sub-ambient concentrations of oxygen also play an important role in leaf elongation during submergence (Voesenek *et al.*, 1996). Furthermore, Stünzi and Kende (1989) have also demonstrated that the carbon dioxide concentration in deepwater rice increases when shoots are submerged.

As a first step in elucidating the molecular regulation of the flooding response mediated by ethylene in *Rumex* species, we isolated a cDNA clone that is similar to the *ERS* gene of *A. thaliana*, and named it RP-ERS1 (*Rumex palustris* ethylene response sensor). Here, we show that the transcription level of the *RP-ERS1* gene is modulated by flooding, and is up-regulated by high concentrations of ethylene and carbon dioxide and low concentrations of oxygen.

Results

Cloning and genomic organization of a Rumex palustris *ERS homolog*

A cDNA library constructed from leaf mRNA of *R. palustris* plants that had been submerged for 24 h was screened with a probe derived from the *ETR1* gene and consisting of most of the exon regions (Chang *et al.*, 1993). By this procedure, we isolated a full-length cDNA clone (RP-ERS1; 2421 bp long) which shares 66% nucleotide homology with *ETR1*. Upon alignment of the deduced amino acid sequence with other known ethylene-response genes (Figure 1), *RP-ERS1* appeared to be a homolog of the recently isolated *ERS* (Hua *et al.*, 1995) and TXTR-14, a cDNA corresponding to the *NR* gene (Wilkinson *et al.*, 1995). RP-ERS, ERS and NR are 103–125 amino acids shorter than ETR1, because they lack the carboxy-terminal domain which contains the putative response regulator in ETR1 (Chang *et al.*, 1993). In Figure 1, boxes are drawn around the five conserved sequences that are characteristic of sensor histidine protein kinase domains (HPK) found in the bacterial two-component systems (Chang *et al.*, 1993; Parkinson and Kofoid, 1992). The amino acids within these domains are 61% identical in all five predicted proteins. The underlined hydrophobic regions (potential transmembrane segments; Bleecker and Schaller, 1996) found in ETR1 have 69% identical amino acids. This alignment revealed two additional highly conserved domains between the hydrophobic and histidine kinase domains (bold in Figure 1). These domains, amino acids 129–184 and amino acids 291–316, consist of 80% and 87% identical amino acids, respectively. The four amino acids that when mutated lead to dominant ethylene insensitivity in the *Arabidopsis etr1* mutants are conserved. The Pro36 residue, which is substituted to Leu in the NR protein of the tomato *Never-Ripe* mutant, is also conserved in all five proteins. The presence of *RP-ERS1*-related genes in the *Rumex* genome was investigated by Southern analysis. As shown in Figure 2, a DNA blot probed with the RP-ERS1 cDNA under very stringent conditions revealed only a few hybridizing bands, suggesting the presence of a single-copy gene or a small gene family. Hybridization at lower stringency showed no additional bands (data not shown).

RP-ERS1 *gene expression is highly regulated during submergence*

In order to analyse *RP-ERS1* transcript accumulation under flooding stress, we performed RNA blot hybridizations with RNA isolated from shoots of 26- to 30-day-old *R. palustris* plants. Figure 3 shows that the level of *RP-ERS1* transcript (2.4 kb) in untreated *R. palustris* plants was low but detectable. In contrast, when the plants were submerged for 24 h, the amount of transcript increased approximately eightfold. When the water level was lowered to just above the roots (de-submergence), an immediate decrease in *RP-ERS1* gene expression in the shoot was observed, and 2 h after de-submergence, accumulation of the transcript reached the basal level. In conclusion, our results show that *RP-ERS1* transcript accumulation is up-regulated during submergence.

However, endogenous gas composition also changes during submergence. In general, when plants are exposed to flooding stress, ethylene and carbon dioxide concentrations increase and the oxygen level declines (Stünzi and Kende, 1989). The impact of these changes on the expression level of *RP-ERS1* was investigated by exposing *R. palustris* to mixtures of these gases at different concentrations. Northern blot analysis of RNA isolated from leaves of treated plants showed that gas mixtures containing 5 μl l^{-1} ethylene stimulated an increase in the transcript level of *RP-ERS1* (Figure 4). Exposure of the plants to 5% carbon dioxide resulted in a threefold increase in the concentration of the transcript, and low oxygen level (3%) caused a 15-fold increase in accumulation of the *RP-ERS1* transcript in the leaf after 24 h. The combination of 5 μl l^{-1} ethylene with 3% oxygen had the strongest stimulating effect, stronger than the effect of these gases separately. This indicated that not only submergence but also high levels of ethylene and carbon dioxide or low oxygen concentrations were able to induce the accumulation of the *RP-ERS1* transcript. In order to test whether low oxygen or high carbon dioxide levels have an effect on the synthesis rate of ethylene, which in turn generates the increase in *RP-ERS1* transcript level, we measured the ethylene production rate after treatment with low oxygen and high carbon dioxide concentrations. The average ethylene production by control plants was

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R. palustris RP-ERS1  ----------------MNTCDCVDT-QWFADDLLIKSQYISDLFIVLAYFSIPLELIYFVQK   45
A. thaliana ERS       ----------------MES.D.FET-HVNQDD..V.Y..I..AL.AL......L......Q.   45
A. thaliana ETR1      ----------------MEV.N.IEP-QWPADE..M.Y..I..FF.AI......L......K.   45
L. esculentum eTAE1   MGSLLRMNRLLSSIV-ES.N.IIDPQLPADD..M.Y..I..FF.AL......V......K.   60
L. esculentum TXTR-14 ----------------MES.D.IEA-LLPTGD..V.Y..L..FF.AV......L......H.   45

RP-ERS1 SALFPYRWVLIQFGAFIVLCGTTHLINLFTFSMHSKTLAVVMAIAKMSTAAVSCITALMLVHIIPDLLSVKMRES  120
ERS     ..F...K...M......I......F.N.WMFFM.SKAV.I..TIA.VSC.V...A..L.............N..L  120
ETR1    ..V...R...V......V......L.N.WTFTT.SRTV.L..TTA.VLT.V...A..L.............T..L  120
eTAE1   ..V...R...V......V......L.N.WTSTP.TRTV.M..TTA.FST.A...A..V.............T..L  135
TXTR14  ..C...R...M......V......F.S.WTFFM.SKTV.V..TIS.MLT.A...I..L.............T..L  120

RP-ERS1 ILRRRADELDREMGLILSQEETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLGLEECSLWMPSQTGMTLKLSH  195
ERS     F.KKK.DE..R...L.LT.............G..R.......R.......K..C.E..A....SQS.LY.Q..H  195
ETR1    F.KNK.AE..R...L.RT.............E..S.......K.......R..A.E..A....TRT.LE.Q..Y  195
eTAE1   F.KKK.AQ..R...I.RT.............E..S.......K.......R..A.E..A....TRT.LE.Q..Y  210
TXTR-14 F.KTR.EE..K...L.IR.............E..S.......K.......R..D.A..A....CQG.LT.Q..H  195

RP-ERS1 ALQNLTPVGSCVSLNLPVITEIFNNPGVVRISHTCPLVSTRPISGRYLAPEVAAIRVPLLNLSNFQINDWPDVSG  270
ERS     T.SHKIQ.GSS.PIN..I.NEL.NSAQAMH.PHSC.LAKIGPPVGR.SPP..VSV.....H.....GS..SDL.G  270
ETR1    T.RHQHP.EYT.PIQ..V.NQV.GTSRAVK.SPNS.VARLRPVSGK.MLG..VAV.....H.....IN..PEL.T  270
eTAE1   T.RHQNP.GLT.PIQ..V.NQV.GTNHVVK.SPNS.VARLRPA-GK.MPG..VAV.....H.....IN..PEL.T  284
TXTR-14 N.NNLIP.GST.PIN..I.NEI.SSPEAIQ.PHTN.LARMRNTVGR.IPP..VAV.....H....-TN..AEL.T  269

RP-ERS1 RTYAVMVLVLPCDGLRKFRKHELELIEVVADQVAVALSHAAILEESMRAHDQLLEKNVALDLARQEAELAIRARN  345
ERS     KG..I...I..TDGA.KWRD.....VEN................E..H.R.Q.M.Q.F...K..Q...M.VH...  345
ETR1    KR..L...M..SDSA.QWHV.....VEV................E..R.R.L.M.Q.V...L..R...T.IR...  345
eTAE1   KR..L...M..SDSA.QWHV.....VEV................E..R.R.L.M.Q.V...L..R...M.VR...  359
TXTR-14 RS..V...V..MNGL.KWRE.....VQV................D..R.H.Q.M.Q.I...V..Q...M.IR...  344

RP-ERS1 DFLAVMNHEMRSAMHAIITLSSFLLETELTPEQRVMIEIILRSSNILATLINDVLDLSRLEDGSLELDSLTFNLH  420
ERS     .....|......TP|..I.S.S.L.L..E.S....V.I....K...LV...IS..........S.L.ENEP.S.Q  420
ETR1    .....|......TP|..I.A.S.L.Q..E.T....L.V....K...LL...MN..........S.Q.ELGT.N.H  420
eTAE1   .....|......TP|..I.A.S.L.Q..D.T....L.V....K...LL...IN..........S.Q.DIGT.N.H  434
TXTR-14 .....|......TP|..V.A.C.L.L..D.T....V.I....K...LL...IN..........I.E.ENGT.N.H  419

RP-ERS1 AVFKKVINLLKPITCVKKLSLTLAVA-PEAICVVGDEKRLVQTILNVVGNAVKFTKVGKISIIVSVAKPDSLSSW  494
ERS     AIFEEVIS.I...ASV...STN.ILSADLPTYAI..E..|.M.TI..IM...|...T.E.YI.IIASIM.PESL-QE  494
ETR1    TLFREVLN.I...AVV...PIT.NLAPDLPEFVV..E..|.M.II..IV...|...S.Q.SI.VTALVT.SDTR---  492
eTAE1   ALFREVHS.I...ASV...FVT.SLSSDLPEYVI..E..|.M.IL..VV...|...S.E.NV.ISAFVA.SDSL-RD  508
TXTR-14 GILREAVN.I...ASL...SIT.ALALDLPILAV..A..|.I.TL..VA...|...T.E.HI.IEASVA.PEYA-RD  493

RP-ERS1 QPADVIYAAVSKGQFYLRVQVKDTGCGINPQEIPHIFDKFYQSQ-NCRRNSTDSGLGLAICKRFVNLMGGQIWVE  568
ERS     LPSPEFFPVLSDSH...C.QVK.T.C..HT.D..LL.T..VQPRTGTQRNHSGG....|.LCK..VG..G.YM.I.  569
ETR1    --AADFFVVPTGSH...R.KVK.S.A..NP.D..KI.T..AQTQSLATRSSGGS....|.ISK..VN..E.NI.I.  565
eTAE1   PRAPEFFAVPSENH...R.QIK.T.I..TP.D..NL.S..TQSQALATTNSGGT....|.ICK..VN..E.HI.I.  583
TXTR-14 CHPPEMFPMPSDGQ...R.QVR.T.C..SP.D..LV.T..AESRPTSNRSTGGE....|.ICR..IQ..K.NI.I.  568

RP-ERS1 SEGLDKGSLVTFVVKMGICRNPDERSINQAASTGHMNQSSTDLLGDSSMSFSLKRFEKSC                  628
ERS     .E.LE..CTAS.IIRL..CNGPSSSSGSMALHLAAKSQTRPWNW                                  613
ETR1    .D.LG..CTAI.DVKL..SERSNESKQSGIPKVPAIPRHSNFTGLKVLVMDENGVSRMVTKGLLVHLGCEVTTVS   640
eTAE1   .E.LG..STAI.IIKL..PGRANESKLPFVTKLPANHTQMSFQGLKVLVMDENGVSRMVTKGLLTH.G.D....G   658
TXTR-14 .E.PG..TTVT.VVKL..CHHPNALPLLPMPPRGRLNKGSDDLFRYRQFRGDDGGMSVNAQRYQRS.          635

ETR1    SNEECLRVVSHEHKVVFMDVCMPGVENYQIALRIHEKFTKQRHQRPLLVALSGNTDKSTKEKCMSFGLDGVLLKP  715
eTAE1   .RD......T......I...S.Q.IDC.EV.VV...R.G.-..G...I...T....RV...N..RV.M...I...  732

ETR1    VSLDNIRDVLSDLLEPRVLYEGM                                                      738
eTAE1   ..VYKM.S...E...HG.VL.S                                                       754
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Figure 1. Comparison of the RP-ERS1 amino acid sequence of *R. palustris* with other ethylene response sensor sequences.
The sequences were obtained from GenBank. Gaps (–) have been introduced to maximize the alignment. The hydrophobic transmembrane regions are underlined, conserved regions are printed in bold and boxes are drawn around the HPK domains. (ETR1, Chang *et al.*, 1993; ERS, Hua *et al.*, 1995; TXTR–14, Wilkinson *et al.*, 1995; eTAE1, Zhou *et al.*, 1996).

4.8 nl g^{-1} DW h^{-1} (SE 1.3 nl g^{-1} DW h^{-1}) during the first 24 h. Ethylene production by plants exposed to 3% oxygen was 3.8 nl g^{-1} DW h^{-1} (SE 0.6 nl g^{-1} DW h^{-1}), and in the presence of 5% carbon dioxide this was 5.5 nl g^{-1} DW h^{-1} (SE 1.3 nl g^{-1} DW h^{-1}). In conclusion, both carbon dioxide and oxygen exerted their effect on the *RP-ERS1* transcript level independently of ethylene.

To test the kinetics of *RP-ERS1* expression, we submerged *R. palustris* plants and analysed leaf RNA at several time points during the first 48 h (Figure 5). As early as 1 h after

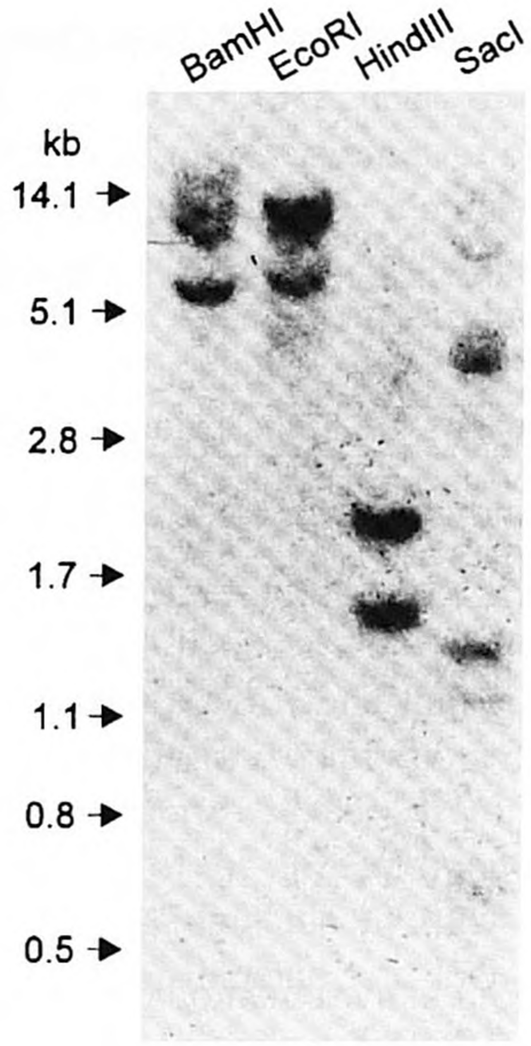

Figure 2. Hybridization of RP-ERS1 cDNA to digested *R. palustris* genomic DNA.
According to the sequence, the cDNA contains one *Bam*HI site, one *Hin*dIII site and two *Sac*I sites. Molecular length standards are indicated at the left in kilobases (kb).

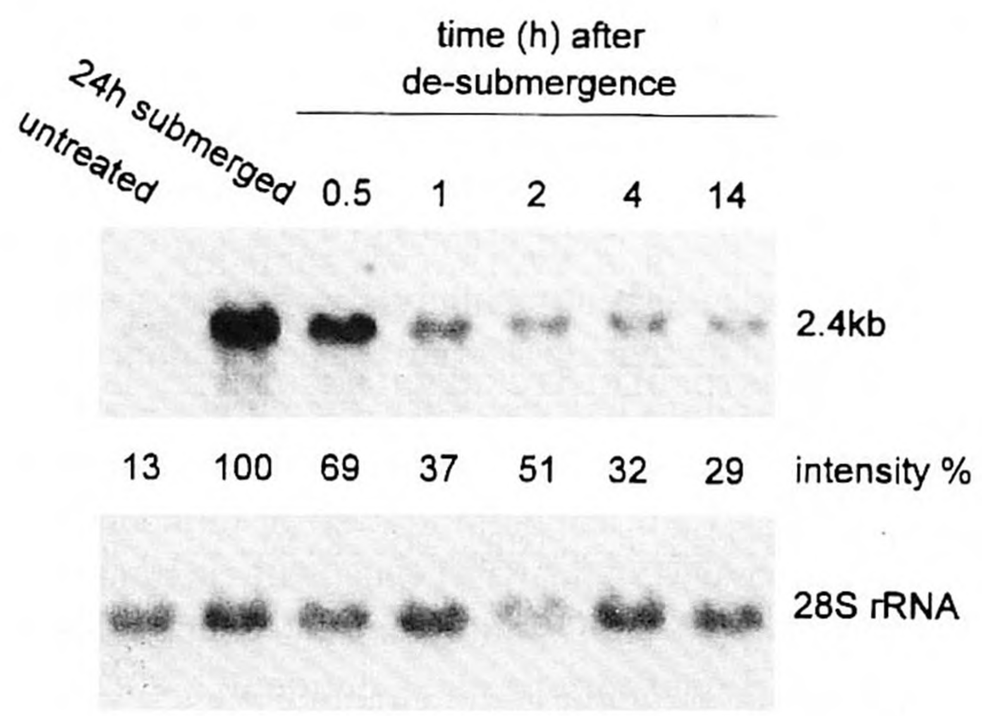

Figure 3. Expression of the *RP-ERS1* gene in *R. palustris* shoots upon flooding and de-submergence.
The blot was reprobed with 28S rRNA as a control for the loaded amount of total RNA. The values given represent the *RP-ERS1* transcript level relative to the highest signal detected.

submergence, there was an increase in the level of the *RP-ERS1* transcript; a maximum was observed after 4 h. Hereafter, the transcript level decreased, but remained higher than the level of mRNA before induction.

Discussion

Rumex palustris leaves elongate during submergence. This response is a part of the resistance mechanism of this species to flooding and is initiated by the hormone ethylene (Voesenek and Blom, 1989). The first step of the ethylene-

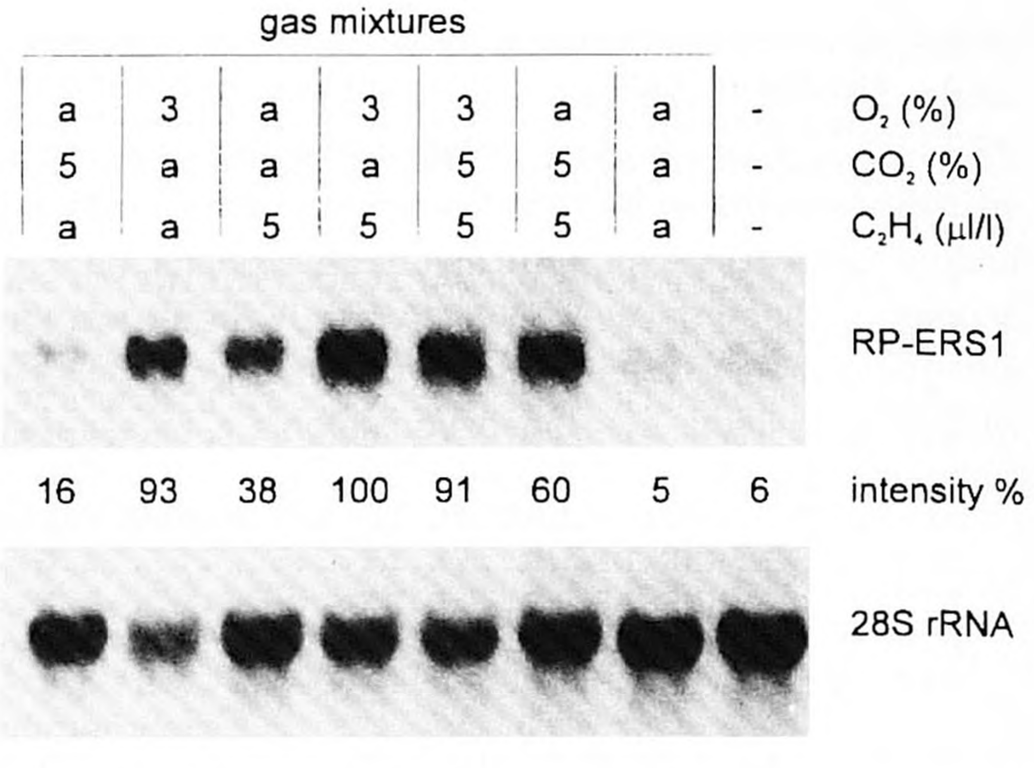

Figure 4. Expression of the *RP-ERS1* gene in leaf material after exposure of *R. palustris* plants for 24 h to different gas mixtures of carbon dioxide, oxygen and ethylene in nitrogen gas.
a, the same concentration as in air (21% O_2; 0.033% CO_2; < 0.005 µl l^{-1} ethylene); –, untreated plants. The blot was reprobed with 28S rRNA as a control for the loaded amount of total RNA. The values given represent the *RP-ERS1* transcript level relative to the highest signal detected (100%).

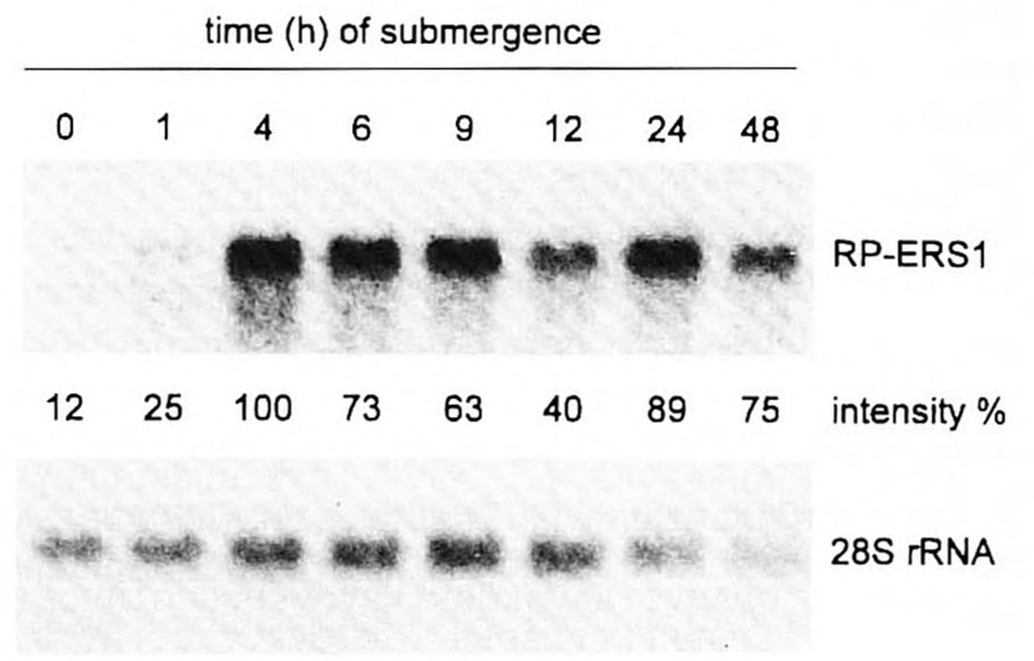

Figure 5. Expression of the *RP-ERS1* gene in *R. palustris* shoots during submergence.
The blot was reprobed with 28S rRNA as a control for the loaded amount of total RNA. The values given represent the *RP-ERS1* transcript level relative to the highest signal detected (100%).

response pathway in *Arabidopsis thaliana* is mediated by the ethylene-binding protein ETR1 (Roman *et al.*, 1995; Schaller and Bleecker, 1995), which consists of several conserved domains. The domain located at the amino-terminal part of these proteins comprises the hydrophobic ethylene-binding transmembrane regions. A second domain in the core of the protein shares homology with the HPK (histidine protein kinase) domains of the prokaryotic two-component systems. The carboxyl-terminal domain of ETR1 is homologous to the receiver domain of the response-regulator component that is also a part of the prokaryotic two-component system (Chang *et al.*, 1993). ERS lacks this domain. We isolated an *R. palustris* (*RP-ERS1)* homolog of the *ERS* gene of *A. thaliana*. The predicted protein, RP-ERS1, contains in the first domain the amino acid sequence characteristic of ETR1/ERS, the cysteines necessary for dimerization (Schaller *et al.*,

1995) and the hydrophobic transmembrane regions. In all five aligned sequences (Figure 1), these regions contain the amino acids Ala31, Ile62, Cys65 and Ala102, which are substituted for in the ETR1 protein of the ethylene-insensitive *etr1* mutants. The Pro36 residue that is converted to Leu in the mutant NR protein is also conserved. The HPK domain is also present in RP-ERS1, whereas the carboxyl-terminal receiver domain is absent, as it is in the ERS protein of *A. thaliana* (Hua *et al.*, 1995) and in NR of *L. esculentum* (Wilkinson *et al.*, 1995). Alignment of ETR1 and ERS amino acid sequences with RP-ERS1 revealed two additional conserved domains between the hydrophobic and the HPK domains. The first domain (amino acids 129–184) and a part of the downstream domain (amino acids 288–295) are hydrophilic (Kyte–Doolittle method; Kyte and Doolittle, 1982) and probably lie at the surface of the protein (Emini surface probability; Emini *et al.*, 1985). The downstream domain, amino acids 291–316, has 83% similarity with a bacterial chromatic adaptation sensor (Kehoe and Grossman, 1996). A plausible explanation for these conserved regions is that they may play a role in the recognition or binding of a second messenger in the ethylene-signalling pathway.

Southern blot analysis of *R. palustris* DNA indicates the presence of one or two *RP-ERS1* genes. However, it is also possible that *R. palustris* possesses both receptor types, as has been found in *A. thaliana*. It is conceivable that in addition to the ETR1 ethylene-receptor type with the two components joined together in one protein, plants normally also contain a ERS ethylene-receptor type with separate HPK and response-regulator domains. Isolation of a gene encoding a homolog of the response-regulator component is required to confirm this hypothesis.

The expression pattern of the genes encoding for proteins with (ETR1-like) and without (ERS-like) a response-regulator domain suggests a differential function. The *ETR1*-like genes are constitutively expressed (Chang *et al.*, 1993; Zhou *et al.*,1996), while the expression of the *ERS*-like gene (Wilkinson *et al.*, 1995) is inducible. This observation is strengthened by our finding that *RP-ERS1*, an *ERS* homologue, is induced by stress such as flooding. The possibility that both the ethylene biosynthesis and the signal transduction pathway may be induced makes the plant capable of regulating tissue-specific ethylene-controlled processes. If a higher accumulation of *RP-ERS1* transcript under stress conditions corresponds to a higher concentration of receptor, this could imply that the plant becomes more sensitive to ethylene (the same response with less ethylene). Two alternative models for the regulation of primary signal transduction have been proposed (Bleecker and Schaller, 1996). The first model assumes ethylene to be a positive regulator of the histidine kinase activity of the receptor, while the second assumes that ethylene has the opposite effect. If the plants were sensitized by more receptor, this would be more consistent with the first model. However, further experimental evidence should be obtained to elucidate the mode of action of the ethylene receptors.

During submergence, the endogenous ethylene concentration reaches the level of 5 $\mu l\ l^{-1}$ within the first 12 h (Voesenek *et al.*, 1993). However, the petiole-growth response is saturated at an ethylene concentration of 1 $\mu l\ l^{-1}$. (Voesenek *et al.*, 1996). Therefore, ethylene does not seem to be the limiting factor in the *R. palustris* leaf-elongation response. In contrast, the concentration of the receptor could be the limiting factor. We observed that the expression of the *RP-ERS1* gene in *R. palustris* during submergence (Figure 5) remained high, at least during the first 48 h of submergence. De-submergence after 24 h led to an immediate decrease in the expression level of *RP-ERS1*. The concentration of the messenger decreased within 1 h to a level approximately 37% of that found in submerged plants (Figure 3). These findings agree with the growth kinetics of *R. palustris* leaves during and after submergence (Voesenek *et al.*, 1993). *RP-ERS1* expression level was induced by flooding and also by ethylene and low oxygen and high carbon dioxide concentrations (Figures 3 and 4). It has been shown that partial oxygen shortage promotes ethylene production in some species (Jackson, 1985). However, our measurements on *R. palustris* plants incubated in 3% oxygen or 5% carbon dioxide showed that the production rate of ethylene was approximately the same as or lower than that of plants in air. Nevertheless, 3% oxygen does have a stimulating effect on leaf growth (Voesenek *et al.*, 1996), and in the present investigation we have shown that it induced a higher accumulation of *RP-ERS1* transcript, while ethylene production itself was not stimulated. It is not clear how this concentration of oxygen stimulated gene expression, but it is evident that a number of physiological changes take place in the cell under low oxygen conditions (Sachs *et al.*, 1996). Interestingly, 5% carbon dioxide only slightly increases transcript accumulation and does not stimulate leaf elongation in *R. palustris* (Voesenek *et al.*, 1996). Taken together, our results suggest that high levels of *RP-ERS1* transcript correlate with high growth rates of *R. palustris* leaves under flooding conditions.

We have used *Rumex palustris* to study the regulation of the genes encoding the ethylene response sensor in response to submergence. Ethylene and low oxygen concentration both stimulate the leaf-elongation response and the mRNA accumulation of the *R. palustris* ethylene receptor RP-ERS1. Low oxygen stimulates the accumulation of *RP-ERS1* messenger independently of ethylene and seems therefore to enhance leaf elongation by increasing input into the ethylene signal transduction pathway.

Experimental procedures

Plant material

Achenes of *Rumex palustris* Sm. were collected from river areas near Millingen in the Netherlands. Germination and growing

conditions were as described by Banga *et al.* (1996). All of the plants used for experiments were 26–30 days old and just starting to develop their fifth leaf.

Plant treatments

During all experiments, and for 24 h before, plants were grown under constant light (Photosynthetic photon flux density (PPFD): 65 μE m^{-2} sec^{-1}, 400–700 nm) at 22°C. Submergence took place in an open tank with 25 cm tapwater at 22°C. At several time points after submergence, petioles and leaf blades were cut from the youngest three leaves (10 plants per sample). During the gas treatments, plants were incubated in 10.8 litre desiccators and were flushed (10 l h^{-1}) with different gas mixtures (Hoekloos, the Netherlands) as described in the legend of Figure 4. Each gas treatment lasted 24 h, and samples were taken from leaves 3, 4 and 5 (10 replicates per sample). There was no difference in the expression level of *RP-ERS1* in leaf blades and petioles of the youngest three leaves.

Ethylene production

The effect of 3% oxygen or 5% carbon dioxide on the ethylene production of *R. palustris* plants was measured with laser-driven photoacoustic spectroscopy (Voesenek *et al.*, 1990). The soil was gently washed off the roots of six plants, and three plants were placed together in 40 ml glass vials filled with tapwater. Two glass vials with three plants each were placed in separate airtight cuvettes (600 ml) that were flushed with air for 24 h. After that, the airflow (1 l h^{-1}) was changed to 3% oxygen (v/v) or 5% carbon dioxide (v/v). The ethylene production was measured every h, and all experiments were repeated at least four times.

cDNA library construction and screening

Leaves 4 and 5 from 20 plants submerged for 24 h were used in a single-step method for RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was isolated using PolyATtract mRNA Isolation Systems (Promega, USA). Double-stranded cDNA was prepared from 2.5 μg mRNA using a Stratagene cDNA Synthesis Kit and a ZAP-cDNA Synthesis Kit. The library was packaged using a ZAP-cDNA Gigapack II Gold Cloning Kit (Stratagene, USA). Approximately 150 000 plaques were screened with an [α-^{32}P]dATP-labelled 1500 bp *Xho*I fragment of the *ETR1* gene using Statagene's protocol. Filters were prehybridized for 4 h and hybridized overnight at 62°C with a solution containing 6 × SSC, 5×Denhardt's reagent, 0.1% SDS and 100 μg ml^{-1} denatured, fragmented salmon sperm DNA . Membranes were then washed twice in 2 × SSC plus 0.1% SDS at 65°C for 15 min each, and twice in 0.2 × SSC plus 0.1% SDS for 15 min. The blots were exposed to Valca HPX44 X-ray film with two intensifying screens at –80°C for 16–48 h. Three positive plaques from the first round of screening were chosen for further analysis. After a second round of screening, only one plaque hybridized to the probe. The pBluescript SK(–) Phagemid containing the only positive cDNA was excised from the Uni-ZAP XR vector and cloned into *Escherichia coli* XL-1 Blue MRF' cells (ExAssist/SOLR *in vivo* excision system, Stratagene, USA).

DNA manipulations and sequence analysis

Plasmid DNA was isolated by the alkaline lysis method (Sambrook *et al.*, 1989). Probes were labelled in low-melting-point agarose with [α-^{32}P]dATP by the random-priming method (Church and Gilbert, 1984). DNA sequencing was carried out with single-stranded plasmid DNA (Russel *et al.*, 1986) by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the T7 DNA polymerase system of Pharmacia.

RNA manipulations

For RNA gel blots, total RNA was isolated (van Eldik *et al.*, 1995) and separated on a 1% agarose gel containing 0.4 M formaldehyde and 0.1 μg ml^{-1} ethidium bromide. After electrophoresis, the gel was inspected by UV exposure and photographed to ensure that equal amounts of RNA (10 μg) were present in each lane. RNA was transferred overnight to a nylon membrane by capillary transfer in 20 × SSC. RNA was fixed to the membrane as described in the Hybond-N nylon membrane (Amersham, UK) handbook. Prehybridization, hybridization and washing conditions were the same as described for the library screening. Hybridization was performed overnight at 65°C using RP-ERS1 as a probe. The blots were stripped and rehybridized with a tobacco ribosomal cDNA (kindly provided by Dr K. Weterings) to ensure an equal transfer during the blotting procedure. The autoradiographs were scanned with a Bio-Rad densitometer, and the signals were quantified with Molecular Analyst software (Bio-Rad, USA). The value for the intensity of the RP-ERS1 signals is the density of the band on the autoradiograph corrected for the density of the 28S band, relative to the highest signal detected on that blot (which has a value of 100%).

Genomic Southern analysis

Genomic DNA was isolated (van Eldik *et al.*, 1995), digested (10 μg) with *Bam*HI, *Eco*RI, *Hin*dIII or *Sac*I (Boehringer Mannheim GmbH, Germany) and electrophoretically separated on an 0.8% agarose gel. Southern blotting, DNA fixation and hybridization were performed on a Hybond-N nylon membrane according to Amersham's handbook. The full-length RP-ERS1 cDNA was used as a probe. Hybridizations were performed at high (65°C) and low (62°C) stringency in 6 × SSC, 0.1% SDS, 5 × Denhardt's reagent and 100 μg ml^{-1} denatured salmon sperm DNA. Washings were performed in 0.1 × SSC + 0.1% SDS at 65°C and in 2 × SSC + 0.1% SDS at 62°C, respectively.

Accession numbers

Sequences used for the alignment have the following GenBank accession numbers: *A. thaliana ETR1*, L24119; *A. thaliana ERS*, U21952; *L. esculentum* eTAE1, U41103; *L. esculentum* TXTR–14, U38666; *R. palustris* RP-ERS1, U63291.

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