Planta (2007) 225:575–588
DOI 10.1007/s00425-006-0373-2

ORIGINAL ARTICLE

The barley ERF-type transcription factor HvRAF confers enhanced pathogen resistance and salt tolerance in *Arabidopsis*

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Received: 14 April 2006 / Accepted: 4 August 2006 / Published online: 26 August 2006

Abstract We isolated *HvRAF* (*Hordeum vulgare* root abundant factor), a cDNA encoding a novel ethylene response factor (ERF)-type transcription factor, from young seedlings of barley. In addition to the most highly conserved APETALA2/ERF DNA-binding domain, the encoded protein contained an N-terminal MCGGAIL signature sequence, a putative nuclear localization sequence, and a C-terminal acidic transcription activation domain containing a novel mammalian hemopexin domain signature-like sequence. Their homologous sequences were found in AAK92635 from rice and RAP2.2 from *Arabidopsis*; the ERF proteins most closely related to HvRAF, reflecting their functional importance. RNA blot analyses revealed that *HvRAF* transcripts were more abundant in roots than in leaves. *HvRAF* expression was induced in barley seedlings by various treatment regimes such as salicylic acid, ethephon, methyl jasmonate, cellulase, and methyl viologen. In a subcellular localization assay, the HvRAF-GFP fusion protein was targeted to the nucleus. The fusion protein of HvRAF with the GAL4 DNA-binding domain strongly activated transcription in yeast. Various deletion mutants of HvRAF indicated that the transactivating activity was localized to the acidic domain of the C-terminal region, and that the hemopexin domain signature-like sequence was important for the activity. Overexpression of the *HvRAF* gene in *Arabidopsis* plants induced the activation of various stress-responsive genes, including *PDF1.2*, *JR3*, *PR1*, *PR5*, *KIN2*, and *GSH1*. Furthermore, the transgenic *Arabidopsis* plants showed enhanced resistance to *Ralstonia solanacearum* strain GMI1000, as well as seed germination and root growth tolerance to high salinity. These results collectively indicate that HvRAF is a transcription factor that plays dual regulatory roles in response to biotic and abiotic stresses in plants.

Keywords ERF-type transcription factor · *Hordeum vulgare* root abundant factor · Resistance to *Ralstonia* · Salt-stress tolerance

Abbreviations

ABA	Abscisic acid
AP2	APETALA2

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ERF	Ethylene responsive factor
ET	Ethylene
MeJA	Methyl jasmonate
MV	Methyl viologen
SA	Salicylic acid
SmGFP	Synthetic modified green fluorescent protein

Introduction

During growth and development, plants respond to a wide range of environmental stresses by multiple defense mechanisms such as transcriptional activation of defense genes, cell wall enforcements, induction of programmed cell death, and production of antibiotic compounds such as phytoalexins (Jenk et al. 1994; Glazebrook 2001; Xiong et al. 2002). In particular, it has been well established that transcriptional activation of diverse defense genes, such as pathogenesis-related (PR) genes, by the accumulation of phytohormones including salicylic acid (SA), ethylene (ET), and methyl jasmonate (MeJA), enhances resistance to a variety of pathogen attacks (Reymond and Farmer 1998). Systemic acquired resistance (SAR) is a well-known SA-mediated defense response following pathogen infections (Ryals et al. 1996). This response involves transcriptional activation of a subset of acidic PR genes, such as *PR1*, *PR2* (*BGL2*), and *PR5*. Apart from the SA-mediated SAR pathway, MeJA and ET also play important roles against a variety of pathogen attacks by activating the basic PR genes such as *PR3*, *PR4*, and *PDF1.2* (Kunkle and Brooks 2002; Turner et al. 2002). Abscisic acid (ABA) is a major phytohormone involved in the defense responses against abiotic stresses as well as in plant development. Upon exposure to various abiotic stresses, plants quickly accumulate ABA in their vegetative tissues, causing the activation of many stress-responsive genes, including *COR* genes (Leung and Giraudat 1998). However, ABA-independent, abiotic stress-responsive signaling pathways have also been studied (Chinnusamy et al. 2005).

A considerable number of genetic and molecular approaches have demonstrated that stress-responsive signaling pathways in plants involve a complex network of various components, including receptors, kinases, phosphatases, and transcription factors (Reymond and Farmer 1998; Gutterson and Reuber 2004). In *Arabidopsis*, various transcription factor families, containing functional domains such as AP2, bZIP/HD-ZIP, WRKY, MYB, and several classes of zinc-fingers, have been studied in this context (Chen et al. 2002; Li et al. 2004; Zhu et al. 2005).

Among the transcription factors mentioned above, the AP2 family includes 122–145 and 139 gene members in *Arabidopsis* and rice, respectively (for review see Riechmann et al. 2000; Nakano et al. 2006). The encoded proteins, containing the conserved domain that was first described in the homeotic gene APETALA (AP2; Jofuku et al. 1994), have been proposed to play important roles in response to diverse developmental processes and environmental signals (Banno et al. 2001; Dubouzet et al. 2003; Ohto et al. 2005).

AP2 family proteins are classified into several groups, such as ethylene response factor (ERF), dehydration-responsive element-binding (DREB)/C-repeat (CRT)/DRE-binding factor, RAV, and AP2 subfamilies (Sakuma et al. 2002; Gutterson and Reuber 2004). ERF-type transcription factors bind to the GCC box, an essential *cis*-acting element in ET-, MeJA-, and SA-responsive genes, and also CRT/DRE of several cold-, high salt-, and drought-inducible genes, thus regulating the expression of both biotic and abiotic stress-responsive genes (Ohme-Takagi and Shinshi 1995; Park et al. 2001; Lee et al. 2004; Yi et al. 2004). Meanwhile, DREB/CBF-type transcription factors bind to CRT/DRE only, thus regulating the expression of the abiotic stress-responsive genes (Stockinger et al. 1997). Both the ERF- and DREB/CBF-type proteins act as either transcriptional activators or repressors (Song et al. 2005; Yang et al. 2005).

Barley (*Hordeum vulgare* L.), one of the major food and feed crops in the world, is more widely adaptable to unfavorable growth conditions, such as cold, dry, and saline environments, as compared to other cereal crops such as rice. Therefore, novel genes that are responsive to environmental stresses and are also involved in subsequent developmental changes are likely to exist in barley. Barley has an estimated genome size of 5,000 Mb ($2n = 14$ chromosomes), which is approximately 40- and 10-folds larger than those of *Arabidopsis* and rice, respectively (Arumuganathan and Earle 1991). Therefore, it is thought that there are at least 100 members of the AP2 family transcription factors in barley, considering that more than 100 AP2 family transcription factors exist in *Arabidopsis* and rice, respectively. However, only a few transcription factors belonging to this family have been characterized in barley (Choi et al. 2002; Xue 2003; Xue and Loveridge 2004), and most of their functions remain to be determined.

Here, we report the cloning and characterization of a cDNA encoding a novel AP2/ERF transcription factor, designated *HvRAF (H. vulgare* root abundant AP2/ERF transcription factor). The transcripts of *HvRAF* are more abundant in the roots than in the leaves of young barley seedlings.

Materials and methods

Plant materials and treatments

Seeds of barley (*H. vulgare* L. cv Jinkwang, obtained from National Institute of Crop Science, Suwon, Republic of Korea) were soaked in distilled water at room temperature for 1 h, and then germinated and grown in a controlled growth chamber, either in half-strength-modified Hoagland nutrient solution or on vermiculite at 21°C under long-day conditions (16 h light/8 h dark cycle) at about 100 μmol photons m^{-2} s^{-1}. Chemical treatments were performed in a Magenta box by either spraying barley seedlings until run-off with a 0.01% TritonX-100 solution containing 5 mM SA, 100 μM MeJA, 2 mM ethephon, or 100 μM ABA, or by dipping seedling roots into distilled water containing 0.1% cellulase or 10 mM methyl viologen (MV). After treatment for various times, barley plants were harvested and frozen in liquid nitrogen for further analyses.

RT-PCR, BAC library screening, and cloning of *HvRAF* genomic DNA

The full-length *HvRAF* cDNA was isolated by RT-PCR. PCR analysis was carried out with a one-step RT-PCR kit (Qiagen, Hilden, Germany) using 500 ng total RNA as a template. Amplification was performed with the forward primer (5′-CAGGAAGATAAAAC AATGTGT-3′) matching the 5′ end of the barley EST BF 256780 and the reverse primer (5′-GATCAAT TCGTAGGACTATTG-3′) matching the 3′ end of the partial *HvRAF* cDNA fragment. PCR products were separated on a 1% agarose gel and eluted using an Accuprep Gel Purification kit (Bioneer, Yusung, Republic of Korea). The DNA fragment of the correct size was cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA), and nucleotide sequences were verified through sequencing analysis.

To obtain genomic DNA of *HvRAF*, 17 high-density filters of the barley (*H. vulgare* L.) BAC library were screened as described by Yu et al. (2000) using a 567-bp *HvRAF*-specific DNA probe labeled with [α-^{32}P] dCTP (Amersham, Little Chalfont, UK), which was generated by PCR amplification using primers designed from the C-terminal and 3′-untranslated regions of *HvRAF* (forward: 5′-ACACGATGCC GAGGGTCGAC-3′; reverse: 5′-AGTACAGAGAG TACCGAGCA-3′). Putative-positive clones were further verified by Southern-blot analyses. The *HvRAF* genomic DNA was cloned by PCR with the adaptor-specific forward and the *HvRAF* cDNA-specific reverse primers, using the Universal Genome Walker kit and its protocol (Clontech, Palo Alto, CA, USA). Nucleotide sequences of the amplified DNA fragments were confirmed by sequencing analysis.

DNA and RNA gel blot hybridizations

For Southern-blot analysis, total genomic DNA was extracted from 7-day-old barley seedlings according to the method of Doyle and Doyle (1987). Either *Bam*HI or *Hin*dIII–cut total genomic DNA (20 μg) was separated on 0.8% agarose gels and transferred to Hybond-N+ nylon membranes (Amersham). The blot was hybridized with the *HvRAF*-specific DNA probe. After hybridization, the membrane was washed in 0.1× SSC buffer containing 0.1% SDS at 55°C and exposed to X-ray film at −70°C.

Total RNA for RNA blot analysis was obtained by modified LiCl/ethanol-mediated precipitation. The 10 or 20 μg of total RNA from each sample was separated by electrophoresis on a 1.1% formamide/agarose gel and blotted onto a nylon membrane. Hybridization was done by adding various [α-^{32}P] dCTP-labeled DNA probes for *HvRAF*, *HvJIP*, 18S ribosomal RNA, and some stress-inducible genes from *Arabidopsis*. Primer sets used in the RNA hybridization experiments are listed in Table 1. Radioactivity was detected by autoradiography.

Detection of subcellular localization of the HvRAF-smGFP fusion protein

The full-length *HvRAF*-coding region was fused to the 5′ end of the soluble-modified green fluorescent protein (smGFP) cDNA using *Bam*HI sites (David and Viestra 1996). *Arabidopsis* protoplasts were transiently transformed with the resulting constructs under the control of the cauliflower mosaic virus 35S promoter using a modified PEG-mediated transformation (Abel and Theologis 1994). After transformation, the protoplasts were kept at 23°C in darkness for 24 h and fluorescent photographs were captured using confocal laser-scanning microscopy (Carl Zeiss-LSM510, Thornwood, USA).

Transactivation assay in yeast cells

To test the acidic region of the *HvRAF* gene as a transactivation domain, diverse deletion mutant constructs were made by PCR amplification using the primer pairs listed in Table 2. The forward and reverse primers contained *Eco*RI and *Pst*I restriction sites (underlined), respectively. The PCR constructs were fused in

Table 1 Primer sets for PCR products as probes for RNA hybridization experiments

Gene name	Primer sequence (5′–3′)	
PDFI.2(*AY133787*)	F[a]:	ATGGCTAAGTTTGCTTCCATC
	R[b]	AATACACACCATTTAGCACCA
PRI(*AY117187*)	F:	ATGAATTTTACTGGCTATTCT
	R	GTATGGCTTCTCGTTCACATA
PR5(*ATU83490*)	F:	CCAGTATTCACATTCTCTCTTCTTCGT
	R	GTGGTTTTATCCCCATCTTTACATT
KIN2(*X62281*)	F:	GAGAGGAGAAGAGCAATGT
	R	TGTCCTTCACGAAGTTAACAC
JR3(*Y13577*)	F:	CATTCGGTCAAGTGAGCTCGA
	R	CTCGCCATTGGTGGTGAACGAGCT
COR6.6(*X62281*)	F:	GAGAGGAGAAGAGCAATGT
	R	TGTCCTTCACGAAGTTAACAC
GSHI(*AF06299*)	F:	GAATGGGAAAAAGTAATGGAAGGTG
	R	CTCTGGGAATATTGTTGTCAGATGG
At18S(*X16077*)	F:	AAATTAGAGTGCTCAAAGCAAGCCT
	R	CGTCAATTCCTTTAAGTTTCAGCCT
HvJIP(*X98124*)	F:	TAACGGAATAGCTGTAAAATGCCTCCT
	R	TTGTAGATATAATCCCAGCTTCCCACA

[a] Forward primer
[b] Reverse primer

Table 2 Primers used to construct partial genes for transcriptional activation assay

Primer name	Sequence (5′–3′)
HvAP2-acti-full-F (*Eco*RI)	GAATTCATGTGTGGCGGCGGCCATCCTAG
HvAP2-acti-full-R (*Pst*I)	CTGCAGTCAGAAAATGGCGCTGTCC
HvAP2-acti-N1 (*Eco*RI)	GAATTCGAGGAGGACTTCGATGACCACTT
HvAP2-acti-N2 (*Eco*RI)	GAATTCGAGCTGATGGAGTTTTTCAA
HvAP2-acti-N3 (*Eco*RI)	GAATTCGCCCTCAGCGTGGACAGTG
HvAP2-acti-C1 (*Pst*I)	CTGCAGGAAGATGCTGTCGGCGGATTC
HvAP2-acti-C2 (*Pst*I)	CTGCAGGCAGGTCGAAGTGGTCATCG

frame to the sequence of the yeast GAL4 DNA-binding domain in the yeast expression vector, pGBK-T7 (Clontech), using the corresponding restriction sites. Transformation of these recombinant plasmids into the yeast strain AH109 was then performed using the modified lithium acetate-mediated method (Gietz et al. 1992). The β-galactosidase activities were quantified using liquid culture and colony filter-lift assays, using o-nitrophenyl β-D-galactopyronoside (ONPG) as a substrate according to the procedures provided by the manufacturer (yeast protocol handbook PT3024-1, Clontech).

Arabidopsis transformation

The full-length *HvRAF* cDNA was inserted into the binary vector pBI111L, a derivative of pBI121 (Clontech). The recombinant plasmid was introduced into *Agrobacterium tumefaciens* strain CS58C1 by electroporation. *Arabidopsis* transformation was performed using the floral dip method (Clough and Bent 1998). Transformants were selected on MS medium containing 30 μg mL^{-1} kanamycin. For further experiments, T3 single-copy homozygous transformants were selected based on Southern blotting and the segregation ratio for kanamycin resistance.

Root inoculation with *Ralstonia solanacearum* strain GMI1000

Transgenic *Arabidopsis* plants overexpressing *HvRAF* and wild-type plants were grown for 3 weeks in soil and inoculated with *R. solanacearum* strain GMI1000, a bacterium that infects plants via root wounds and spreads through the vascular system (Vasse et al. 1995). Intact roots of the plant were wounded in the soil by cutting about 2 cm from the root tips, followed by dipping for 2 h in a suspension of 10^8 colony-forming units (cfu) per milliliter of *R. solanacearum* strain GMI1000 in 10 mM $MgCl_2$. Inoculated plants were kept in a growth chamber at 25°C with a 16 h light/8 h dark cycle. Disease severity and progress were evaluated daily, beginning at 4 days after inoculation, using a 0–4 disease scale (0, no wilting; 1, 1–25% wilted; 2, 26–50% wilted; 3, 51–75% wilted; and 4, 76–100% wilted or dead; Tans-kersten et al. 2001). Each assay was performed with 9 or 12 plants and repeated three times.

Germination and root growth assay

For the germination test, at least 100 sterilized seeds were surface-sterilized as described previously (Weigel

and Glazebrook 2002) and sown on half-strength MS medium containing 1% phytoagar and 0.05% Mes-KOH (pH 5.7; Duchefa, Haarlem, The Netherlands), supplemented with either 0 or 200 mM NaCl. After stratification at 4°C in the dark for 4 days, seeds were transferred under light conditions at 23°C. Germinated seeds with emerged radicles were counted at various time points.

For the root growth assay, plants were grown vertically on MS medium containing 0 or 100 mM of NaCl for 2 weeks after the seeds were planted in a growth chamber under long-day conditions (16 h light/8 h dark) at 23°C.

Results

Isolation of the *HvRAF* cDNA and genomic clone

To isolate the environmental stress-induced *ERF* genes in barley, EST clones encoding either GCC- or C-repeat/DRE-binding factors were searched for in The Institute for Genomic Research (TIGR) Barley Unique Gene database (http://www.tigr.org/). Among several candidates, TC131838 showed the highest sequence similarity to genes encoding several unknown AP2 domain-containing proteins. BF256780, the corresponding EST (846 bp in size; isolated from roots of young barley seedlings) in GenBank, contained a 141-bp longer sequence at the 3′ region, as compared to TC131838, thereby encoding 191 amino acid downstream of the 5′ UTR. Therefore, to isolate the full-length cDNA of the corresponding gene, the DNA fragment of BF256780 was cloned and used as a probe to screen the barley cDNA library that had been constructed from poly(A)$^+$ RNA of 7-day-old barley seedlings. However, all positive clones obtained were partial cDNAs with various sequence length overlaps with the EST clone. The longest partial cDNA (734 bp), containing polyadenylation sequences, showed a 186-bp sequence overlap at its N-terminus region with the C-terminus of the EST clone. Consequently, through RT-PCR using the primers corresponding to the N- and C-termini regions of the expected cDNA, we obtained the full-length cDNA, including both the 5′ and 3′ UTR sequences. It was designated as *HvRAF* (*H. vulgare* root abundant AP2/ERF transcription factor; Fig. 1a; GenBank accession no. DQ102383). The open reading frame of this gene encodes a polypeptide of 328 amino acids (GenBank protein ID: AAZ14086; predicted MW 35 kDa; pI 5.6), containing a novel MCGGAIL motif at the N-terminus, a putative nuclear localization sequence (NLS; RKKR), a highly conserved AP2/ERF domain, and an acidic domain with a mammalian hemopexin domain signature-like sequence (Tolosano and Altrude 2002) at the C-terminus (Fig. 1).

To clone the *HvRAF* genomic DNA, a barley BAC library (Yu et al. 2000) was first screened using *HvRAF* cDNA as a probe. Seven positive BAC clones obtained (HV_MBa0154O14, HV_MBa0277E06, HV_MBa0527P04, HV_MBa0604L12, HV_MBa 0673O09, HV_ MBa 0811I08, and HV_MBa 0811I09) showed very similar hybridizing band patterns when subjected to Southern blot analysis using the *HvRAF* cDNA probe (data not shown). Identical *HvRAF* genomic DNA (GenBank accession no. DQ102384) was obtained from two randomly selected clones, HV_MBa0154O14 and HV_MBa0811I08, based on PCR cloning (see Materials and methods). Comparison of the sequences between *HvRAF* genomic clone and its cDNA revealed the presence of an intron 35 nt upstream of the sequences encoding the putative NLS. In addition, several putative *cis*-regulatory sequences, including root-specific, ET-, SA-, MeJA-, and elicitor-responsive elements, were found in its promoter region by searching the PlantCARE (http://www.bioinformatics.psb.ugent.be/webtools/plantcare/html) and PLACE (http://www.dna. affrc.go.jp/PLACE/) databases (Fig. 1a).

Phylogenic analysis of the AP2/ERF domain of the encoded HvRAF with other related domains of the AP2 family proteins, using the program CLUSTALX, confirmed that HvRAF belongs to the ERF subfamily according to the classification by Sakuma et al. (2002; Fig. 2a). Sequence alignment showed that HvRAF has the highest homology of the AP2/ERF domain to those of AAK92635, a putative ERF transcription factor from rice, and RAP2.2 from *Arabidopsis* (identity: 90 and 82%, respectively; similarity: 96 and 94%, respectively; Fig. 2b). In addition to the AP2/ERF domain, the MCGGAIL/I motif at the N-terminus, putative NLSs at several amino acid upstream of the AP2/ERF domain, and hemopexin domain signature-like sequences at the end of the acidic C-terminus were also observed in both AAK92635 and RAP2.2. No other particular sequence similarities were found among these three proteins (Fig. 2c).

Spatial expression of the *HvRAF* gene

Genomic Southern analysis was performed to estimate the number of copies of the *HvRAF* gene in barley. Because of the high conservation of AP2/ERF domains as mentioned above, the *HvRAF*-specific DNA probe flanking the C-terminal and 3′ UTR regions was used as a probe. A single hybridizing band was obtained

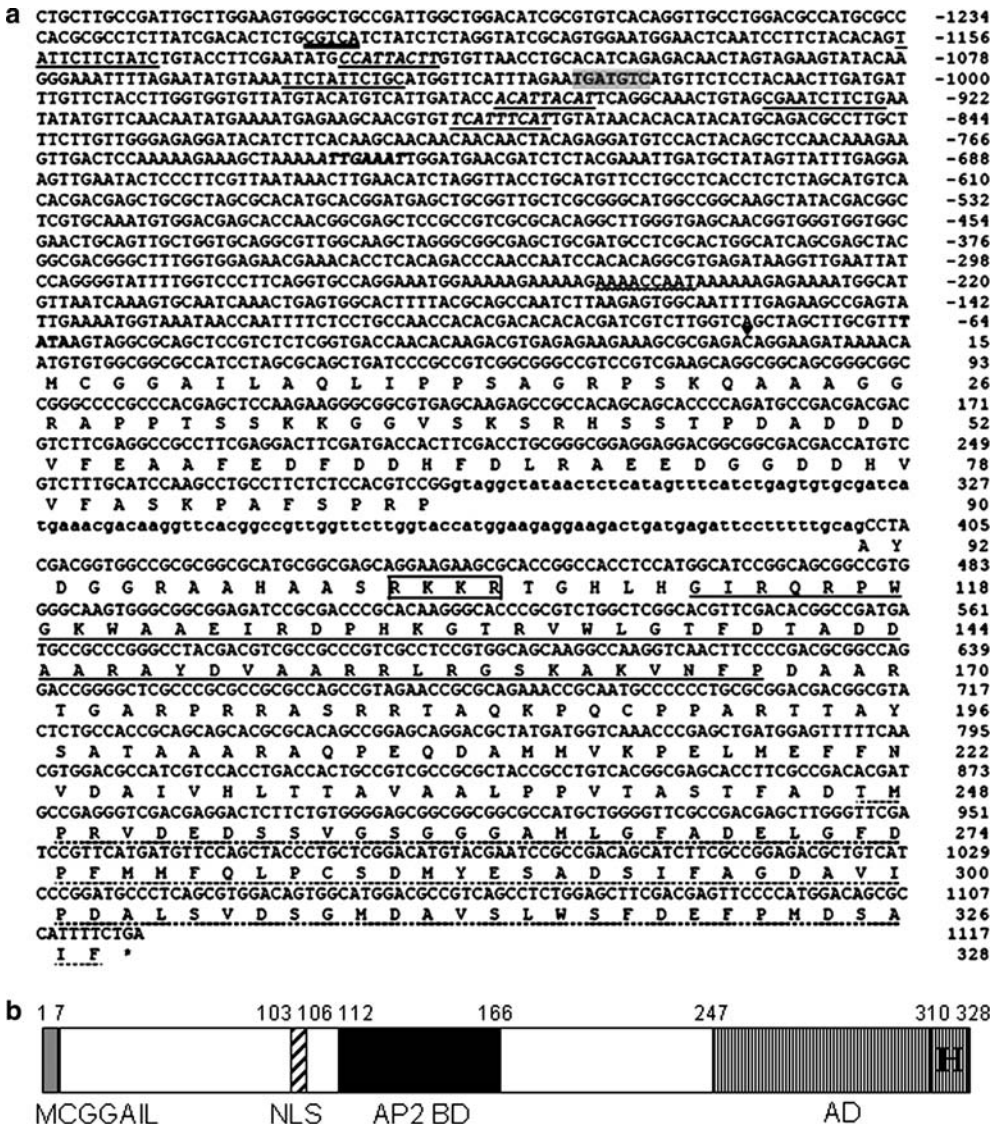

Fig. 1 Sequence analysis of the *HvRAF* gene and its promoter from barley. **a** Nucleotide and deduced amino acid sequence of the *HvRAF* gene and its promoter region. The putative nuclear localization sequence (NLS) is shown in the *box*. The AP2/ERF DNA-binding and acidic C-terminal domains are designated by *solid* and *dashed lines*, respectively. The root-specific *cis*-element is shown in *shaded letters*, and the MeJA-responsive element is *double-underlined*. SA-responsive elements are *underlined* and ET-responsive elements are shown in *bold italic letters*. Elicitor-responsive elements are marked by *wavy underlines*. The intron sequence is indicated by *small letters*. The potential TATA box and the transcription initiation site are marked as *bold letters* and an *inverted triangle*, respectively. The 3′ UTR sequences are not shown. **b** Schematic representation of the 328 amino acids of the HvRAF protein (AAZ14086). The conserved AP2/ERF DNA-binding domain (AP2 BD) and other putative functional domains are marked; MCGGAIL (unknown signal peptide), NLS, AD (acidic domain), and H (hemopexin domain signature-like sequence)

from either *Bam*HI- or *Hin*dIII-digested genomic DNA, indicating that the *HvRAF* gene is likely present as a single copy in the barley genome (data not shown).

To examine the spatial expression of *HvRAF*, RNA blot analyses were performed using the total RNA obtained from either roots or serial leaf sections (from the base to the top) of 7-day-old barley seedlings. The mRNA was much more abundant in roots than in leaves (Fig. 3). Interestingly, however, a gradual decrease in the accumulation of *HvRAF* mRNAs was observed from the basal to apical parts of the leaves.

Expression of the *HvRAF* gene in response to phytohormones, cellulase, and MV

To investigate whether the induction of the *HvRAF* gene is regulated by the key signaling phytohormones in response to various environmental stresses in higher

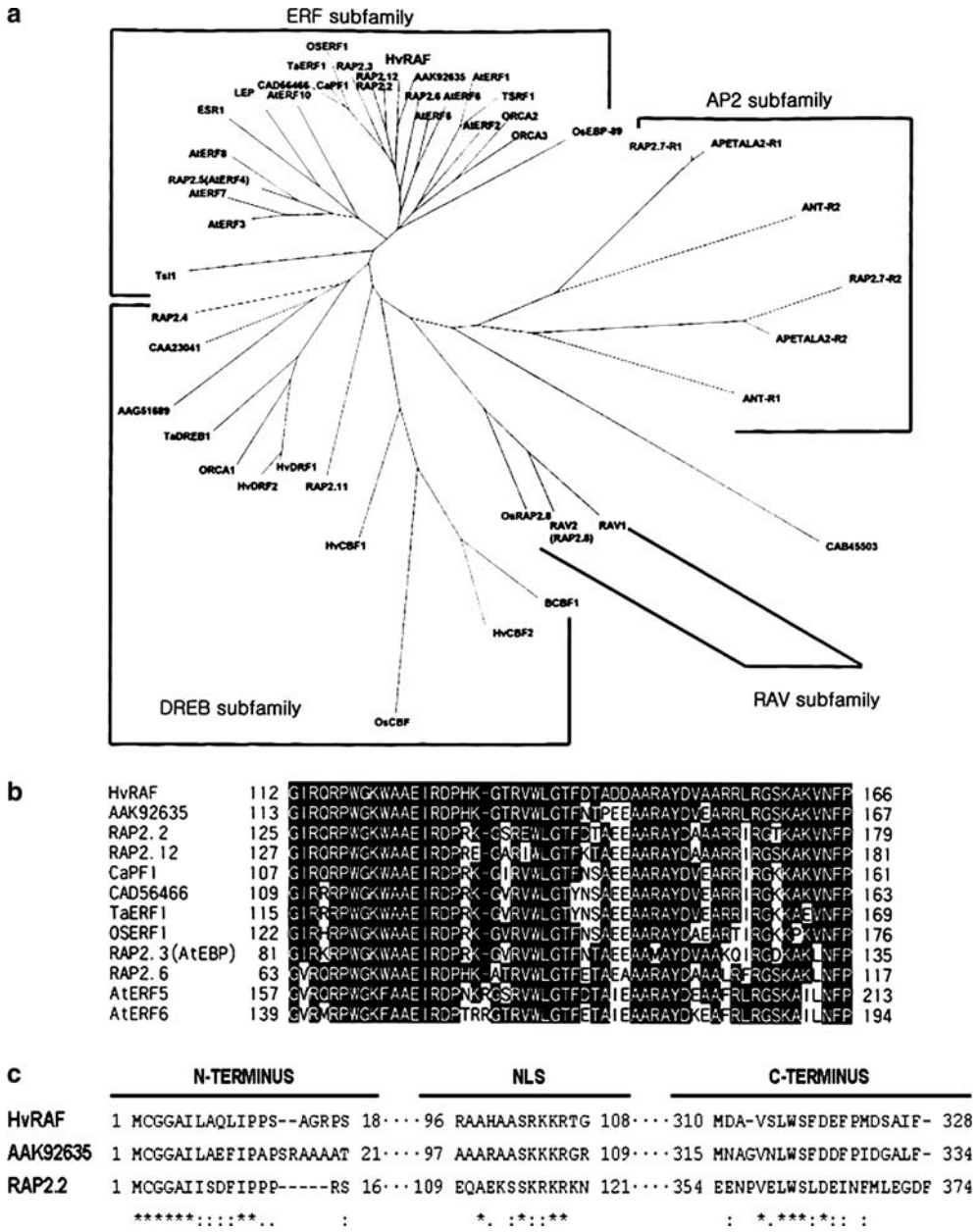

Fig. 2 Relationship between HvRAF and other representative AP2 family proteins. **a** Phylogenic analysis of AP2/ERF domains for HvRAF and other representative AP2 family proteins. An unrooted phylogenic tree was drawn using TreeView based on alignments by CLUSTALX. AP2 family proteins used in this analysis are HvRAF (AAZ14086), a putative rice ERF transcription factor (AAK92635), RAP2.2 (At3g14230), RAP2.3 (At3g16770), RAP2.4 (At1g78080), RAP2.5 (At3g15210), RAP2.6 (At1g43160), RAP2.7 (At2g28550), RAP2.11 (At5g19790), RAP2.12 (At1g53910), AtERF1 (At3g23240), AtERF2 (At5g47220), AtERF3 (At1g50640), AtERF5 (At5g47230), AtERF6 (At4g17490), AtERF7 (At3g20310), AtERF8 (At1g53170), AtERF10 (At1g03800), OsEBP-89 (CAC83122), Tsi1 (AAC14323), putative wheat ERF protein (CAD56466), CaPF1 (AAP72289), ESR1 (At1g12980), ORCA1 (CAB93939), ORCA2 (CAB93940), ORCA3 (CAB96900), TaDREB1 (ABA08424), BCBF1 (AAK01088), HvCBF1 (AAL84170), HvCBF2 (AAM13419), OsCBF (AAR88625), RAV1 (At1g13260), RAV2 (RAP2.8; At1g68840), OsRAP2.8 (BAB21218), APETALA2 (At4g36920), ANT (AAA91040), LEP (At5g13910), TSRF1 (AAN32899), HvDRF1 (AY223807), HvDRF2 (AAO27884), OsERF1 (XP479169), TaERF1 (AAP80852), and several putative AP2 transcription factors (AAG51689, CAA23041, and CAB45503). **b** Alignment of AP2/ERF domains of HvRAF and several representative ERF transcription factors in the ERF subfamily by CLUSTALW. Identical amino acid residues are shown in *black*. **c** Comparison of the deduced amino acids in the N-terminal, NLS, and C-terminal regions of HvRAF, AAK92635 (rice), and RAP2.2 (*Arabidopsis*). Identical amino acids are indicated by *asterisks*, conserved amino acids by *colons*, and semiconserved amino acids by *dots*

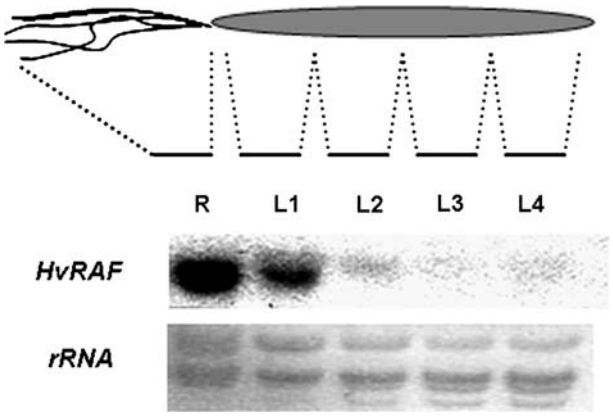

Fig. 3 Spatial expression of *HvRAF*. Total RNA was isolated from roots (*R*) and leaves (*L*) of 7-day-old barley seedlings. Leaves were divided into four segments and RNA was extracted. Equal amounts of RNA loading in each lane were verified by visualizing rRNA on a gel stained with ethidium bromide

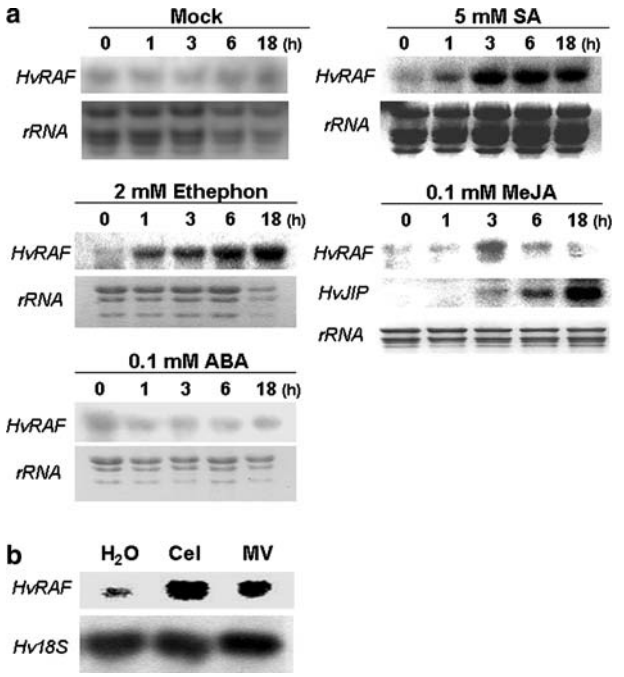

Fig. 4 Expression of the *HvRAF* gene in response to several exogenous stresses. **a** Total RNA was prepared from 7-day-old barley seedlings treated with the phytohormones SA, ET, MeJA, and ABA at indicated time points. The results for *HvJIP* encoding a jasmonate-inducible protein are shown as a control for gene induction by MeJA treatment. **b** The 12-day-old barley seedling roots were dipped into distilled water containing 0.1% cellulase and 10 mM MV, respectively. Samples were harvested from roots of seedlings 6 h after treatment, respectively

plants, we monitored the expression patterns of the *HvRAF* mRNAs in barley seedlings subjected to several stress-responsive phytohormones, cellulase, and MV treatments that would mimic both biotic and abiotic stresses. As shown in RNA blot analyses (Fig. 4), *HvRAF* gene expression was induced in 7-day-old barley seedlings within 1–3 h after SA, ethephon, and MeJA treatments, although the accumulation kinetic patterns differed. However, little induction was observed by either mock or ABA treatment. Expression was also induced in the roots of 14-day-old barley seedlings by cellulase and MV treatments.

Targeting of HvRAF to the nucleus

To determine the subcellular localization of HvRAF, we fused the stop-codon deleted open reading frame of *HvRAF* cDNA to the start-codon of the *smGFP* reporter gene and expressed it under the control of the 35S promoter of cauliflower mosaic virus. The resulting construct was introduced into *Arabidopsis* protoplasts by PEG-mediated transformation (Abel and Theologis 1994). As expected, the HvRAF::smGFP fusion protein was targeted to the nucleus of *Arabidopsis* protoplasts, whereas only smGFP was distributed throughout the cytosol (Fig. 5).

HvRAF as a potential transcriptional activator

To investigate the possible functional role of the acidic C-terminal region of HvRAF (82 amino acids, from amino acids 247–328; pI = 3.17) as a transactivation domain, we fused various deletion mutants of the cDNA to the GAL4 DNA-binding domain expression vector and assayed each construct in yeast. The intact HvRAF fused to the GAL4 DNA-binding domain activated transcription of the *lacZ* reporter gene in liquid culture assay (Fig. 6a), indicating the capability of HvRAF as a transcriptional activator in yeast. The N-terminus deletion mutants of the *HvRAF* cDNA containing the intact acidic C-terminal region (HvRAFΔN1; HvRAFΔN2) showed slightly higher ß-galactosidase activity. The mutant containing only one-third of the downstream part of the acidic C-terminal region (26 amino acids, including the hemopexin domain signature-like sequence; HvRAFΔN3) showed approximately half the ß-galactosidase activity of the intact *HvRAF* construct. Furthermore, the deletion of the 34 amino acids in its C-terminal downstream region (HvRAFΔC1) resulted in the loss of most of the ß-galactosidase activity. Similar results were also obtained from the colony filter-lift assay (Fig. 6b). These results suggest that the acidic C-terminus, especially the 34 amino acids containing the hemopexin domain signature-like sequence in the C-terminal downstream region of HvRAF, is critical for the ability of HvRAF as a potential transcriptional activator.

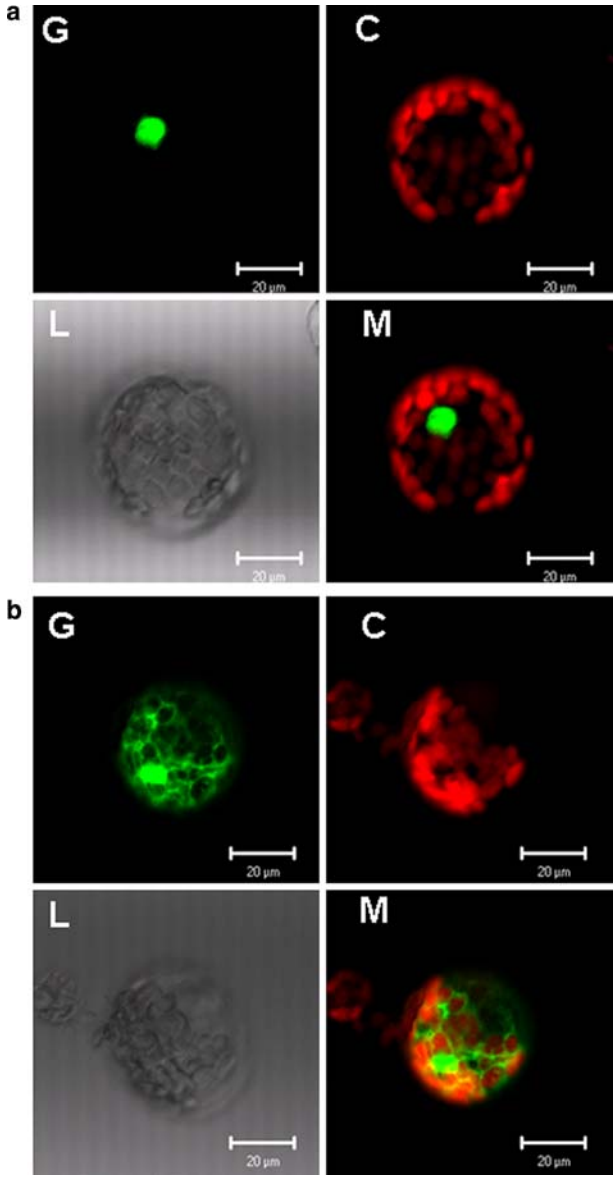

Fig. 5 Subcellular localization of the HvRAF::smGFP. **a** The complete sequence of the *HvRAF* gene was fused to the 5′ end of the *smGFP* reporter gene under the control of the 35S CaMV promoter; the chimeric vector was then introduced into *Arabidopsis* protoplasts by PEG-mediated transformation. The HvRAF::smGFP fusion protein showed green fluorescence in the nucleus. **b** The smGFP protein alone showed fluorescence throughout the cytosol. *G* GFP fluorescence; *C* chlorophyll fluorescence; *L* light microscopic image; *M* merged image *G* and *C*

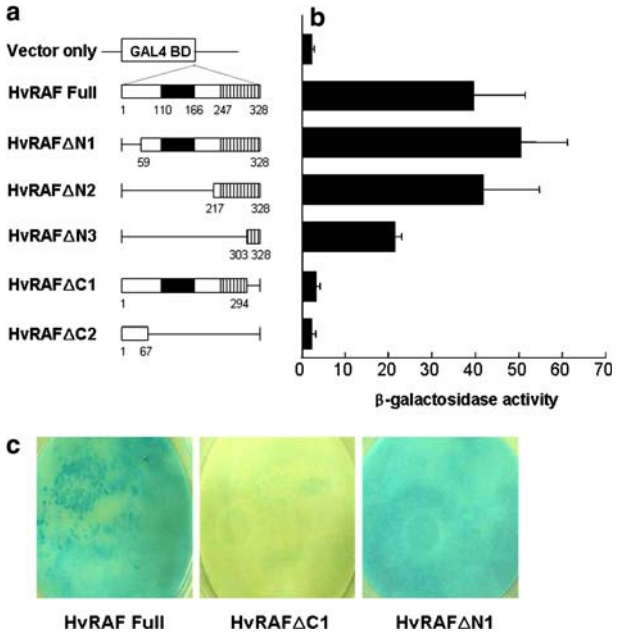

Fig. 6 Transactivation assay in yeast cells. **a** The *HvRAF* and several partial deletion constructs of *HvRAF* were fused to the GAL4 DNA-binding domain expression vector pGBKT7 and transformed into yeast cells. *Black* and *hatched boxes* indicate the conserved AP2/ERF DNA-binding domain and the acidic potential transactivation domain, respectively. **b** To investigate whether HvRAF and its partial proteins act as a transcriptional activator in yeast, a β-galactosidase enzyme assay using yeast liquid culture was performed in triplicate using o-nitrophenyl β-D-galactopyranoside (ONPG) as the substrate. One unit of β-galactosidase activity is defined as the amount that hydrolyzes 1 μmol of ONPG to o-nitrophenol and D-galactose per minute per cell. **c** A colony filter-lift assay was performed for further functional confirmation of several constructs tested in **b**

Overexpression of *HvRAF* activates several biotic and abiotic stress-responsive genes

As described earlier, many ERF transcription factors activate the transcription of biotic stress-responsive genes, which most often contain a GCC-box in their promoters, such as *PDF1.2* and *PR* genes. Therefore, to test HvRAF as an ERF-type transcription factor, transgenic *Arabidopsis* plants overexpressing *HvRAF* (35S::*HvRAF*) were produced, and the expression patterns of several representative stress-responsive genes were monitored in 2-week-old transgenic plants. In contrast to wild-type plants, 35S::*HvRAF Arabidopsis* plants showed significant activation of several biotic stress-responsive genes, such as *PDF1.2* (Penninckx et al. 1998), *JR3* (Titarenko et al. 1997), and *PR1* and *PR5* (Ukness et al. 1992), and also abiotic stress-responsive genes, including *KIN2* (Kurkela and Borg-Frank 1992) and *GSH1* (Xiang and Oliver 1998; Fig. 7).

Enhanced resistance to bacterial pathogenesis and tolerance of seed germination and root growth against high salinity in transgenic *Arabidopsis* overexpressing *HvRAF*

Due to the activation of several typical biotic stress-responsive genes in 35S::*HvRAF* transgenic plants, the possible involvement of HvRAF in defense responses to bacterial pathogens was first examined. For this purpose,

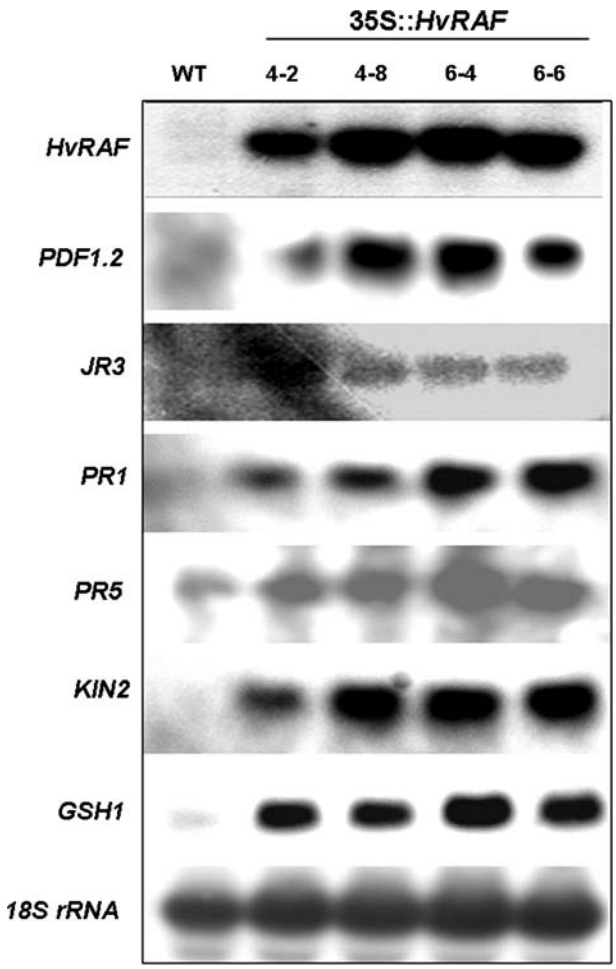

Fig. 7 Upregulation of several stress-responsive genes in transgenic *Arabidopsis* plants overexpressing the *HvRAF*. RNA blot analyses were performed using total RNA isolated from 2-week-old transgenic plants grown in MS medium. Transgenic plants showed induction of several representative stress-responsive genes, including *PDF1.2*, *JR3*, *PR1*, *PR5*, *KIN2*, and *GSH1*. An *Arabidopsis* 18S rRNA probe was used as a control

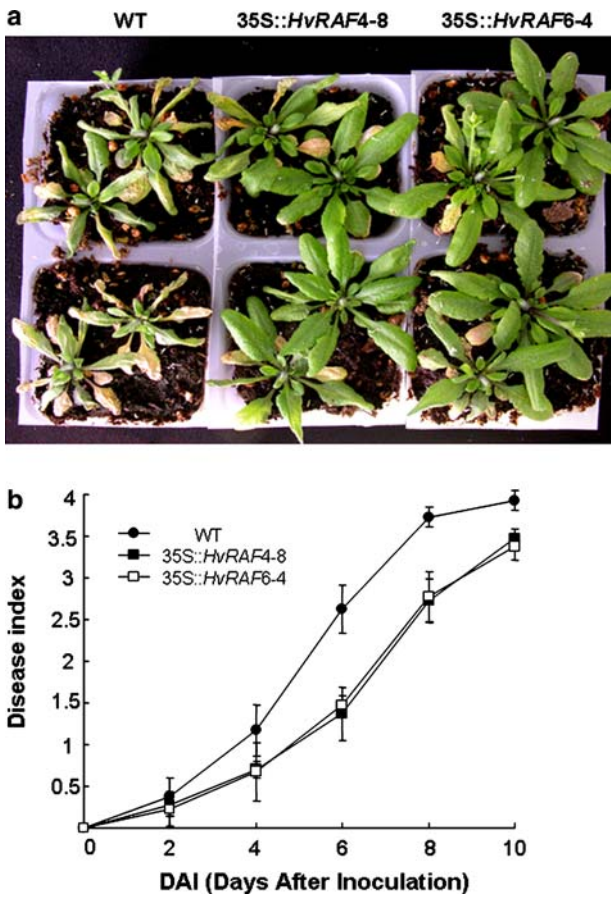

Fig. 8 Enhanced resistance of 35S::*HvRAF* transgenic *Arabidopsis* to *R. solanacearum* strain GMI1000. **a** Roots of 3-week-old *Arabidopsis* Col-0 and 35S::*HvRAF* transgenic plants were inoculated with a culture suspension of 10^8 colony-forming units (cfu) per milliliter of *R. solanacearum* strain GMI1000. The photograph was taken 6 days after pathogen inoculation. **b** Plants were scored every 2 days on a disease index ranging from 0 to 4: 0 (no wilting), 1 (1–25% wilted), 2 (26–50% wilted), 3 (51–75% wilted), and 4 (76–100% wilted or dead). Each data point represents the mean ± SE from triplicate experiments (9–12 individual plants were used in each experiment)

we inoculated 3-week-old *Arabidopsis* plants with the root-invading bacterial wilting agent *R. solanacearum* strain GMI-1000 (Vasse et al. 1995). At 6 days after inoculation (DAI), the 35S::*HvRAF Arabidopsis* plants showed ~50% wilting relative to that of wild-type plants. This relatively reduced wilting of transgenic plants was observed until 10 DAI (~73% at 8 DAI; ~85% at 10 DAI compared to wild-type plants; Fig. 8), suggesting that the disease resistance conferred by *HvRAF* overexpression is effective against bacterial pathogen attacks.

To investigate whether HvRAF-induced accumulation of abiotic stress-responsive genes, including *KIN2* and *GSH1*, could enhance plant tolerance to abiotic stresses such as high salinity, we examined seed germination and root growth inhibition of 35S::*HvRAF Arabidopsis* plants under high salt conditions. First, we compared the germination rates of transgenic *Arabidopsis* seeds to those of wild-type seeds. Most wild-type and transgenic seeds germinated in MS media at 3 days after transfer (DAT) under light conditions at 23°C from a cold treatment under dark conditions. However, in MS media supplemented with 200 mM NaCl, approximately 60% of the transgenic seeds germinated, whereas none of the wild-type seeds germinated at 3 DAT (Fig. 9a). At 5 DAT, most of the transgenic seeds germinated in the high salt-containing media, while almost none of the wild-type seeds germinated. Subsequently, for the root growth inhibition study, 35S::*HvRAF Arabidopsis* plants were grown in MS media containing 100 mM NaCl for 2 weeks after planting; their root growth was then compared to wild-type plants. As shown in Fig. 9b, the

transgenic plants showed slightly shorter root growth compared to wild-type plants in MS medium only. There were no differences in other phenotypes including rosette size, arrangement of lateral roots, and flowering time (data not shown). However, in the presence of 100 mM NaCl, the transgenic plants showed less severe inhibition of root growth than did wild-type plants. These results collectively indicate that the overexpression of *HvRAF* led to less sensitivity toward high salinity during germination and root growth in *Arabidopsis*.

Discussion

Plant-specific AP2 transcription factors are well-characterized proteins that are involved in a variety of stress and developmental responses. We isolated *HvRAF*, a gene encoding an ERF-type transcription factor, from young seedlings of barley. Based on the analysis of the deduced amino acid sequence and phylogenic relationship, HvRAF is thought to be a novel member of the ERF subfamily (Sakuma et al. 2002; Tournier et al. 2003), containing an unknown signal sequence (MCGGAIL), a putative nucleus localization signal (NLS: RKKR), and an acidic transactivation domain, in addition to the highly conserved AP2/ERF DNA-binding domain (Figs. 1, 2). MCGGAIL at the N-terminus is a novel sequence of unknown function, which is highly conserved in some of the ERF proteins found in various plant species, including AAK92635 from rice and RAP2.2 from *Arabidopsis*, which are the proteins most closely related to HvRAF based on phylogenic analysis of the AP2/ERF domains (Fig. 2a). Another novel feature of HvRAF is that it has a hemopexin domain signature ([LIFAT]-x(3)-W-x(2,3)-[PE]-x(2)-[LIVMFY]-[DENQS]-[STA]-[AV]-[LIVMFY])-like sequence at the end of the acidic transactivation domain. Hemopexin is a mammalian serum glycoprotein that binds heme and transports it to the liver (Tolosano and Altrude 2002). Similar sequences were also observed in the C-terminal acidic domains of the AAK92635 and RAP2.2 proteins, reflecting the functional importance of this conserved sequence motif (Fig. 2c). This sequence motif turned out to be important for the transactivation of the reporter gene in yeast cells (Fig. 6). Therefore, the hemopexin domain signature-like sequence is thought to be involved in protein–protein interactions, although the precise function has yet to be determined. The relative position of the constituting domains and sequence motifs revealed that HvRAF, along with AAK92635 and RAP2.2, is a transcription factor belonging to class IV of the ERF subfamily (Tournier et al. 2003). The characteristics of HvRAF as a transcription factor were well demonstrated in this study by translocation of the protein into the nucleus, transactivation of the *lacZ* reporter gene in yeast, and activation of stress-responsive genes in 35S::*HvRAF* transgenic *Arabidopsis* (Figs. 5, 6, 7).

HvRAF expression was induced in response to several phytohormones such as SA, ET, and MeJA that control plant responses to various stresses. In addition, *HvRAF* expression was induced by other treatment regimes such as cellulase and MV, which mimic both biotic and abiotic stresses. These results indicate that *HvRAF* is probably regulated by certain components of multiple stress-signaling pathways. A complex network of signaling pathways, which is often regulated by crosstalk, is involved in plant responses to biotic and abiotic stresses (Cheong et al. 2002; Lorenzo et al. 2003; Anderson et al. 2004).

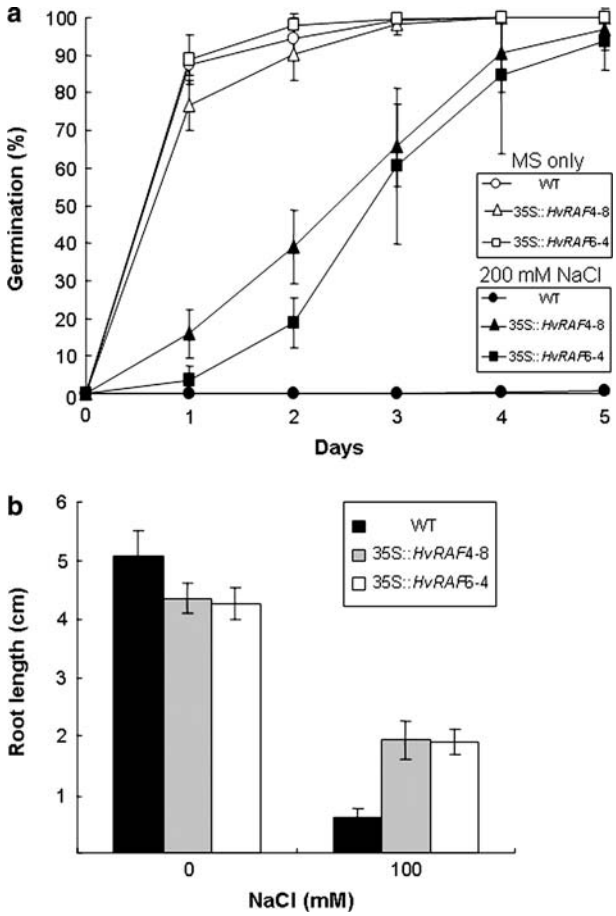

Fig. 9 Salt tolerance of 35S::*HvRAF* transgenic *Arabidopsis*. **a** After stratification at 4°C for 4 days, seeds of wild-type (WT) and two 35S::*HvRAF* transgenic *Arabidopsis* lines were germinated on 0.5× MS media containing either 0 (*open symbols*) or 200 mM (*filled symbols*) of NaCl. Seeds showing radicle emergence were counted daily. The germination percentage represents the averages ± SE of triplicate samples. **b** For the root growth assay, plants were grown vertically on MS medium containing 0 or 100 mM of NaCl for 2 weeks after sowing. Independent experiments were performed in triplicate; *error bars* indicate ±SE

Many ERF-type transcription factors bind to the GCC-box in promoter regions of PR genes induced by SA, ET, or MeJA, such as *PDF1.2* and *PR* genes, thus activating the gene expression for the disease defense response (for review see Gutterson and Reuber 2004). However, some ERF transcription factors also additionally bind to CRT/DRE sequences in abiotic stress-inducible promoters, thereby acting as dual stress-responsive regulators (Park et al. 2001; Lee et al. 2004; Yi et al. 2004). Although the conserved AP2/ERF DNA-binding domain of HvRAF has a high similarity to other GCC box-binding AP2/ERF domains, we failed to demonstrate an interaction of HvRAF with the GCC box because soluble recombinant HvRAF protein could not be produced in *Escherichia coli*. However, overexpression of the *HvRAF* gene clearly activated a subset of representative PR genes, such as *PDF1.2*, *JR3*, *PR1*, and *PR5*, in *Arabidopsis*. Interestingly, in addition to the GCC box-containing genes, typical abiotic stress-responsive genes such as *KIN2* (*COR6.6*) and *GSH1*, which contain CRT/DRE elements in their promoters, were also activated by *HvRAF* overexpression (Fig. 7). Taken together, these results support the idea that HvRAF is functionally relevant in *Arabidopsis*, thus interacting with downstream partners of various stress-responsive genes, which would subsequently confer defense responses against both biotic and abiotic stresses.

HvRAF transcripts in barley seedlings were more abundant in roots than in leaves (Fig. 3). In addition, *HvRAF* transcription was activated in roots of barley seedlings that were treated with cellulase, a cell-wall-degrading enzyme, and MV, an oxidative-stress-causing chemical (Fig. 4b). These results, along with the activation of the defense-related genes in 35S::*HvRAF* transgenic plants, suggest that HvRAF may be an important regulator in the adaptation of roots in response to biotic and abiotic stresses, especially given the fact that plant roots are surrounded by soil in which microorganisms are abundant. Therefore, we first examined whether the overexpression of *HvRAF* could affect protection against bacterial root infection. For this purpose, we exploited a virulent soil pathogenic bacterium, *R. solanacearum* strain GMI1000, which invades plants by infection through root wounds or at sites of secondary root emergence (Hayward 1991; Deslandes et al. 1998). Upon root inoculation, the bacterial population was found in the xylem vessels and spread systemically throughout the plant, leading to wilting symptoms of the host plant within 5–10 days (Vasse et al. 1995). Therefore, the enhanced resistance of the transgenic *Arabidopsis* to the pathogenic bacteria, manifested by the relatively reduced wilting of the leaves (Fig. 8), is likely to have occurred primarily due to the overaccumulation of the defense-related proteins in the infected roots, including *PDF1.2*, *JR3*, *PR1*, and *PR5* gene products, conferred by the function of the ectopically expressed HvRAF.

The idea that HvRAF may also act as an abiotic stress-responding regulator in roots was tested by growing 35S::*HvRAF Arabidopsis* plants under high-salt conditions for 2 weeks. Considering the positive effect on root growth under high salinity for a relatively long period (Fig. 9a), HvRAF may be a stress-responding regulator involved in the long-term root adaptation process under high salt conditions. In addition to less severe inhibition of root growth, 35S::*HvRAF* plants also showed dramatically lower sensitivity to seed germination inhibition in response to a high concentration of salt that was almost completely inhibitory to the germination of wild-type seeds (Fig. 9b). The tolerance of high salinity by transgenic *Arabidopsis* was most likely conferred by the activation of salt stress-responsive genes, including *KIN2*, which is induced by high salinity as well as drought and cold stresses (Kurkela and Borg-Franck 1992). However, the tolerance of 35S::*HvRAF* plants to abiotic stress was specific to high salinity. In other words, the transgenic plants did not show tolerance to other abiotic stresses such as drought or cold (data not shown). Considering the lack of induction of *HvRAF* by ABA treatment (Fig. 4), HvRAF may act as a regulator in ABA-independent signaling pathway(s) for both root growth and seed germination under high salinity in transgenic *Arabidopsis*. *Arabidopsis* DREB2A and DREB2B act as regulators in ABA-independent signaling pathways in response to high salinity (Chinnusamy et al. 2005). In addition, similar tolerance responses to high salinity have also been observed in terms of the reduced inhibition of root growth and seed germination in transgenic tobacco plants that overexpress CaERFLP1 and JERF1, the ERF-type transcription factors from hot pepper and tomato, respectively (Lee et al. 2004; Zhang et al. 2004).

In summary, HvRAF is proposed as a novel, root-abundant ERF-type transcription factor that is likely to have a dual functional role, possibly acting in multiple signaling pathways in response to both biotic and abiotic stresses, such as pathogen attack and high salinity. For a more detailed elucidation of the biological function of HvRAF, especially in the context of the possible crosstalk between biotic and abiotic stress signaling, further studies will be necessary through various approaches, including microarray analyses with *HvRAF*-overexpressing transgenic plants.

Acknowledgments We thank Je Il Tak for helping in this study. This study was supported by research grants (CG1520 to Minkyun Kim, CG1412 to Ingyu Hwang) from the Crop Functional Genomics Center of the 21st Century Frontier Research Program funded by the Korean Ministry of Science and Technology. Jinwook Jung, So Youn Won, and Yeonhwa Jeong were supported by the Korean Ministry of Education, through the Brain Korea 21 Project.

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