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RESEARCH PAPER

Functional characterisation of an intron retaining K^+ transporter of barley reveals intron-mediated alternate splicing

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ABSTRACT

Intron retention in transcripts and the presence of 5′ and 3′ splice sites within these introns mediate alternate splicing, which is widely observed in animals and plants. Here, functional characterisation of the K^+ transporter, *HvHKT2;1*, with stably retained introns from barley (*Hordeum vulgare*) in yeast (*Saccharomyces cerevisiae*), and transcript profiling in yeast and transgenic tobacco (*Nicotiana tabacum*) is presented. Expression of intron-retaining *HvHKT2;1* cDNA (*HvHKT2;1-i*) in *trk1, trk2* yeast strain defective in K^+ uptake restored growth in medium containing hygromycin in the presence of different concentrations of K^+ and mediated hypersensitivity to Na^+. *HvHKT2;1-i* produces multiple transcripts *via* alternate splicing of two regular introns and three exons in different compositions. HKT isoforms with retained introns and exon skipping variants were detected in relative expression analysis of (i) *HvHKT2;1-i* in barley under native conditions, (ii) in transgenic tobacco plants constitutively expressing *HvHKT2;1-i,* and (iii) in *trk1, trk2* yeast expressing *HvHKT2;1-i* under control of an inducible promoter. Mixed proportions of three HKT transcripts: *HvHKT2;1-e* (first exon region), *HvHKT2;1-i1* (first intron) and *HvHKT2;1-i2* (second intron) were observed. The variation in transcript accumulation in response to changing K^+ and Na^+ concentrations was observed in both heterologous and plant systems. These findings suggest a link between intron-retaining transcripts and different splice variants to ion homeostasis, and their possible role in salt stress.

INTRODUCTION

Salt tolerance is a complex polygenic character, and the high affinity potassium transporter (*HKT*) gene family is reported as an important pathway in imparting salinity tolerance to plants (Berthomieu *et al.* 2003; Huang *et al.* 2006, 2008; Horie *et al.* 2008, 2009; Hauser & Horie 2010; Plett *et al.* 2010; Baek *et al.* 2011; Mian *et al.* 2011). Plant HKT proteins comprise a family of cation transporters, together with prokaryotic KtrB (Durell & Guy 1999), TrkH (Cao *et al.* 2011; Domene & Furini 2012), fungal Trk proteins and KdpA transporter subunits (Maser *et al.* 2002). The cDNAs of *HKT* genes have been isolated from many species of plant (Durell & Guy 1999; Fairbairn *et al.* 2000; Takahashi *et al.* 2007; Ardie *et al.* 2009) and have been functionally characterised as K^+ or Na^+ transporters and/or K^+/Na^+ symporters (Schachtman & Schroeder 1994; Rubio *et al.* 1995; Uozumi *et al.* 2000; Fairbairn *et al.* 2000; Horie *et al.* 2001; Garciadeblas *et al.* 2003; Su *et al.* 2003; Ren *et al.* 2005). Besides the presence of glycine/serine residues in the first pore loop, the K^+ and Na^+ selectivities of HKTs are also reflected in gene organisation and intron–exon structure (Platten *et al.* 2006). At the functional level, strong differences in relative permeability to Na^+ and K^+ were observed between rice HKTs, some of them being Na^+ selective, *e.g. AtHKT1*, while others displayed high permeability to both Na^+ and K^+ or high permeability to K^+ (Jabnoune *et al.* 2009; Horie *et al.* 2011; Oomen *et al.* 2012; Sassi *et al.* 2012). Differences in Na^+/K^+ permeability is well correlated with overall sequence differences, which led to the sorting of HKT into two subfamilies corresponding to the two main phylogenetic branches of the rice family: subfamily 1 regroups Na^+-selective HKT, and subfamily 2 comprises transporters displaying permeability to K^+ (Platten *et al.* 2006).

The division of HKTs into two groups also corresponds to the presence of two introns in HKT, group 1 HKTs have significantly longer introns than group 2 (Platten *et al.* 2006). Alternate splicing of two regular introns and three exons in *HKT* genes produce mixed proportions of intron-retaining and incompletely spliced transcripts in different plant species (Golldack *et al.* 2002; Takahashi *et al.* 2007). Changing proportions of spliced and un-spliced transcripts have also been reported in dehydrin proteins (Xiao & Nassuth 2006) that, like HKTs, are characterised by the presence of glycine and play a role in salt and osmotic stress tolerance (Saavedra *et al.* 2006). Alternate splicing with different transcript splice junctions results in tran-

scripts with shuffled exons, alternative 5′ or 3′ splicing sites, retained introns and different transcript termini in *Arabidopsis* (Mehlgarten & Schaffrath 2004), where alternative 3′ splice site type of alternate splice forms is the most common (Koralewski & Krutovsky 2011). Evidence suggests a critical role for these events in functional importance in response to stress conditions in plants that impacts domestication and trait selection (Zhang & Gassmann 2007; Barbazuk *et al.* 2008). The majority of alternate splicing events in plants have not yet been functionally characterised. Accumulation of Na^+ in specific leaf blades in rice depends on transcript abundance and correct splicing of *OsHKT1;4* (Cotsaftis *et al.* 2012). Alternate splicing has not been observed in studies where full-length HKT cDNAs from barley or other grass species were expressed in heterologous and/or plant systems (Haro *et al.* 2005; Hoopen *et al.* 2010; Mian *et al.* 2011; Qiu *et al.* 2011). Previous findings in plant HKT demonstrated the splicing events, incomplete splicing (Golldack *et al.* 2002), intron retention and exon skipping events (Takahashi *et al.* 2007), but to date no report has shown expression of any plant HKT with introns and expression profiling in yeast or in a plant system at the same time. Our findings with *HvHKT2;1-i* (*HvHKT2;1* with introns) expression in yeast and plant systems suggests a mechanism for intron-mediated alternate splicing that has not been previously explored for HKT. In this study, cDNA-encoding HKT was isolated from barley leaves and a kallar grass leaf cDNA library under normal and salt-stressed conditions. Isolated HKT cDNA from both grass species belongs to subfamily 2 (GGGG type) and was found to contain insertions corresponding to two introns. *HvHKT2;1* with these insertions was expressed in the wild type and K^+ uptake-deficient mutant yeast strains (*trk1, trk2*) for functional characterisation. Relative expression patterns of *HvHKT2;1-i* transcripts were evaluated in barley under native conditions, expression under galactose-inducible promoter in *trk1, trk2* and wild-type yeast strains, and under the constitutive promoter in tobacco plants. The current work aimed to examine whether (i) introns stably integrate in *HKT* transcripts on expression of *HvHKT2;1-i* in yeast and transgenic tobacco, (ii) *HvHKT2;1-i* still complements the growth function in yeast, (iii) introns in the transcripts affect *HvHKT2;1-i* transport properties and K^+ uptake activity, and (iv) *HvHKT2;1-i* produces correctly spliced transcripts and HKT protein directed to the target site.

MATERIAL AND METHODS

Plant material, growth conditions and stress treatments for *HvHKT2;1-i* expression analysis

Barley (*Hordeum vulgare*) and kallar grass (*Leptochloa fusca* L. Kunth) seeds were collected from field-grown plants at the Biosaline Research station Pakka anna (Toba Tek Singh, Faisalabad, Pakistan). Barley seeds were planted in pots and irrigated with normal tap water for 40 days and then grown for a further 10 days with 0, 50 or 100 mM NaCl solution. Total RNA was extracted from these salt-treated barley leaves using the RNeasy plant extraction Mini Kit (Qiagen, Germantown, MD, USA) according to manufacturer's instructions. RNA was treated with RNase-free DNase I (Qiagen) and 2 μg RNA was reverse-transcribed for amplification of the *HKT* gene and relative quantification of transcripts. Using 100 ng of the reverse transcription product, the relative abundance of transcripts was tested using *18S* rRNA and *elf1α* as internal controls under different Na^+ concentrations.

Total RNA extraction and kallar grass cDNA library construction

Total RNA was extracted from kallar grass leaves using the RNeasy Plant Extraction Mini Kit according to the manufacturer's instructions (Qiagen). The CloneMine II cDNA Library Construction Kit (Invitrogen, Waltham, MA, USA) was used to produce a kallar grass leaf cDNA library. Total RNA was processed for first strand cDNA synthesis using the SuperScriptR III RT enzyme. Using first strand cDNA as template, second strand cDNA was synthesised with *E. coli* DNA polymerase 1. The *att*B adapter sequences were ligated using T4 DNA ligase. *att*B-flanked cDNA fragments were cloned into pDONR 222 using the BP Clonase II enzyme mix. The reaction was inactivated with proteinase K and transformed into competent *E. coli* cells through electroporation. cDNA samples were divided into six aliquots and transformed separately through electroporation. M13 forward and M13 reverse primers flanked the cloned cDNAs; therefore internal primers (within the conserved region of the gene) were used in combinations with flanking M13 primers to isolate the *HKT* gene.

Cloning *HvHKT2;1* and sequence confirmation

High Fidelity Platinum Taq DNA polymerase (Invitrogen) was used to amplify HKT cDNA from barley and kallar grass. HKT genes from both grass species were cloned into the Gateway entry vector pENTR D-TOPO (Invitrogen). Fidelity of clones was checked through sequencing. Gene sequences were confirmed as *HKT* clones from a BLAST (Basic Local Alignment Search Tool) search at NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). Gateway HKT entry clones were used for sub-cloning in Gateway expression vectors using LR clonase II enzyme mix (Invitrogen). HKT gene integration into expression vectors was checked through restriction digestion and sequencing using gene-specific primers (Table 1).

Yeast transformation and functional complementation analysis

The *HvHKT2;1-i* construct and control vector (empty vector) were expressed under the galactose-inducible promoter in pYES-DEST52 and pYES2/CT (Fig. 1; Invitrogen) in wild-type (WT) and *trk1, trk2* (K^+ uptake deficient mutant) *S. cerevisiae*. Ura^+ transformants were grown overnight in minimum medium containing 0.17% yeast nitrogen base (without amino acids or ammonium sulphate; Invitrogen), 0.67% SC–U powder, 2% glucose, 0.5% ammonium sulphate (Invitrogen) and 100 mM KCl. Cells were pelleted and recultured in galactose-inducible media (2% galactose + 2% raffinose) for 8 h. OD_{600} was adjusted to 1.00 and cells were pelleted and rinsed three times with sterile distilled deionised water. Serial dilutions were made and used for ion complementation analysis on yeast peptone dextrose medium (1% yeast extract, 2% peptone, 2% dextrose). For each assay, 5 μl of serially diluted yeast cell suspension were spotted on hygromycin ($\mu g \cdot \mu l^{-1}$) supplemented solid YPD medium with milimolar concentrations of KCl or NaCl.

Table 1. Primers used for cloning and relative quantification. HKT Fwd/Rev were used for isolation and cloning of full-length *HvHKT2;1*. Three primer sets within the *HKT* gene were used for transcript profiling in yeast, tobacco and barley. F1/R1, first exon region *HvHKT2;1-e*; F2/R2, *HvHKT2;1-i1* and F3/R3, *HvHKT2;1-i2* from intron–exon junction of first and second intron, respectively.

primer name	primer sequence (5 –3)
HKT fwd. full	ATGAGCTCGGTGAAAAGATTTTAC
HKT rev. full	TCATACTTTCCAGGATTTACCCATGGTCA
KG forward	ATGGGTCGGGTGAAAAGATTTTAC
F1	TTAGGTTACTGGTATGGTTCCTGG
R1	GCCATCAAACACGGAAGAATTCCAA
M13 forward	GTAAAACGACGGCCAGTCTTAAGCTCGGGC
M13 reverse	CCAGGAAACAGCTATGACCATGTAATACGA
F2	GCCATTATAGTACTATTCATTGTCATGATG
R2	TGCAAATGTTGCTGATGGTGGGCA
F3	ACCTTAAACATGATATTCGAGGTCATCA
R3	TGGATAACCCTACATTGCCATATGC
35S forward	TTGATGAAGTGACAGATAGCTGG
35S reverse	GGCGAACAGTTCATACAGAGTCT
18S forward	AGCAAGCCTACGCTCTGTATAC
18S reverse	GCTTTCGCAGTTGTTCGTCT
elf1α forward	TTCTTGAGGCTCTTGACCAGATC
elf1α reverse	TCGTGGTGCATCTCAACAGAT

Growth conditions and growth rate measurements in *trk1, trk2*

To study K^+ uptake activity of *HvHKT2;1-i*, fresh dropout liquid medium (SC–U) was used to re-culture *trk1, trk2* yeast cells (expressing *HvHKT2;1-i* and the empty control lacking *HvHKT2;1-i*) with 2% glucose (non-inducing conditions) and 2% galactose + raffinose (induced) separately at 0, 10, 25, 50, 75, 100, 150 and 200 mM K^+. OD_{600} of the inoculum was set to 1 for sub-culturing yeast cells to standardise growth conditions for comparative transcript profiling under inducing and non-inducing conditions. Growth rates were monitored by measuring OD_{600} after 12, 24, 36 and 48 h. Samples were run in triplicate and statistical analysis was undertaken on growth rate differences of *trk1, trk2* expressing *HvHKT2;1-i* and the empty vector under inducing and non-inducing conditions at different K^+ levels.

Stable transformation in tobacco

Tobacco (*Nicotiana tabacum*) plants were transformed using the *Agrobacterium*-mediated transformation method. *HvHKT2;1-i* was cloned into the plant expression vector pSPDK-1677 (Fig. 1) followed by transformation into the GV3101 *Agrobacterium* strain using the electroporation method. Tobacco leaf discs were infected with *Agrobacterium* culture and subsequently incubated on plates containing MS medium (Murashige & Skoog 1962) supplemented with spectinomycin (30–40 $\mu g \cdot \mu l^{-1}$) for selection of transformants. Wild-type and transgenic tobacco plants were grown in a growth chamber with a 12-h photoperiod at 350 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 28/22 °C. Expression levels of *HvHKT2;1-i* in transgenic plants were determined with quantitative RT-PCR.

Relative quantification of *HvHKT2;1-i*

For relative quantification of the alternatively spliced HKT transcripts in barley under native promoter conditions, total RNA was isolated from barley leaves watered with 0, 50 or 100 mM NaCl. Wild type and K^+ uptake-deficient yeast strain *trk1, trk2* expressing *HvHKT2;1-i* and the empty vector grown in dropout liquid media (SC–U) under both inducing and non-inducing conditions were also used for relative quantification of HKT transcripts at 100 mM KCl. To investigate the regulation of gene expression in *trk1, trk2* yeast strain expressing *HvHKT2;1-i*, transcript analyses were done with RNA extracted from plants exposed to 0, 10, 25 or 100 mM KCl. Induced gene expression was also studied by growing *trk1, trk2* under both inducing and non-inducing growth conditions for further RNA extraction and quantitative PCR (qPCR) analysis. Transgenic tobacco plants transformed with the *HvHKT2;1-i*-pSPDK construct were also used for relative quantification analysis of the HKT transcript. Two housekeeping genes, *18s* RNA and *elf1α*, were used as internal control for relative transcript analysis to avoid bias. In all cases of relative quantification of *HKT* transcripts, 2 μg RNA was reverse-transcribed with 100 ng of each reverse transcription product for relative quantification in a 20-μl reaction on a 7500 Fast Real-Time PCR system (software version 2.0.6) using SYBR Green dye (Life Technologies Corporation, Carlsbad, CA, USA) according

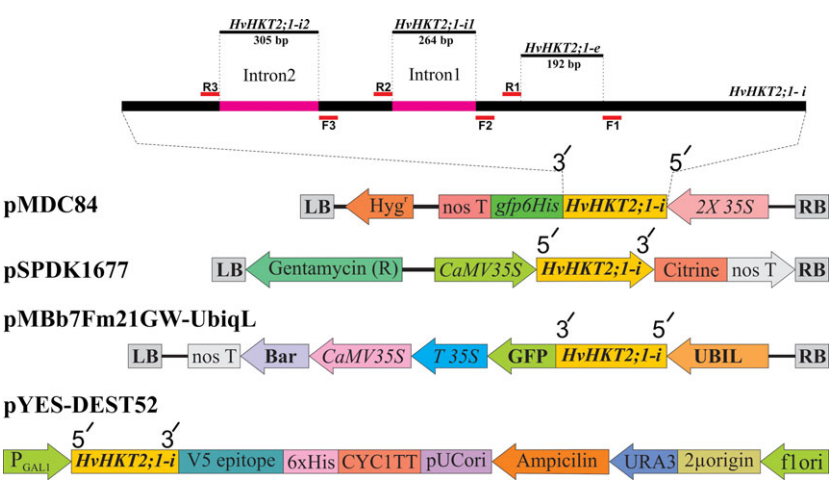

Fig. 1. Cloning *HvHKT2;1-i* with both introns (in pink) in yeast and plant expression vectors. LB, left border; RB, right border; CaMV35S, Cauliflower 35S promoter; Tnos or nos T, nopaline synthase terminator; citrine, citrine synthetase; T 35S, 35S terminator; 2X 35S, double 35S promoter; gfp/GFP, green fluorescent protein; MZ-Ubiq, maize ubiquitin promoter; PGAL1, galactose promoter; CYC1, transcription termination signal; Amp, Ampicillin resistance gene; URA3, gene for prototrophic selection; 2μ origin, for maintenance and high copy replication in yeast and f1 origin, to rescue single-stranded DNA.

to manufacturer's instructions. In all the cases for relative quantification, three primer sets were used: one from the first exon and the other two from the intron–exon junction of the first and second intron, respectively (Table 1).

Transient gene expression in *Nicotiana benthamiana*

Nicotiana benthamiana plants were raised in a growth chamber with a light/dark cycle of 16 h/8 h at 25 °C and 60–70% relative humidity. The 5–7-week-old plants were used for transient gene expression analysis. *A. tumefaciens* strain GV3101 containing *HvHKT2;1-i* cloned into pMDC-84 (Curtis & Grossniklaus 2003; Karimi *et al.* 2007), and the pMBb7Fm21 GW-UbiqL construct was grown overnight in Luria-Bertani broth supplemented with 10 mM MES and 25 μM acetosyrigone. Cell cultures were then centrifuged and resuspended in infiltration medium (250 mg D-glucose, 5 ml 500 mM MES, 5 ml 20 mM $Na_3PO_4{\cdot}12H_2O$), with acetosyrigone added at the end to a final concentration of 150 μM. OD_{600} of the infiltration culture was adjusted to 0.8 for leaf infection. *Agrobacterium* culture was infiltrated into the abaxial side of fully expanded young leaves of *N. benthamiana* using a 5-ml syringe without a needle. Infiltrated leaves were analysed for *GFP::HvHKT2;1-i* localisation under a confocal microscope (Leica, Solms, Germany; SP2 laser scanning spectral confocal microscope) 72 h following the above procedure (Levy *et al.* 2005).

Statistical analysis

All experiments were run in triplicate. Means and SD of triplicate experiments were calculated and ANOVA at $P \leq 0.05$ considered significant. An least significant differences (LSD) test was done for comparison between growth conditions, growth rates and relative quantification of transcripts using a factorial designs for quantitative data analysis at $P \leq 0.05$ with STATISTIX software version 8.1 (Analytical Software, Tallahassee, FL, USA).

RESULTS

Primers deduced from the conserved regions of *HKT* gene sequences and standard reverse transcription (RT)-PCR methods were used to clone *HvHKT2;1* from barley leaves. The amino acid sequence of *HvHKT2;1* showed 92% identity to wheat HKT1 (AAA52749.1), 42% to AtHKT1 (NP_567354.1), 70% to PhaHKT1 (BAE44384.1), 44% to ScTrk1 (EDV12642.1) and 58% to ScTrk2 (CAA82128) transporters. BLAST alignment of nucleotide sequences of *HKT*, *HKT* clones and cDNAs revealed a gene structure of three exons and two introns in barley and kallar grass *HKT2* genes (Figures S1 and S2). Introns were located at positions previously described for *HKT* in rice (Garciadeblas *et al.* 2003). Relative expression analysis detected *HKT* isoforms, *HvHKT2;1-e* (*HvHKT2;1* with first exon), *HvHKT2;1-i1* (*HvHKT2;1* with first intron) and *HvHKT2;1-i2* (*HvHKT2;1* with second intron) in barley expressing *HvHKT2;1-i* under native conditions, in transgenic tobacco plants and heterologous expression in yeast. Mixed transcripts accumulated to different levels in barley leaves and were found to correlate with Na^+ concentrations in the soil (Fig. 2). The expression patterns also varied with growth rate, growth conditions (non-inducing and inducing) and K^+ concentrations in the heterologous system (Figs 2 and 3).

Cloning splice variants of *HKT2* from barley and kallar grass

Kallar grass plants were grown in pots with NaCl treatment to enhance expression of drought- and salinity-related genes to capture RNA and construct a leaf cDNA library. Barley line PK-38, was grown in parallel under similar conditions for relative quantification of HKT transcripts and cloning of barley *HvHKT2;1*. A variable proportion of HKT isoforms accumulated with abundance of intron-retaining transcripts (Figs 2

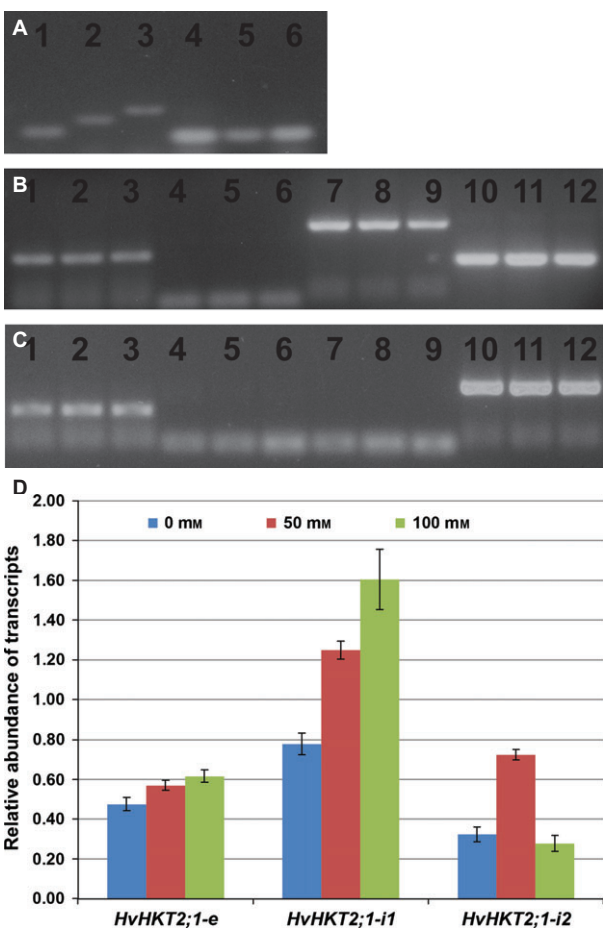

Fig. 2. Detection of relative expression levels of HvHKT2;1 in barley and yeast with quantitative and semiquantitative PCR. A: *HvHKT2;1-i* expression patterns analysed in *Hordeum vulgare*; Lane 1–3, amplification from *HvHKT2;1-e*, *HvHKT2;1-i1* and *HvHKT2;1-i2*; Lane 4–6, 18S rRNA as reference transcript. B: Expression patterns of *HvHKT2;1i* in trk1, trk2; Lane 1–3, *HvHKT2;1-i1*; Lane 4–6, EV; Lane 7–9, *HvHKT2;1-i2*; Lane 10–12, 18S. C: Expression patterns of HKT2 from barley cDNA with introns; Lane 1–3, *HvHKT2;1-i1* cDNA with introns; lane 4–6, barley control cDNA without introns for transcript analysis of *HvHKT2;1-i1*, Lane 7–9, barley control cDNA without introns for transcript analysis of *HvHKT2;1-i2*; Lane 10–12, *HvHKT2;1-i2* amplification from barley cDNA with introns. D: Quantitative RT-PCR analysis of intron retaining cDNA from barley grown under salt stress treatments; 0 mM (control), 50 mM or 100 mM NaCl. *X*-axis represents different transcripts detected while *Y*-axis shows relative abundance of transcripts. Data are presented as mean + SE (n = 3).

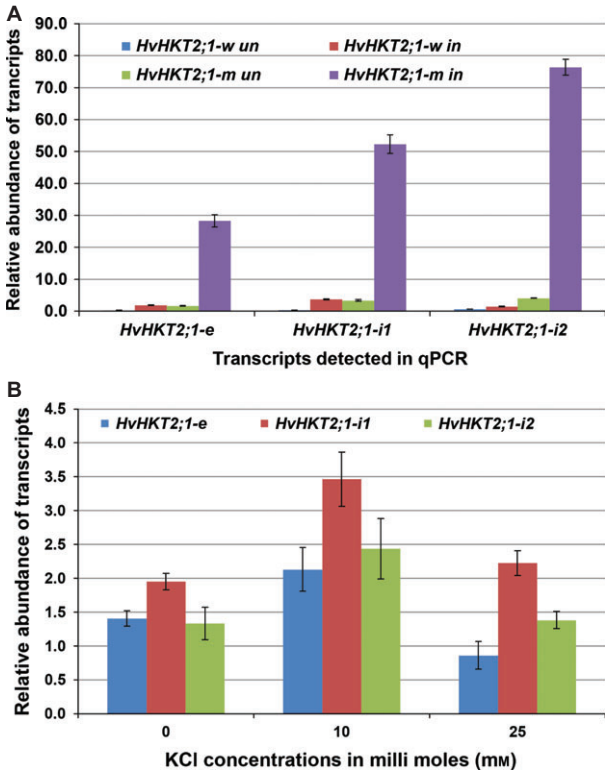

Fig. 3. Transcript analysis of *HvHKT2;1-i* in yeast and tobacco. Three set of primers were used for transcript analysis; F1R1, to amplify *HvHKT2;1-e*. F2R2, to amplify fragment from first exon-intron junction, *i.e. HvHKT2;1-i1*; F3R3, from second exon-intron junction, *i.e. HvHKT2;1-i2*. A: *HvHKT2;1i* transcript analysis of wild type and trk1, trk2 yeast expressing EV (empty vector: control) and *HvHKT2;1-i* grown at 100 mM KCl. B: trk1, trk2 yeast expressing EV and *HvHKT2;1*-i under induced growth conditions at 0, 10 and 25 mM KCl. Wild type yeast strain expressing *HvHKT2;1-i*; under non-inducing conditions, HvHKT2;1-w un; induced (HvHKT2;1-w un) growth conditions. *trk1, trk2* mutant expressing *HvHKT2;1-i*; under induced, HvHKT2;1-m in and HvHKT2;1-m un non-inducing growth conditions. Data are presented as mean ± SE (n = 3).

and 3). Two short fragments of 1200 and 471 bp of *HKT2* from both grass species were detected with semi-quantitative PCR (data not shown). Sequencing results showed that neither intron 1 nor intron 2 was correctly spliced (Figures S1 and S2). The shorter HKT fragment contained the second intron, which was 34 bases shorter than the regular second intron at the 3 -end and lacked the first and second exon. The amplified product of the shorter 471-bp fragment (Figure S3) was always more abundant in comparison to amplifications from the full-length gene. In all experiments, there was no amplification from *HvHKT2;1* cDNA (control) using primers from intron–exon junctions. As a second control, *HKT* cDNA (with introns) cloned from a kallar grass leaf cDNA library was also used to clone a full-length vacuolar proton inorganic pyrophosphatase, *AVP1* (GQ387485.2), and Na^+/H^+ antiporter *LfNHX1* (Rauf *et al.* 2014) genes. Both *AVP1* and *LfNHX1* genes contained complete open reading frames (ORF) without introns. This served as a control to eliminate the possibility of DNA contamination in kallar grass cDNA libraries and that intron retention was due to the presence of genomic DNA. Additionally, to further remove the possibility of DNA contamination in the RNA, rDNase was added to RNA preparations, as suggested by the manufacturer (Qiagen). Moreover, the primers designed for amplification of the full-length gene and relative quantification experiments showed homology to *HKT* gene sequences available at NCBI (Table 1), with 100% similarity to reported *HKT* gene sequences of *H. vulgare* (JN5477409.1) and *L. fusca* (JN5477411.1), but no resemblance to any gene sequence in tobacco, yeast or *A. thaliana*.

Transcript analysis of *HvHKT2;1* in barley and alternative splicing

It is well established that *HKT* genes are up-regulated by Na^+ ions (Kader *et al.* 2006; Ardie *et al.* 2009; Mian *et al.* 2011). Salt stress conditions induced *Thellungiella salsuginea TsHKT1;2* whereas *AtHKT1* was down-regulated at the level of transcription (Ali *et al.* 2013). As the effect of salt stress, or more specifically Na^+ stress, conditions is different in *HKT* transcripts, different Na^+ stress conditions were used in experiments to investigate transcriptional regulation. Expression patterns of *HvHKT2;1* under control and salt stress conditions were determined in the barley line PK-38. Different alternate forms of *HvHKT2;1* transcripts were detected with real-time qPCR (Fig. 2A,D). The results showed up-regulation of *HvHKT2;1-i* transcripts in barley leaves under increasing Na^+ stress (Fig. 2D), which is consistent with previous findings of *HvHKT2;1* transcript up-regulation in shoots under Na^+ stress (Mian *et al.* 2011). Up-regulation of gene expression led to significantly higher transcript levels of *HvHKT2;1-i1* type in barley plants irrigated with 100 mM NaCl rather than with 0 or 50 mM NaCl (Fig. 2D). The exon-containing transcripts (*HvHKT2;1-e*) showed the next highest accumulation after *HvHKT2;1-i1* type, maintaining similar trends in transcript accumulation at each NaCl level (0, 50 and 100 mM), showing significant differences in transcript abundance (Fig. 2D). Relative to the control (0 mM NaCl), the cryptic transcripts containing the second intron accumulated to higher levels in 50 mM NaCl, while transcript levels of this type decreased in plants irrigated with 100 mM NaCl (Fig. 2D). Overall, higher relative accumulation of transcripts containing the first intron was observed than transcripts of either the first exon or second intron (Fig. 2D), suggesting a mixed proportion of truncated transcripts (Figure S4). Three amplification products were detected with real time semi-quantitative PCR: *HvHKT2;1-e* (192 bp), *HvHKT2;1-i1* (264 bp) and *HvHKT2;1-i2* (305 bp), corresponding to F1R1, F2R2 and F3R3 primer combinations, respectively (Fig. 2). *HvHKT2;1* (cDNA without any intron as control) showed no amplification using primers from the intron–exon junction (Fig. 2C), while the same primers in *HvHKT2;1-i* detected fragments of the exact size from intron regions (Fig. 2C). Semi-quantitative PCR with primers from intron–exon junctions using *HvHKT2;1* and *HvHKT2;1-i* amplified larger PCR fragments for intron-containing transcripts compared to the control. The primer combinations to determine transcript sizes were F1R1, F2R2, F3R3, F1R3 and F2R3 (see Table 1). The F1R3 primer combination amplified two products, *i.e.* a shorter fragment (356 bp) corresponding to a splice variant without the intron and a larger upper fragment (560 bp) with intron retention in *HvHKT2;1-i* (data not shown). These isoforms of the *HKT* gene detected with semi-quantitative and qPCR are evidence for the occurrence of

spliced, un-spliced and incompletely spliced transcripts, indicating the presence of alternate structure in *HvHKT2;1* in barley.

Differential regulation and splicing of *HvHKT2;1-i* in yeast

Yeast strains were grown under both inducing and non-inducing growth conditions for determination of comparative splicing and differential splicing patterns in the WT and K^+ uptake-deficient mutant strain expressing *HvHKT2;1-i* or the empty vector. Dropout medium was supplemented with KCl (100 mM) for culturing *trk1, trk2* expressing *HvHKT2;1-i* or the empty vector. Different proportions of the three transcripts were observed in *HvHKT2;1-i* transcript analysis of barley under the native promoter, and also detected in WT and *trk1, trk2* yeast expressing *HvHKT2;1-i* (Fig. 3). No amplification product was detected in the yeast strain expressing the empty vector, as seen in amplification plots from real-time qPCR. Relative abundance of *HvHKT2;1* transcripts was more significant in the *trk1, trk2* mutant yeast strain than the WT under inducing growth conditions, suggesting strong induction of the galactose-inducible promoter (Fig. 3A). Enhanced expression of *HvHKT2;1-i* in *trk1, trk2* under inducing conditions as compared to non-inducing conditions corresponds to the observations from growth rate analysis (Figs 3A and 5A). Furthermore, statistical analysis using three-way interaction LSD tests gave highly significant results, with relatively more *HvHKT2;1-i2* than *HvHKT2;1-e* type transcripts, indicating an abundance of intron-retaining transcripts. Semi-quantitative PCR confirmed the fragment lengths of amplified products from *trk1, trk2* expressing *HvHKT2;1-i*. No amplification was seen in the *trk1, trk2* yeast strain expressing the empty vector with primers corresponding to the exon region or the intron–exon junction, while a fragment of exact length was detected in *trk1, trk2* yeast expressing *HvHKT2;1-i* (Fig. 2A–C). Results of relative qPCR in yeast showed the presence of different proportions of intron-retaining (Figs 2B and 3A), incompletely spliced and correctly spliced transcripts, much as found for *HvHKT2;1-i* in barley.

Changes in K^+ ion concentration regulates *HvHKT2;1-i* transcripts

The mutant yeast strain *trk1, trk2* expressing *HvHKT2;1-i* was grown under inducing and non-inducing growth conditions at various KCl concentrations (0, 10 and 25 mM) to investigate transcriptional regulation of *HvHKT2;1-i*. Statistical analysis of relative quantification showed no significant differences in transcript accumulation between 0 and 10 mM or between 10 and 25 mM KCl, whereas there were significant differences between 0 and 25 mM, with more transcripts at 25 mM KCl (Fig. 3B). The LSD test revealed significant differences between inducing and non-inducing growth conditions, with a two to threefold increase in transcripts under inducing growth conditions (Fig. 3B). Statistical tests to compare growth activity under different growth conditions and K^+ concentrations showed transcript abundance under inducing growth conditions at low K^+ concentrations (10 mM). *HvHKT2;1-e* transcripts differed significantly, with less transcripts than either *HvHKT2;1-i1* or *HvHKT2;1-i2*, while under inducing growth conditions, significantly more transcripts of *HvHKT2;1-i1* were observed. Furthermore, mixed proportions of *HvHKT2;1-e*, *HvHKT2;1-i1* and *HvHKT2;1-i2* transcripts were detected, consistent with the results of transcript analysis in barley (Figs 2 and 3B). Significantly higher *HvHKT2;1-i2* type transcripts under non-inducing growth conditions might indicate leaky expression of the galactose promoter.

Potassium uptake function of *HvHKT2;1-i* in *trk1, trk2*

The ability of *HvHKT2;1-i* to complement the K^+ transport function in the *trk1, trk2* yeast mutant defective in K^+ uptake was tested using growth assays in solid YPD medium in the presence of the antibiotic hygromycin B. *trk1*, *trk2* yeast mutants had a hyperpolarised cell membrane due their limited ability to take up K^+ and therefore present a hypersensitive phenotype after hygromycin treatment (Madrid *et al.* 1998). Indeed hygromycin, a cationic aminoglycoside, accumulates much more readily in yeast cells with abnormally high negative membrane potentials and inhibits cell growth (Perlin *et al.* 1988). So, in theory, any transport mechanism that depolarises the membrane potential (by taking up cations such as K^+, Na^+, etc.) will at least partially if not completely rescue *trk1*, *trk2* yeast growth in hygromycin YPD medium, as shown previously with other membrane cation transporters (Seto-Young *et al.* 1996; Ali *et al.* 2006; Rajagopal *et al.* 2007). The *trk1, trk2* yeast mutant expressing *HvHKT2;1-i* or the empty vector grew well on solid YPD in the absence of added hygromycin or KCl/NaCl (Fig. 4A1). Adding hygromycin (100 $\mu g \cdot \mu l^{-1}$) while still in YPD medium with no added KCl/NaCl resulted in complete loss of growth of *trk1, trk2* yeast cells transformed with either *HvHKT2;1-i* or the empty vector (Fig. 4A2). Increasing KCl concentration while keeping 100 $\mu g \cdot \mu l^{-1}$ hygromycin clearly reversed the growth trend, especially of *trk1, trk2* yeast cells transformed with *HvHKT2;1-i* even at 10 mM KCl (Fig. 4A3). The *trk1, trk2* yeast cells transformed with the empty vector partially recovered only with 75 mM KCl (Fig. 4A6); no growth was seen with either 25 mM (Fig. 4A4) or 50 mM KCl (Fig. 4A5). In the absence of hygromycin, *trk1, trk2* yeast cells transformed with either *HvHKT2;1-i* or the empty vector grow better as KCl concentration increased (Fig. 4A7–A9) but those transformed with *HvHKT2;1-i* grow much better with 10 mM KCl (Fig. 4A7). Finally, addition of increasing concentrations of hygromycin (10, 25 or 50 $\mu g \cdot \mu l^{-1}$) in YPD medium with no added KCl or NaCl resulted in total loss of growth activity of *trk1, trk2* yeast cells expressing the empty vector, while *trk1, trk2* yeast cells expressing *HvHKT2;1-i* showed some growth (Fig. 4A10–A12).

Hygromycin concentrations of 10, 25 or 50 $\mu g \cdot \mu l^{-1}$ proved lethal for *trk1, trk2* yeast growth (Fig. 4A10–A12), so we further tested growth recovery after addition of different concentrations of K^+ in incremented concentrations of hygromycin-supplemented medium (Fig. 4B1–B12). At these concentrations of hygromycin (10, 25, 50 or 75 $\mu g \cdot \mu l^{-1}$), expression of the empty vector in *trk1, trk2* yeast showed no growth activity, except for a few yeast cells at 10 $\mu g \cdot \mu l^{-1}$ with 50 mM K^+ (Fig. 4B3), 25 $\mu g \cdot \mu l^{-1}$ with 25 mM K^+ (Fig. 4B5) and 25 $\mu g \cdot \mu l^{-1}$ with 50 mM K^+ (Fig. 4B6). In contrast, under the same conditions, growth recovery was seen on expression of *HvHKT2;1-i* in *trk1, trk2* yeast cells under all conditions (Fig. 4B1–B12). These results point to the fact that when supplemented with KCl (as low as 10 mM), the *HvHKT2;1-i* transformed *trk1, trk2* yeast

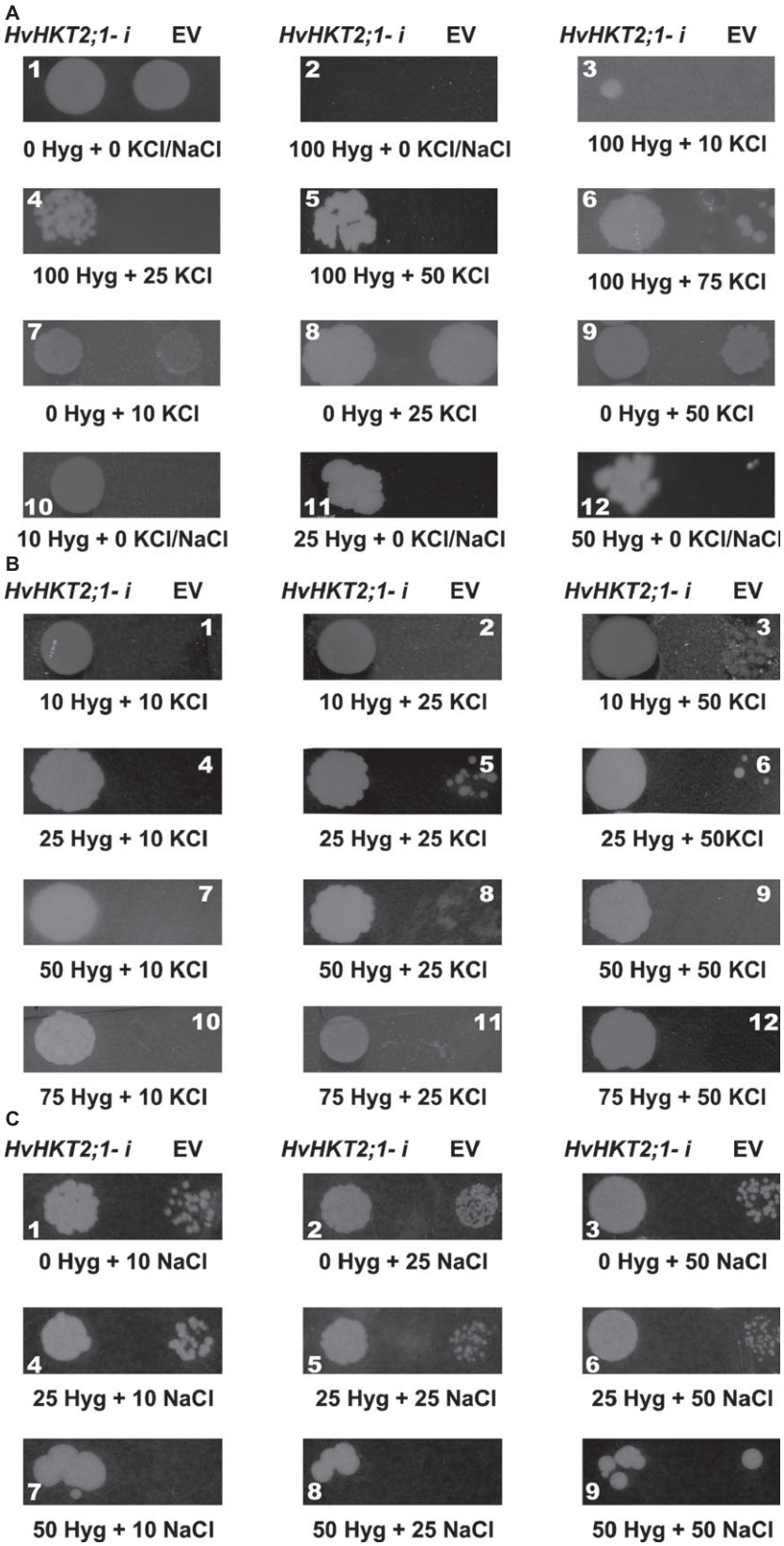

Fig. 4. Functional complementation analysis in *trk1, trk2* yeast expressing EV (empty vector) and *HvHKT2;1-i* on solid YPD medium. A: Effect of hygromycin without added KCl/NaCl and growth complementation at higher K^+ condition in hygromycin ($\mu g \cdot \mu l^{-1}$) supplemented medium. B: Complementation at different K^+ conditions (mM) under various hygromycin ($\mu g \cdot \mu l^{-1}$) concentrations. C: Effect of addition of Na^+ in hygromycin supplemented media with different concentrations of NaCl (mM).

mutant grew much better than the *trk1, trk2* yeast mutant transformed with the the empty vector only.

Increased cell sensitivity to Na^+ uptake and K^+/Na^+ symport function of *HvHKT2;1-i*

To study Na^+ uptake activity of *HvHKT2;1i*, *trk1, trk2* expressing *HvHKT2;1-i* or the empty vector were grown on hygromycin (0, 25 and 50 $\mu g \cdot \mu l^{-1}$) YPD solid medium supplemented with different Na^+ concentrations (10, 25 and 50 mM; Fig. 4C). In the absence of hygromycin, *trk1, trk2* yeast mutants expressing *HvHKT2;1-i* grew better than those expressing only the empty vector when supplemented with increasing concentrations of NaCl (Fig. 4C1–C3); although cells expressing *HvHKT2;1-i* and even the empty vector did not grow as efficiently as when supplemented with similar concentrations of KCl (compare Fig. 4C1–C3 with Na^+ to Fig. 4A7–A9 with K^+). The growth sensitivity of *trk1, trk2* yeast expressing the empty vector was enhanced on addition of 25 $\mu g \cdot \mu l^{-1}$ hygromycin with 10, 25 or 50 mM NaCl (Fig. 4C3–C6), while growth recovery was still observed in *trk1, trk2* yeast cells expressing *HvHKT2;1-i* (Fig. 4C3–C6). The same trend was seen when increasing the concentration of hygromycin to 50 $\mu g \cdot \mu l^{-1}$ at 10 or 25 mM NaCl, *i.e.* total loss of growth activity in *trk1, trk2* yeast cells expressing the empty vector while expression of *HvHKT2;1-i* led to better growth activity compared to the empty vector. A further increase in NaCl concentration to 50 mM (while keeping hygromycin at 50 $\mu g \cdot \mu l^{-1}$) helped growth recovery of a few *trk1, trk2* yeast cells expressing the empty vector, but *trk1, trk2* yeast cells expressing *HvHKT2;1-i* showed even better growth activity than this control. The growth is clear evidence of Na^+ uptake on expressing *HvHKT2;1-i.* Also, the growth assays (Fig. 4A–C) shown here all evidence the possible K^+/Na^+ symport function of *trk1, trk2* yeast cells expressing *HvHKT2;1-i.*

Potassium transport function of *HvHKT2;1-i* in *trk1, trk2*

To check K^+ transport properties, *trk1, trk2* yeast mutants expressing *HvHKT2;1-i* or the empty vector were grown at different KCl concentrations under both inducing and non-inducing growth conditions in minimal medium (uracil dropout). Transformed *trk1, trk2* yeast strains were grown in liquid SC–U (uracil deficient) dropout medium under inducing (2% galactose) and non-inducing (2% glucose) growth conditions with 0, 10, 25, 50, 75, 100, 150 or 200 mM KCl (Fig. 5A); growth rates were measured at 12-h intervals (Fig. 5B). Yeast under different K^+ concentrations showed better growth of *trk1, trk2* expressing *HvHKT2;1-i* at low K^+ (best at 10 mM) compared to growth at higher K^+ concentrations (50, 75, 100, 150 and 200 mM). The growth activity of *trk1, trk2* yeast cells expressing *HvHKT2;1-i* always remained higher than that of the empty vector under both inducing and non-inducing conditions. Interaction of KCl concentration with growth of *trk1, trk2* yeast cells showed significantly better growth of the mutant strain expressing *HvHKT2;1-i* under galactose-induced rather than non-inducing growth conditions (Fig. 5A). Furthermore, growth rate measurements indicated better growth of *trk1, trk2* expressing *HvHKT2;1-i* than in the mutant expressing the empty vector (Fig. 5B). Results from these growth assay experiments showed better growth of *trk1, trk2* yeast cells expressing

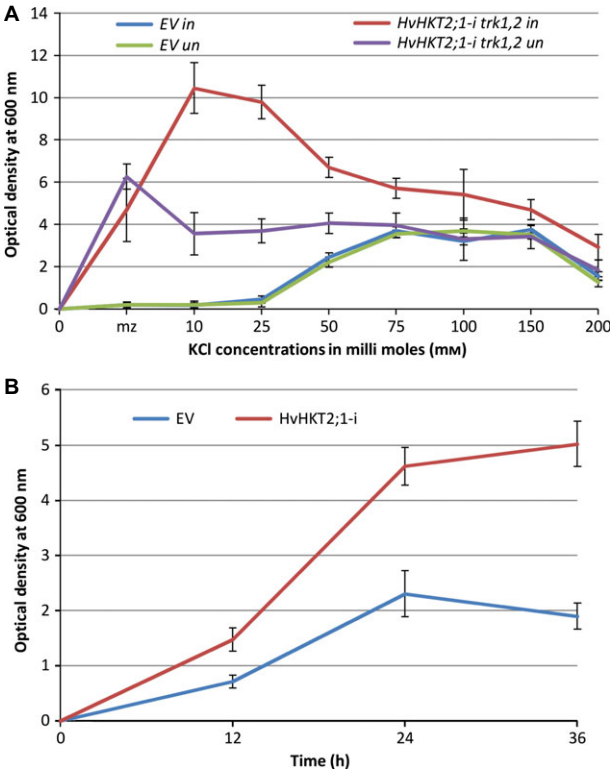

Fig. 5. Comparative growth analysis of trk1, trk2 expressing EV (empty vector) and HvHKT2;1-i. Yeast strains were grown under induced (2% galactose + 2% raffinose) and un-induced (2% glucose) conditions in selective drop out medium (SC–U) and data were taken after 12, 24 and 36 h. Minimal zero (mz) is considered as minimum KCl concentration as the initial inoculum used for re-culturing was taken from an overnight grown culture containing 100 mM KCl. A: Comparative analysis of growth activity of trk1, trk2 mutant expressing EV and HvHKT2;1-i. B: Growth rate analysis of trk1, trk2 mutant expressing EV and HvHKT2;1-i. Diagram of trk1, trk2 expressing: EV un, Empty Vector un-induced; EV in, Empty Vector induced; HvHKT2;1-i trk1,2 un, trk1,trk2 expressing HvHKT2;1-i under un-induced growth conditions; HvHKT2;1-i trk1,2 in, trk1,trk2 expressing HvHKT2;1-i under induced growth conditions; mz, minimal zero (along *x*-axis). Data are presented as mean + SE (n = 3).

HvHKT2;1-i under induced conditions, and especially under low K^+ conditions, suggesting a high affinity K^+ transport mechanism of *HvHKT2;1-i.*

Transcript analysis in *N. tabacum* expressing *HvHKT2;1-i* and salt stress analysis

Transgenic *N. tabacum* plants were generated with the *Agrobacterium* leaf disc transformation method to analyse transcript levels. RNA was isolated from leaf tissues of 50 independent transgenic plants and the reverse transcription product was used for relative quantification (qPCR) of *HvHKT2;1-i.* Relative expression analysis detected various compositions of HKT isoforms in different transgenic events, whereas no amplification was seen in samples from control tobacco plants. Control (seventh event) and transgenic (sixth event) plants were used for the salt stress experiment (Fig. 6). Relative quantification using real-time qPCR showed significant differences in transgenic plants expressing *HvHKT2;1-i.* Transgenic and control

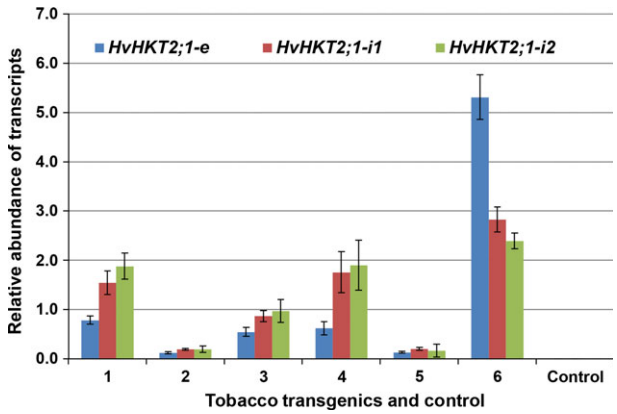

Fig. 6. Transcript profiling of *Nicotiana tabacum* plants ectopically expressing HvHKT2;1-i. Relative abundance of transcripts presented in graphical form as primer pairs: F1R1, to amplify HvHKT2;1-e; F2R2, to amplify fragment from first exon-intron junction, *i.e.* HvHKT2;1-i1; F3R3, from second exon-intron junction, *i.e.* HvHKT2;1-i2. Data presented as mean ± SE (n = 3).

plants were exposed to 100 mM NaCl to determine salt-related effects on plant growth. After watering with 100 mM NaCl solution, control plants showed symptoms of physiological drought, wilting and yellowing of older leaves (Figure S5A). This effect first resulted in drying out of older leaves and this eventually progressed to younger leaves (Figure S5A,B). However, the putative transgenic plant, even after 2 weeks of salt stress treatment, showed normal growth (Figure S5C). These results were also observed in transgenic tobacco plants with low *HvHKT2;1-i* expression, however, a few transgenic plants showed growth recovery after normal watering (without salt) but control plants were unable to recover (data not shown). Transgenic events showed similar patterns of intron retention and alternate splicing as observed in barley and yeast. Increased transcript levels for *HvHKT2;1-e* were found as compared to either *HvHKT2;1-i1* or *HvHKT2;1-i2* in some transgenic plants (Fig. 6; see event 6).

Transient expression of *HvHKT2;1-i* in *N. benthamiana*

Nicotiana benthamiana plants were used to transiently express GFP (green fluorescent protein) in leaf tissue using *Agrobacterium* infiltration to determine if correctly spliced transcripts are produced along with the HKT protein at the predicted target site. *HvHKT2;1-i* was cloned without a stop codon in two expression vectors, pMDC84 and pMBb7Fm21GW-UbiqL (Fig. 1), in-frame with GFP and was transiently expressed in *N. benthamiana.* Confocal studies revealed that *HvHKT2;1* and the GFP signals co-localised in the plasma membrane (Fig. 7C,E, F), whereas no signal was detected in control samples (Fig. 7B). Detection of GFP fluorescence in the plasma membrane indicate the presence of correctly spliced transcripts besides alternate splice variants, as seen in relative quantification analysis from *HvHKT2;1-i* in stably transformed *N. tabacum.*

DISCUSSION

In this study, cDNAs were cloned for *HvHKT2;1* and *LfHKT2;1* that encode high affinity K^+ transporters from salt-tolerant barley (*H. vulgare*) and kallar grass (*L. fusca*), respectively. Both cDNAs contained two insertions corresponding to introns previously reported for *PhaHKT1-u* from *Phragmatis australis* (Takahashi *et al.* 2007). The occurrence of introns in transcripts, their role in gene regulation, proteome diversity, complexity and, importantly, their role in adaptation to a particular stress condition has been reported previously (Pagani *et al.* 2000; Ner-Gaon *et al.* 2004; Reddy 2007; Zhang & Gassmann 2007; Kesari *et al.* 2012). Alternative splicing has

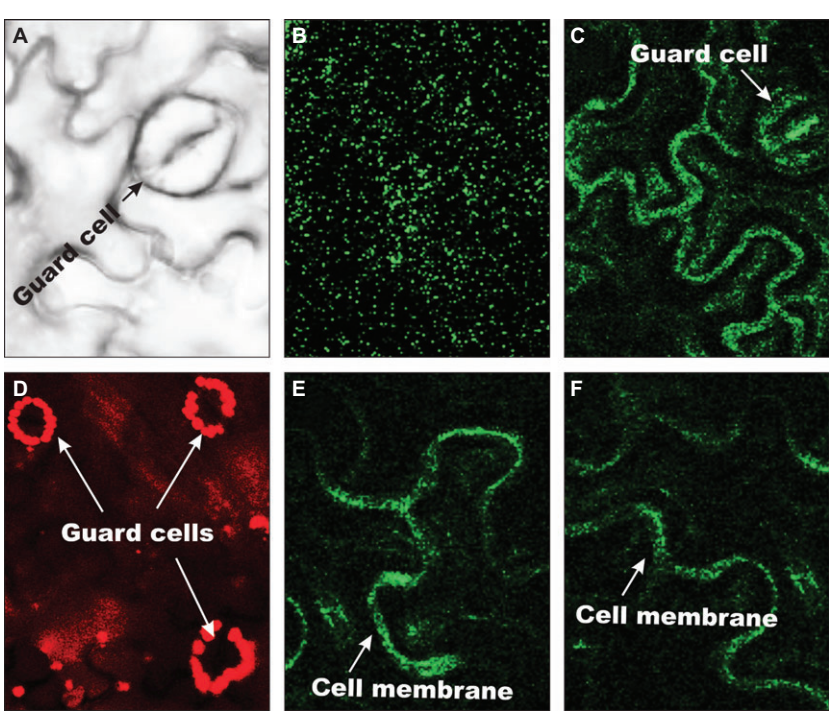

Fig. 7. Transient expression of HvHKT2;1-i in *Nicotiana benthamiana*. Fluorescence signals were examined after 48 h in *Agrobacterium* infection leaves. A: Image under bright field, (B) control, (C): samples from leaves infected with HvHKT2;1-i-pMDC84, (D): image under red fluorescence, (E–F): leaf samples infected with HvHKT2;1-i-pMBb7Fm21GW-UbiqL showing GFP signals in plasma membrane.

not been observed in studies where ORFs of barley encoding HKT1 or HKT2 were expressed in heterologous or plant systems (Haro *et al.* 2005; Banuelos *et al.* 2008; Mian *et al.* 2011).

Presence of two SNPs in second intron in *HvHKT2* in Tibatian barley is associated with shoot and root K^+ concentration under salinity stress (Qiu *et al.* 2011). The abundance of multiple transcripts and their relative proportion may help in mediating resistance responses, since different transcripts may have distinct functions, much as observed for the *RPS4* gene involved in disease resistance to *Pseudomonas syringae* (Zhang & Gassmann 2007). We propose a similar hypothesis based on the current work on *HvHKT2;1*, postulating that the dynamic changes in transcripts produced *via* alternate splicing may confer tolerance to Na^+ stress conditions in barley (Fig. 2D). Different proportions of alternate forms of HKT transcripts were detected in relative quantification analyses. Similar HKT transcript patterns were detected in relative quantification analysis in transgenic tobacco plants as in barley under native conditions and on heterologous expression in yeast with mixed proportions of *HvHKT2;1-e*, *HvHKT2;1-i1* and *HvHKT2;1-i2* (Fig. 3). Intron retention and exon skipping events create complexity in gene regulation and proteome diversity (Graveley 2001). Accumulation of intron retaining transcripts to higher levels has also been reported in dehydrin protein under drought and cold conditions (Xiao & Nassuth 2006). Enhanced expression of *HvHKT2;1-i* transcripts in barley in response to increased Na^+ concentrations (Fig. 2D) and under changing K^+ ion concentrations in the *trk1, trk2* yeast mutant (Fig. 3A,B) indicates the regulation of *HKT* transcripts by Na^+ and K^+ ions. Up-regulated expression of *HvHKT2;1-i* with changing K^+/Na^+ ratios is consistent with previous findings in HKT genes (Shao *et al.* 2008; Zhang *et al.* 2008; Chen *et al.* 2011). Differential gene expression in response to changing K^+ and Na^+ ion concentrations in both heterologous and plant system is consistent with the hypothesis that RNA processing of these *HKT* isoforms is linked to salt tolerance (Qiu *et al.* 2011).

The presence of glycine/serine residues in HKT proteins at specific positions in the first pore loop (Figure S6) determine K^+ and Na^+ selectivities (Rubio *et al.* 1995, 1999; Diatlo *et al.* 1998; Kato *et al.* 2001; Maser *et al.* 2002; Tholema *et al.* 2005). Conserved glycine residues at conserved positions in all the pore loops indicates that genes amplified from kallar grass and barley are GGGG type K^+ transporters (Figure S6) and belong to subfamily 2 of HKT transporters (Platten *et al.* 2006). Here, we used yeast hygromycin sensitivity as a functional assay to test the K^+ and Na^+ uptake activities of *HvHKT2;1-i*. Yeast hygromycin sensitivity assays have been used to characterise membrane proteins involved in passive and/or active ion transport, such as HKT, Trk or H^+-ATPases (Seto-Young *et al.* 1996; Calero *et al.* 2000; Ali *et al.* 2006). A similar approach was utilised in this study to determine the K^+/Na^+ symport activity of yeast cells expressing *HvHKT2;1-i*. Even though introns were present in the ORF, heterologous expression of *HvHKT2;1-i* in yeast helped growth recovery of yeast cells under hygromycin-sensitive growth conditions. Growth recoveries were presumably seen due to increased concentrations of K^+ ions inside the cells, which in turn results in reduced hygromycin uptake and toxicity. This effect operates under all conditions where an increased concentration of K^+ resulted in growth recovery of yeast cells expressing either the empty vector or *HvHKT2;1-i*. Growth recovery of a few yeast cells expressing the empty vector under high hygromycin conditions can be attributed to limited K^+ influx through plasma membrane non-selective cation channels (NSCC). As an addition to this uptake mechanism, the growth recoveries of *HvHKT2;1-i* expressing yeast cells under higher hygromycin conditions on addition of K^+ might likely result also from additional K^+ uptake activity of *HvHKT2;1-i* (Fig. 4A). The growth recovery of yeast cells at higher hygromycin concentrations under increased KCl concentrations suggests improved uptake of K^+ as compared to the empty vector (Fig. 4B). Although Na^+ taken up by cells is toxic, it may be less so than hygromycin and therefore allows some growth recovery of yeast cells expressing *HvHKT2;1-i* (Fig. 4C). Functional complementation analyses in yeast suggest that Na^+/K^+ ions are transported in response to expression of *HvHKT2;1-i* (Fig. 4).

Growth of the *trk1, trk2* yeast strain expressing *HvHKT2;1-i* or the empty vector was monitored in minimum media (inducing and non-inducing conditions) to test growth activity and growth rate under different K^+ concentrations. K^+ is an important ion that regulates numerous cellular processes. The *trk1, trk2* yeast mutant lacks two major K^+ transport pathways, cannot grow in the absence of K^+ and shows reduced growth under K^+ limiting conditions. Observations of significantly higher growth activity of *trk1, trk2* yeast cells expressing *HvHKT2;1-i* under low K^+ conditions clearly suggests better K^+ uptake activity of *HvHKT2; 1-i* (Fig. 5A).

Results from confocal studies suggest appropriately spliced transcripts and HKT protein are correctly directed to the membrane (Fig. 7), which is also the site of expression of other HKT proteins such as PutHKT2;1 from *Puccinellia tenuiflora* (Ardie *et al.* 2009), McHKT1;1 from *Mesembryanthemum crystallinum* (Su *et al.* 2003), AtHKT1;1 (Sunarpi *et al.* 2005), rice OsHKT2;4 (Lan *et al.* 2010) and OsHKT2;1 (Horie *et al.* 2007). It is possible that the completely spliced *HvHKT2;1* transcripts enabled *trk1, trk2* yeast mutants to complement K^+ and Na^+ uptake functions. Alternatively, a truncated version of the protein could still enable K^+/Na^+ symport in *trk1, trk2*. We propose that the occurrence of multiple *HvHKT2;1* isoforms *via* alternate splicing can be attributed to regulation of transcript lengths and types by specific sequence elements (nucleotide) within introns. This notion is supported by intron-mediated alternate splicing in the *P5SC1* gene for proline accumulation in *Arabidopsis* under drought stress, where a G to T transversion in the third intron and TA repeats in the second intron in the *P5CS1* gene were sufficient to promote alternate splicing of this gene (Kesari *et al.* 2012). Furthermore, presence of two SNPs in the second intron in barley is related to K^+ concentration in roots and shoots under salinity stress, which shows a link between intron retention and ion transport (Qiu *et al.* 2011). Specific sequence elements within introns and exons serve as the sites of splicing factors that either enhance or repress alternate splicing (Wang *et al.* 2008; Barash *et al.* 2010). Therefore, it is possible that the presence of a high frequency of retained introns is related to the existence of splice site recognition by introns in plants (Wang & Brendel 2006). We propose that alternate splicing might have regulatory functions beyond nonsense mediated decay. However, the complexity of proteome diversity resulting from alternate splicing, although impor-

tant in adaptations to changing environments, remains only partially resolved.

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SUPPORTING INFORMATION

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

Figure S1. Alignment of intron containing cDNAs and gene sequences from NCBI to locate position of introns and exons in HvHKT2;1.

Figure S2. Nucleotide sequence of *HvHKT2;1-i* gene (total length with introns: 2058 bases).

Figure S3. A short fragment of 471 bases of *HvHKT2;1-i*.

Figure S4. A hypothetical diagram of different alternate forms of *HvHKT2*.

Figure S5. Salt stress treatment (100 mM NaCl) has dramatically less effect on transgenic tobacco plants. A) Control tobacco plant after 1st week of salt stress treatment. B) Control tobacco plant after 2nd week. C) Transgenic tobacco plant after two weeks of salt stress treatment.

Figure S6. Structural model of HKT proteins adapted and modified from Mäser *et al*. (2002).

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