Original Article

Overexpression of two upstream phospholipid signaling genes improves cold stress response and hypoxia tolerance, but leads to developmental abnormalities in barley

Krisztián Gierczik [1, 2], András Székely [1], Mohamed Ahres [1, 2], Zsuzsa Marozsán-Tóth [1], Ildikó Vashegyi [1], Wendy Harwood [3], Balázs Tóth [1], Gábor Galiba [1, 2], Alexandra Soltész [1, ✉, *] and Attila Vágújfalvi [1, ✉, *]

1 Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Martonvásár 2462, Hungary
2 Festetics Doctoral School, Georgikon Faculty, University of Pannonia, Keszthely 8360, Hungary
3 John Innes Centre, Norwich Research Park, Norwich NR4 7UH, United Kingdom
✉ Corresponding author: soltesz.alexandra@agrar.mta.hu and vagujfalvi.attila@agrar.mta.hu; Tel.: +36 22 569 501; Fax: +36 22 569 576
* Alexandra Soltész and Attila Vágújfalvi contributed equally to this work.

Abstract

Phosphatidylinositol transfer protein (PITP) and phosphatidylinositol 4-kinase (PI4K) are very upstream regulatory elements of the phospholipid signaling pathway in the signal transduction network. Unlike in animal systems, their role in stress signaling is poorly understood in plants. To study this area, *PITP* and *PI4K* over-expressing transgenic barley lines were developed. Morphological and developmental abnormalities were surveyed and characterized. It was revealed that the over-expression of the upstream signaling genes led to more phenotypic abnormalities than in other transgenic studies working with effector genes or even transcription factors. We hypothesize that this high level of abnormalities are the consequence of the modulation of the very upstream signal transduction pathway elements. On the other hand, we also revealed that over-expression of the *PITP* and *PI4K* genes increased stress tolerance during hypoxic cold stress, but not during salinity stress. Differences were also found in the level of frost tolerance between the transgenic over-expression plants and the recipient Golden Promise line. Molecular analysis showed that this improvement was not related to the most important cold responsive transcription factors, the *CBF* genes. We conclude that the transgenic method may be useful to prove the role of an upstream signaling element, however, due to the many developmental consequences that occur as side effects, it is a less advisable approach to achieve improved stress tolerance.

Keywords

Phosphatidylinositol transfer protein, Phosphatidylinositol 4-kinase, Signal transduction, Abiotic stress tolerance, Morphogenetic disorders

Introduction

Like any living creature, plants often face environmental extremes, with many of these being life threatening biotic or abiotic stresses. However, being sessile organisms, plants are

unable to escape, so, in many case, their survival depends on the ability to cope and respond appropriately to the received signals *in situ*. The complexity and diversity of the signaling pathways allow them to react with fine-tuned, adequate responses. It has long been well documented that Ca^{2+} plays a crucial role in cellular signaling, not just in animal, but in the plant cells as well. It is one of the most important secondary messenger molecules which regulate many essential physiological processes. Elevated cytosolic Ca^{2+} concentration initiates several signal transduction pathways in plants in response to abiotic stresses, such as salt, cold, osmotic stress or anoxia (reviewed by Lindberg et al. 2012 and Ranty et al. 2016). One of those pathways, in which Ca^{2+} is a crucial component, is the so called 'phosphoinositide signaling pathway', named after the very first signaling molecule: phosphoinositol, which is, as yet, poorly characterized in plants cells.

Besides being structural components of biological membranes, phosphoinositides are involved in intracellular signaling across plasma membranes. They are precursors of second messengers and act as ion channel activators (Xue et al. 2009), thus regulating cellular responses to environmental changes, among which are abiotic stresses (Xue et al. 2007). Phosphatidylinositol (PI) is the first molecule of the phosphoinositide signaling pathway. PI specific lipid transfer protein (PITP) transports the PI between membranes, whereas PI 4-kinase (PI4K) catalyzes the phosphorylation of the PI molecule to PI 4-phosphate (PI4P), which can be further phosphorylated to PI 4, 5-bisphosphate (PIP_2). The membrane bound phospholipase C (PLC) enzyme catalyzes the hydrolysis of PIP_2 producing secondary messenger molecules: diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3). DAG activates ion channels and protein kinases to generate phosphatidic acid, a typical plant second messenger molecule, whereas IP_3 activates receptors on the endoplasmic reticulum membrane, which release Ca^{2+} into the cytoplasm. The phosphoinositide signaling pathway is excellently reviewed recently by Delage et al. (2013), Ruelland et al. (2015) and Hou et al. (2016), however, it should be noted, that the exact function of each and every element is not fully understood yet in plant systems.

Although novel molecular techniques are now available, such as genome editing especially using CRISPR-based methods (Ricroch et al. 2017), overexpression or silencing the gene of interest by transformation methods has proved to be a very useful technique in gene functional analysis research for a long time. To prove the role of genes in plant development or in stress tolerance, transcription factors, but also effector genes have been examined (reviewed by Zhang 2003 and Haak et al. 2017). However, it is less common to determine the role of a gene involved in the signaling pathway using plant transformation techniques, especially not linked to the enhancement of stress tolerance. Only a very few articles have been published studying the role of PI signaling in plant environmental responses. One of them, studied transgenic *Arabidopsis* lines overexpressing the *ZmSEC14p* gene (homologous to *PITP* gene) from a cold-tolerant maize line. These transformant lines conferred tolerance to cold stress and they also up-regulated the expression of *PLC* genes, which are members of the calcium-signaling pathway (Wang et al. 2016). The role of the barley *HvSec14p* gene has recently been determined under water deficit conditions and salinity stress, using susceptible and tolerant barley genotypes (Kiełbowicz-Matuk et al. 2016). It was revealed that the *HvSec14p* expression level was strongly up-regulated under drought stress and sodium treatment in the vegetative organs. In another study with *Arabidopsis pi4k* mutants, the double mutant lines displayed significantly delayed germination under low temperature compared to the wild type, indicating higher cold

sensitivity, thus suggesting the role of the *PI4K* gene in cold tolerance (Delage et al. 2012). These results support the involvement of the PI signaling pathway in stress responses; however there are many missing links in our understanding.

In our previous study, we have demonstrated that the *HvPITP* and *HvPI4K* genes, members of the phospholipid signaling pathway, show circadian rhythm in barley. We also showed that their expression is modulated by the ratio of the illuminating red/far-red light (Gierczik et al. 2017). This experimental result, and also the fact that the PITP and the PI4K enzyme are responsible for the activation of Ca^{2+} channels and the release of secondary messenger molecules, suggested to us that these genes may play a role in the enhancement of certain abiotic stress tolerances, via the facilitation of the stress signal propagation. To test this hypothesis, the *HvPITP* and *HvPI4K* genes were isolated from winter barley and overexpressed in a spring barley genotype. The effect of the transgenes on stress adaptation was analyzed by determining the response to salinity, frost and hypoxic cold stress.

Materials and Methods

Plasmid construction, plant material and transgenic plant production

The *PITP* and *PI4K* gene sequences were obtained from the NCBI nucleotide database as described previously (Gierczik et al. 2017). The AK374523 accession was selected as the target *HvPITP* gene and the AK360859 accession was selected as the target *HvPI4K* gene for transformation. The cDNAs were isolated from winter barley (*Hordeum vulgare* spp. *vulgare*) variety 'Nure'. Genes were reverse-transcribed (M-MLV Reverse Transcriptase, Promega Corporation, Madison, WI, USA) from RNA isolated by TRIzol® Reagent (Invitrogen Corporation, Carlsbad, CA, USA), the samples were treated with DNase I enzyme (Promega Corporation, Madison, WI, USA). The cDNAs were amplified (AccuPrime™ *Pfx* DNA Polymerase, Invitrogen Corporation, Carlsbad, CA, USA) using the primer pairs 'PITP Gateway Fwd' (5'-CAC CAT GGT TCA GAT CAA GGA ATT CCG AAT C-3'), 'PITP Gateway Rev' (5'-CTA CGT GCA GCT CCC CAT GAC TGC-3'), 'PI4K Gateway Fwd' (5'-CAC CAT GTC ACA AGG GAT GAA CAT GTT CGT G-3') and 'PI4K Gateway Rev' (5'-CTA TTT CTC AAT ACC TTG CTG CAA GTA TTG-3'), including the 4 base pair sequences (CACC), which are necessary for directional cloning on the 5' end of the forward primers. The amplicons were cloned into the pENTR/D-TOPO® (Invitrogen Corporation, Carlsbad, CA, USA) Gateway based cloning vector. Then the LR recombination reaction between the cloning (donor) vectors and the pBract214 binary recipient vector (John Innes Centre, Norwich Research Park, Norwich, United Kingdom) was completed. In this construct the maize *ubiquitin* promoter + intron (*Ubi-1*) sequences ensure the constitutive expression of the transgene. Immature embryos of the spring barley (*Hordeum vulgare* spp. *vulgare*) cv. 'Golden Promise' were transformed by *Agrobacterium*-mediated (*A. tumefaciens* strain AGL1) method (Bartlett et al. 2008; Harwood et al. 2009), using pBract214-*HvPITP* and pBract214-*HvPI4K* constructions (**Fig. S1**).

In our experimental system, different transgenic generations were used for the tests. Some tests were run on the segregating T_1 generation, but most of them were carried on homozygous T_2 or T_3 plants.

Molecular analysis of the transgenic lines

In order to prove the presence of each essential element of the transgenes, genomic DNA samples were isolated from leaf tissue from all independent transformant lines (i.e. regenerated from different calli) by DNeasy Plant Mini Kit (Qiagen®, Hilden, Germany) according to the manufacturer's instructions. The DNA quantification was carried out by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, MA, USA). The presence of the transgene was confirmed by PCR, amplifying the *hpt* selection marker gene (Stanley et al. 2011), the *ubiquitin* (promoter)/transgene and transgene/*NOS* (terminator) regions of the constructions (as in Soltész et al. 2013). The 'HvPITP Fwd' (5'-CAC CAT GGT TCA GAT CAA GGA ATT CCG AAT C-3'), 'HvPITP Rev' (5'-CTA CGT GCA GCT CCC CAT GAC TGC-3'), 'HvPI4K Fwd' (5'-TAC AGC TGG ACA GCG GTA TTC CTC TCC-3') and 'PI4K Rev' (5'-AAC TGG ATC CTT TTC TGG TGG TGC C-3') primer sequences were designed to amplify a 1214 bp (HvPITP Fwd + NOS), 1013 bp (HvPITP Rev + ubiquitin), 1251 bp (HvPI4K Fwd + NOS) and a 1202 bp (HvPI4K Rev + ubiquitin) PCR product.

The determination of transgene copy number in the T_0 and T_1 generation was carried out at g-Count™ service (IDna Genetics Ltd., Norwich, United Kingdom) using *hpt* as the assay target. The zygosity of T_1 plants was deduced based on the copy number values of the T_0 and T_1 plants and only homozygous T_1 plants were used to produce the genetically stable (i.e. non-segregating) T_2 seeds.

To determine the transgene expression levels, we used quantitative Real-Time PCR. Leaf samples of the T_1 plants were collected from five independent plants and homogenized into one sample for nucleic acid isolation. Total RNA was isolated by RNeasy Plant Mini Kit (Qiagen®, Hilden, Germany) according to the provided protocol, and supplemented with DNA digestion with DNase I Set (Zymo Research Corp., Irvine, CA, USA). RNA quantification was performed with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, MA, USA), while the RNA integrity and the DNA degradation was checked by agarose gel electrophoresis. cDNA syntheses were made with the M-MLV Reverse Transcriptase and oligo$(dT)_{15}$ primer (Promega Corporation, Madison, WI, USA) according to the instructions of the manufacturers. Gene expression levels were determined with CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Hungary Ltd., Budapest, Hungary) with the KAPA SYBR® FAST, Master Mix (2×), Universal qPCR Kit (Kapa Biosystems, Inc., Wilmington, MA, USA). Each cDNA sample was used for Real-Time qPCR in three amplifications and was considered as technical replicates. The *PITP*, *PI4K* and *cyclophilin* gene specific primer sequences were taken from the literature (Burton et al. 2004; Gierczik et al. 2017). The melt curve analysis was performed in every case to confirm the amplification of a single gene product. The relative gene expression values were calculated by the ΔΔCt method (Livak and Schmittgen 2001) using the *cyclophilin* as the reference gene. The untransformed, wild type Golden Promise was used as a control for normalization.

Plant growth and phenotypic evaluation

The homozygous T_2 generation was used for phenotyping. At developmental phase Z13 (Zadoks et al. 1974), i.e. the 3-leaf stage, the plants were placed into a vernalisation chamber for four weeks (0.9-5.9°C, 20-25 µmol m^{-2} s^{-1}, 16 hour photoperiod). After vernalisation, seven plants from each line were planted into plastic pots and placed into a

plant growth chamber (Conviron PGR15; Controlled Environments Ltd.; Winnipeg, Manitoba, Canada). The growing medium was a 2:1:1 (v/v/v) mixture of soil, sand and humus; the temperature was 18/15°C (day/night) with 220 µmol m^{-2} s^{-1} light intensity and 75% RH. After seven weeks, the temperature was raised to 22/20°C (day/night), but the other environmental parameters were not changed.

Heading date and flowering date was recorded from each plant during the growing phase. After full ripening, all plants from each line were evaluated for phenotypic traits, which were plant height, dry weight of shoot biomass, length of head spike, grain number and weight of head spike, spike number per plant, grain number and grain weight per plant as well as thousand grain weight (TGW).

Photosynthetic pigment content

The indirect measurement of the chlorophyll content of the leaves was recorded with a SPAD-502 Chlorophyll Meter (Minolta Co. Ltd., Osaka, Japan) after nine weeks from planting, at the beginning of inflorescence development, i.e. early-heading phase, Z51-52 (Zadoks et al. 1974). The measurements were made on the flag leaves of all seven plants from each line. The average of three SPAD values made from the midpoint of the leaves was calculated.

During the experiments, pale leaves or even albinism was detected in several cases. To quantify the photosynthetic pigment content of these abnormal leaves and every transgenic barley lines (including the pale ones) and to compare them to the Golden Promise control genotype, seeds from T_1 generation were germinated. In the case of the 'pale leaves' phenotype, samples of similar ages were taken. Leaves from the wild type Golden Promise variety and from green plants from each transgenic line were used as controls. Five independent leaves were measured in each case. The chlorophyll *a*, chlorophyll *b* and total carotenoids were extracted with 80% acetone. The maximum absorptions of chlorophyll *a*, chlorophyll *b* and total carotenoids (664 nm, 646 nm and 470 nm, respectively) were measured by Cary 100 Scan UV-Visible spectrophotometer (Agilent Technologies, Santa Clara, California, USA). The chlorophyll *a*, chlorophyll *b* and total carotenoid contents were calculated according to the method of Lichtenthaler and Buschmann (2001). Photosynthetic pigment contents were calculated in µg × g^{-1} fresh weight.

Frost tests

The wild type Golden Promise and seeds from T_1 and T_3 generations were used in two types of frost tests. One type was carried out with a cold hardening period, while a second one was run without it. For both experiments, the seeds were germinated as described above, then 20 individual seedlings were planted randomly into wooden boxes (42×30×13 cm) from each of the barley lines. The growing medium was as described above. The plantlets were grown in plant growth chambers with 16 hour photoperiod, at 20/15°C (day/night) temperature, at 220 µmol m^{-2} s^{-1} light intensity under cool white fluorescent tubes (Sylvania 215 W F96 T12). To prevent inhomogeneity in plant development the wooden boxes were randomized in the plant growth chamber every week. After nineteen days of the growth period, at 5-leaves stage (Z15, Zadoks et al. 1974), two different treatments were applied. For the first type of frost test, the T_1 plants were exposed to cold hardening period before freezing. To ensure the cold hardening, the temperature was gradually decreased over fourteen days to a continuous

+5/5°C (other environmental parameters left unchanged). Then, to reach the freezing temperature, it was decreased gradually (-0.6°C) to -6/-6°C. This -6°C freezing period was maintained for 24 hours. Having finished the freezing process, the temperature was increased gradually (+0.6°C/h) to 18°C for recovery. In the second type of frost test, the T_3 plants were subjected to the frost without any cold hardening period. For freezing, the temperature was decreased dramatically (-3°C/h) to -3°C or to -5°C. The lowest temperature (-3°C or -5°C) was applied for sixteen hours in both cases. In every frost tests, when the temperature reached 0°C, the leaves were sprayed with distilled water to induce ice nucleation. After both types of frost tests, the leaves were completely cut off 2-3 cm above the crown. Then the plants were allowed for stem elongation during at recovery period, which allowed the crown meristems to produce new tillers (Sutka 1981). The temperature in the recovery chamber was 18/13°C in every case, while the other environmental parameters were the same as in the growth period). After three weeks of recovery, the survival percentage (i.e. the ratio of the living and the total number of plants for each barley line) was calculated.

NaCl treatments

Six PITP lines and four PI4K lines from the homozygous T_3 generation and the wild type Golden Promise were used in this test. In three independent biological replicates, ten seeds were germinated on wet filter paper, moistened with half-strength modified Hoagland solution (Table S1) or half-strength modified Hoagland solution supplemented with NaCl to the final concentration 150 mM (Hoagland and Arnon 1950). The seeds were kept in the dark at 26°C for three days. The length of the shoots and the length of the roots were measured at the end of experiment.

In addition to the test above, seeds of six PITP lines and four PI4K lines from the homozygous T_3 generation and the wild type Golden Promise were germinated in Petri dishes wetted with distilled water. After three days at 26°C, 10-10 seedlings were tested in hydroponic-culture with half-strength modified Hoagland solution (see above). Plastic pots were placed into plant growth chamber under 16 hour photoperiods, at 22/20°C (day/night) temperature with 120-130 µmol m^{-2} s^{-1} light intensity. After nine days, salt stress was induced by adding 150 mM NaCl to the nutrient solution. The control plants were grown without salt treatment. The nutrient solutions were renewed every two days and the plastic pots were randomized in the plant growth chamber as well, to prevent inhomogeneity in plant development. The shoot length, shoot weight, root length, root weight, SPAD value (the method has already described in phenotyping for traits section) and relative water content (RWC) were measured after seven days. RWC was determined as RWC [%] = (fresh weight – dry weight) / (turgid weight – dry weight) × 100 (Schonfeld et al. 1988).

Combined hypoxia and cold stress

The homozygous T_3 generation and the wild type Golden Promise barley line as a control were used in this test. The environmental parameters were based on the Complex Stressing Vigour Test (Barla-Szabó and Dolinka 1988). One hundred seeds from each barley line were fully soaked in distilled water and were held at room temperature for two days. On the third day, without changing the water, the seeds were placed at 5°C ± 1°C for another two days. After the combined hypoxia and cold stress, the seeds were aliquoted randomly to four replicates, rolled in paper towels and placed vertically in glass beakers and held in

20°C ± 0.5°C. During the whole growing period the paper rolls were randomized every two days to prevent inhomogeneity in development. After eight days at 20°C, the survival percentage was calculated for each barley line. Following that, the vigour of the seeds was evaluated as well. Seedlings were considered highly vigorous if the length was larger than the 2/3 average of the three longest ones from each replicate.

Statistical analysis

For the statistical analyses, One-Way ANOVA (Analysis of variance) with Least Significant Difference (LSD) or Tukey's-b *post hoc* test (if equal variances assumed), Dunnett's T3 probe (if equal variances not assumed) or Mann-Whitney U nonparametric test was performed with the SPSS 16.0 software. The normality was tested by the Kolmogorov-Smirnov probe, while the homogeneity of the variances was tested by the Levene's probe.

Results and Discussion

Verification of transgenic barley lines

The coding regions of the *HvPITP* and *HvPI4K* genes were cloned into the pBract214 vector, especially designed for gene over-expression in cereal species. Immature Golden Promise spring barley embryos were transformed using pBract214-HvPITP and pBract214-HvPI4K constructs, also carrying the *hpt* selectable marker gene to produce hygromycin resistant transgenic plants. Only those plants, which were originated from different calli lines were considered as independent transgenic lines. Altogether, fifteen independent PITP and thirteen independent PI4K overexpressing lines were regenerated. The integration of the transgene was verified by PCR; all of the candidate transgenic lines proved to be PCR positive for the *hpt* gene and positive for the *ubiquitin* (promoter)/transgene and transgene/*NOS* (terminator) regions in the T_0 generation, thus confirming that all the essential elements were successfully inserted, in the desired orientation, in all transgenic lines.

Molecular characterization of the transgenic lines

The copy number estimation revealed in T_0 generation that the transformant barley lines contained the *PITP* gene in one, two or six copies and the *PI4K* gene in one or two copies (Table S2). Eleven out of fifteen lines (73%) contained the *PITP* gene as a single copy, while eight out of thirteen lines (62%) contained just one copy of the *PI4K* gene. From all the independent transformant plants, only one (4%), i.e. PITP L5 contained the transgene in more than two copies. These low copy numbers are indicating that our choice of *Agrobacterium*-mediated transformation protocol was correct. Our decision was based on the fact that 60% of the transgenic barley lines, which were produced by particle bombardment contained more than eight transgene copies in their genome (Travella et al. 2005); and this very high number could be considered a disadvantage, because the multiple transgene insertion often causes lower gene expression levels (reviewed by Dahleen and Manoharan 2007), and the production of homozygous lines is quite challenging. In a second study, from 260 independent barley transgenic lines produced by *Agrobacterium*-mediated transformation, almost half of the transformed lines contained the *luciferase* marker gene as

a single copy, while only 8% of the transformant lines contained more than three copies of the marker gene (Bartlett et al. 2008). This tendency was also observed in transgenic rice produced by *Agrobacterium*-mediated methods, 30-40% of more than 200 analyzed plants contained the *hpt* marker gene as a single copy (Sallaud et al. 2003).

To determine the un-induced *PITP* and *PI4K* gene expression levels of the transformant plants (**Fig. 1**), leaf samples were collected from control conditions (i.e. without any stress) from the segregating T_1 generation. We found that the *PITP* transgene expression values were much higher (20-60 fold, **Fig. 1/a**) than the *PI4K* transgene values (3-7 fold, **Fig. 1/d**) with the exception of PI4K L6 line (23 fold, **Fig. 1/d**) in the transformant lines compared to the un-transformant Golden Promise. To examine a possible interaction of the two genes, we determined the *PITP* expression levels in the PI4K lines and the *PI4K* expression levels in the PITP lines as well. The results showed that the transformed genes indeed, have an influence on the expression levels of the other gene. The *PITP* transgene was expressed in PI4K lines 2 to 6 fold higher than in wild type (**Fig. 1/b**), while the *PI4K* transgene was expressed in PITP lines 2 to 8 fold higher than in control genotype (**Fig. 1/c**). This evidence raises the possibility that the effects of the expression of the transformed gene influences (increases) the expression of the downstream element (i.e. *PITP* enhances *PI4K*), which, considering that the transporter PITP provides the substrate inositol for the kinase (PI4K), seems to be reasonable. However, it could be also concluded, that the downstream element enhances the activity of the upper-stream element (i.e. PI4K enhances PITP) by an unknown feedback mechanism. These speculations, however, must undoubtedly be supported by other experimental data.

The relationship between the copy number of the transformant lines and the expression level of *HvPITP* or *HvPI4K* genes were also analyzed, however no correlation was observed between target gene expression level and copy number, neither for the PITP lines ($R^2 = 0.4935$) nor for the PI4K lines ($R^2 = 0.0470$). This finding is clearly demonstrated by comparing the PI4K L6 line, which showed the highest expression level among the PI4K lines, however it contains only one transgene copy, with the PITP L9 line, which contained the *PITP* gene in two copies, but its expression level was the lowest of all the examined PITP lines. It has been shown in many studies that there is no clear evidence for strong correlation between transgene expression level and copy number in genetically modified plants. The differences in transgene expression level has often been explained by the chromosomal location of the transgene cassettes, the so called as ‘position effect’ (Matzke and Matzke 1998).

Developmental disorders of the transgenic lines

When T_0 plants were regenerated and progressed in our system, it was observed that some plants, overexpressing either the *HvPITP* or the *HvPI4K* genes exhibited retarded flowering compared to the wild type Golden Promise. We realized that the apex of these plants, although they moved from the vegetative phase into the generative phase, appeared stuck at Z45-50 (Zadoks et al. 1974) (**Fig. S2/a-b**). In these plants, it seemed that the spikelet was not able to develop normally, so normal flower or spikes with seeds were not developed. Beside these developmental disorders, lack of internodes, another abnormal phenotype, also appeared in the T_0 generation (**Fig. S2/c**).

In the segregating T_1 generation, some transgenic barley lines showed chlorophyll deficiency or even albinism (**Fig. S2/e**). Such abnormalities have already been described in several publications for transformant plants (reviewed by Vyroubalová et al. 2011), but we found that the PITP and PI4K transformants showed this abnormality in a relatively higher rate. In the PI4K L6 line 6.5% of the plants showed a pale leaf phenotype, while in the PI4K L4 line 6%, and in the PITP L11 line 5% of the plants showed albinism. Without exception these plants grew slower than the wild type Golden Promise or the rest of the transformant lines. This developmental disorder proved to be lethal from the third week after germination. In the case of the wild type Golden Promise, no chlorophyll deficiency has ever been detected. Considering the fact that in our previous experiment, when transformant lines, carrying the transcription factor coding *CBF* genes, were developed (Soltész et al. 2013) by exactly the same transformation system (vector, transformation method, recipient, etc.) hardly any albinism was recorded (unpublished data), we suppose that this result, found for the upstream regulators (PITP and PI4K), might be the side effect of the transgenes themselves (or it might be 'position effect' as mentioned above).

In order to quantify the above mentioned observations, the chlorophyll *a*, chlorophyll *b* (**Fig. 2/a**) and the total carotenoids content (**Fig. 2/b**) were determined in those T_1 lines which showed yellowish leaf phenotype or even albinism. There were no significant differences between the wild type Golden Promise and those transgenic lines, which showed normal, 'green' leaf phenotype. 'Green' (i.e. non-deficient) plants were also measured from those transgenic lines in which yellowish leaves or albinism had also appeared, but no significant differences were found between the green leaves and the wild type Golden Promise. However, a dramatic decrease was detected in the chlorophyll *a*, chlorophyll *b* and the total carotenoids content in the PI4K L6 line compared to the wild type and the PI4K L6 'green' samples. It is well accepted that the ratio of chlorophyll *a* / *b* is a good indicator of the greenness of leaves. The lower chlorophyll *a* / *b* ratio (i.e. between 2.5-3.5) is typical for the healthy, dark green leaves, while the higher chlorophyll *a* / *b* ratio (i.e. between 4.0-10.0) is specific for etiolated leaves (Lichtenthaler and Buschmann 2001). The chlorophyll *a* / *b* ratio in the wild type Golden Promise and in the green leaves from the transgenic lines were between 2.9-3.3, while in the yellowish leaf samples in the PI4K L6 line the chlorophyll *a* / *b* ratio was 3.8. The chlorophyll *a*, chlorophyll *b* and total carotenoids content were undetectable in case of albino plants in the white plants of the PITP L11 and PI4K L4 lines, showing real albinism.

It is known that many factors might induce albinism during tissue culture methods. Among them are genetic factors, such as the position of the inserted gene in the host genome; several culture conditions; the type of medium used; the species or the genotype of the recipient plant. A detailed study revealed that 45% of transformant callus lines yielded only albino plants. It was shown that the origin of the callus influenced the ratio of albinism, since 55% of the plants, which were regenerated from immature zygotic embryos, were normal green, while only 25% green plants were regenerated from microspore-derived embryos (Wan and Lemaux 1994). In another study, where particle bombardment was used to produce transformant wheat lines, some transformant lines had yellow-green appearance, compared to the dark-green host variety Bobwhite. This trait was noticeable before anthesis, furthermore, the leaves had a narrow and twisted shape (Bregitzer et al. 2006). In transgenic rice plants yellowish-greenish leaves appeared in T_3 and T_4 generations as well, which were in contrast to the dark green progenitor Xiushui 11 line. These transgenic plants grew slower,

developed less tillers and their heights were circa 80% shorter, compared to the non-transformants (Shu et al. 2002).

In the T_0 generation some transgenic lines, both from PITP and PI4K were unable to enter into the developmental phase Z30 (Zadoks et al. 1974), and remained at the tillering phase (**Fig. S2/d**). Without exception, these plants were not able to produce seeds, so they were not sustainable lines. We observed another abnormality among the transgenics: in the homozygous T_2 generation some transgenic barley lines showed branched spike morphology (**Fig. S2/f**). This is not unique; it has been described as naturally occurring several times (reviewed by Terzi et al. 2017); however the domesticated barley has a branchless shaped spike. In this study, we demonstrated that the transgenes increased the number of branched spikes in both PITP and PI4K lines as well (Table S3). In the case of PITP L13 and PI4K L2, the branched shaped morphology, as a developmental disorder of the spikes, was significantly increased due to the transgenes.

In several papers (Wang et al. 2008; Luo et al. 2012; Ku et al. 2013; Shen et al. 2015), the performance of the transgenics are compared not just to the progenitor wild type line, but to a transgenic control line also. These transgenic control lines contained only an empty vector, i.e. only the selection marker gene, but not the gene of interest, so these lines were suitable to study the effect of the transformational procedure itself. It was recorded that these empty vector lines did not show either development differences or higher stress tolerance compared to the wild type. In our previously study (Soltész et al. 2013), where the pBract214 binary vector was used (just like in this study), the thorough experiments revealed that the transgenic control line, carrying an empty vector, showed no differences compared to the wild type Golden Promise in either development or stress tolerance. Hence, in this study, only the wild type Golden Promise was included as the control line in all experiments. We concluded that the revealed differences, in trait phenotype, developmental disorders or stress tolerance, were therefore due to the *HvPITP* and *HvPI4K* transgenes and not because of the transformation event (see below).

Although plant transformation has a several decade-long history, it is still not usually possible to control the location of transgene insertion into the host genome or the precise expression level of the transgene with conventional plant transformation methods. New genome editing techniques offer the possibility for targeted transgene insertion but in the meantime there are still unintended events and unpredictable consequences in phenotype. In the case of *Arabidopsis*, the floral dip method generates minimal variations, on the other hand, when tissue culture must be used, and this is the case for the cereals, the frequency of 'non-intended' consequences appears more often. In monocotyledonous plant species, such as wheat or barley, these unintended consequences can be divided in three main categories: (1) insertion effect, (2) positon effect, and (3) effect of regeneration technique (reviewed by Filipecki and Malepszy 2006). Another source of the (sometimes frequently) unwanted events is the transgene itself. The agronomic performances of transgenic wheat lines containing additional high molecular weight glutenin subunit were assessed, and overall lower performances of the transgenic lines were detected. It was discussed that these deviations may have been caused by the selection marker gene or by somaclonal variation, or, by the altered transgene expression, i.e. because of the transgenes themselves (Bregitzer et al. 2006). However, no molecular evidence supported any of these assumptions. Such a kind of causality was presented for transgenic barley lines, when two

CBF transcription factors (CBF14 and CBF15) were introduced into the recipient Golden Promise line. These transgenes not only increased the level of frost tolerance, but also led to delayed flowering and retardation in development. The molecular analysis of the transgenic lines proved that the increased expression of these regulatory genes enhanced the transcript levels of the catabolic *gibberellic acid 2-oxidase enzyme* gene, which led to the inactivation of bioactive gibberellic acids and the accumulation of DELLA protein (Soltész et al. 2013). So, in this case, the overproduction of a regulatory transgene induced an effector gene, involved in the hormonal system, whose alteration finally led to the unwanted phenotype, i.e. to growth retardation. In our current work we have also shown that the transgenes, coding for very upstream signaling elements, thus influencing the expression of many downstream regulatory and effector genes, might be responsible for the unintended, unwanted phenotypes we have recorded.

Phenotypic analysis of the homozygous T_2 generation

A complex phenotypic evaluation was performed in the PITP and PI4K T_2 transgenic barley lines. Seven transgenic plants were measured from every line for every parameter, listed in Table 1. The PI4K L3 line was the only one from the eleven lines, which had an elevated SPAD value, compared to the wild type Golden Promise. Therefore we concluded that the overexpressed *PITP* and *PI4K* genes have no notable effect on the chlorophyll content. The plant height of two out of eleven transgenic lines, namely PITP L13 and PI4K L6 were lower than the wild type; moreover, the length of head spike was also reduced only in these two transgenic barley lines, compared to the Golden Promise. The heading and the flowering date were not changed dramatically by the influence of the *PITP* and *PI4K* transgenes; only PI4K L5 in heading and PITP L13 in flowering date showed a significant difference compared to the wild type Golden Promise. The total dry weights of shoot biomass were significantly higher in the PITP L10 line than in Golden Promise, while PITP L15 and PI4K L2 showed a significant decrease. Three out of eleven lines, namely PITP L13, PI4K L5 and PI4K L6 showed a significant increase in spike number per plant, compared to the wild type. The grain weight of the head spike was decreased significantly in PITP L13, PI4K L2, PI4K L5 and PI4K L6 lines compared to the Golden Promise; moreover, from these four lines, the PI4K L5 and PI4K L6 showed a significant decrease in the grain number of the spike as well. In PITP L13, PITP L15, PI4K L5 and PI4K L6 lines a significant decrease in grain number per plant was recorded. Furthermore, in PITP L13, PITP L15 and PI4K L5 lines a significant decrease was revealed in the grain weight per plant as well. Above all, the TGW values were reduced significantly compared to the Golden Promise in PITP L13, PI4K L2 and PI4K L6 lines. Taken together, several transgenic barley lines showed significant reduction in the agronomically relevant phenotypic traits, but there were also some which did not differ significantly from the wild type (e.g. PITP L4 or PITP L12).

Similar results were obtained with the TGW reduction among transgenic barley lines. In a complex study, where agronomic characteristics of the Golden Promise based transgenic barley lines were compared, it was revealed (Horvath et al. 2001) that the average TGWs of transgenic lines showed 30-40% weight reduction in two years in field conditions. In this experiment the transgenic barley lines were tested in an irrigated area as well, and it was found that the yield of the transgenic lines decreased to circa 6.0 tha^{-1}, while a 7.7 tha^{-1} yield was harvested for the progenitor Golden Promise line.

In our experiment a detailed comparison of PITP L12 and PITP L13 lines revealed some interesting differences as well. The PITP L13 line showed dwarfism and showed delay in flowering, but this line developed significantly more spikes per plant. Furthermore, this PITP L13 line showed a much more branched spike morphology 16.1% (P=0.006) too. However, it should be also noted that these spikes were much shorter and contained fewer seeds and, also, the grain weight of the head spike was just half of those in PITP L12 lines. The PITP L13 line had fewer grains per plant, had lighter grain weight per plant and the TGW was also lighter compared to the PITP L12 line. Since both lines contained the transgene only in one copy, and there was no difference between the transgene expression levels due to copy number, the molecular explanation for these differences is still unclear. One explanation might be the site of the insertion of the transgenes in the host genome, but the clarification of this assumption requires further work.

Frost tolerance testing

To test whether these transgenes have any effect on plant frost tolerance, the transformant barley lines and the wild type Golden Promise were tested in two kinds of frost tests, based on the method of described by Sutka (1981.(Sutka 1981). Since the two type of frost tests were not performed at the same time, the number of the tested lines was different in the two experiments. In the first frost test, after a growth period, 20-20 plants of the segregating T_1 generation (including null segregants) from each line were cold hardened for four weeks, and then frozen at -6°C. After three weeks of recovery, the plants were scored and the survival percentage was calculated. The PITP L9 and PITP L13 lines proved to be the most frost resistant compared to the wild type Golden Promise; twice as many plants survived this frost test at -6°C than the control line (**Fig. 3/a**). The PITP L9 line was the most frost resistant among the transformant lines, 45% (P=0.041) of the plants survived the -6°C frost period. Among the PI4K lines, two lines (PI4K L4 and PI4K L8) showed a small, but not significant increase in frost tolerance. In the second type of frost test, the T_3 homozygous plants were subjected to the frost without any cold hardening period. These non cold-acclimated plants were frozen at -3°C and -5°C. No significant difference was found between the transformant lines and the wild type Golden Promise when the freezing temperature was -3°C (**Fig. 3/b**). On the other hand the -5°C freezing temperature seemed to be lethal to all the tested transformant lines and the wild type Golden Promise, none of the non-acclimated plants survived this low temperature.

These results indicate that the overexpression of two upstream elements of the phosphoinositol signaling pathway can result in a slightly elevated level of cold stress tolerance. To our best knowledge, very few papers focus on the involvement of PI signaling components in plant abiotic stresses, such as low temperature tolerance. One of them describes that Phospholipase C and D is activated during a chilling period in *Arabidopsis* suspension cells (Ruelland et al. 2002). On the other hand, it became also clear that the overexpression of these phospholipid signaling elements may not be sufficient for increased freezing tolerance. We found that cold - as a signal - is really required to improve cold tolerance in the transformant barley lines. Why is cold as a signal essential? We do not have any experimental explanation at the moment – just a speculation. We suppose that the overexpression of the *PITP* and *PI4K* genes leads to an excess, therefore an easier access to these molecules in the cells. This abundance makes the *de novo* synthesis of these

elements unnecessary, which ensures this early response to be more effective. And this more efficient response leads to an increased level of cold stress tolerance in the end.

The CBF (C-repeat binding factor) transcription factors are one of the most important and well-described cold stress related genes in plants. They show high expression levels in low temperature and they regulate many cold responsive genes, the so called 'CBF-regulon' (this topic is reviewed by Thomashow (1999). In the PITP and PI4K transformant lines the transgenes are regulated by the *ubiquitin* constitutive promoter, which ensures strong transgene expression levels (**Fig. 1**). Since we found several improvements in the frost tolerance of some transgenic lines, we determined the gene expression levels of the most important *HvCBF*s that are known to play a pivotal role in cold stress responses (e.g. *HvCBF4B*, *HvCBF9*, *HvCBF12* and *HvCBF14*). The Real-Time PCR method used was the same as in the molecular analysis of the transgenic lines section. The primer sequences were taken from the literature (Morran et al. 2011; Dhillon et al. 2017; Gierczik et al. 2017). Despite the constitutive promoter, we could not detect any difference between the transgenic lines and the wild type Golden Promise (**Fig. S3/a-d**) for any of the studied genes.

The sudden burst of intracellular Ca^{2+} after a short exposure of cold stress is long known. The fact, that the CBF transcription factors are key players in cold stress regulation has been confirmed by dozens of papers in dozens of plant species but very few papers focus on the role of the upstream or intermediate elements. The direct connection between calcium signaling and the induction of *CBF* genes was suggested by showing that the calmodulin-binding transcriptional activator (CAMTA) was able to bind to the *CBF2* gene promoter in *Arabidopsis* (Doherty et al. 2009). Here we show that the two very upstream Ca^{2+} signaling elements may play roles in the increased level of frost tolerance, however, since no induced *CBF* expression levels were recorded, this response is not due to the activation of the CBF-regulon.

Salt stress responses

Among the few papers published on the effect of PI pathway elements on abiotic stress tolerance, Kiełbowicz-Matuk et al. (2016) recently tested salt stress susceptible and tolerant barley genotypes. It was revealed that the barley *PITP* homologue gene, *HvSEC14p* was up-regulated not only at the transcriptional, but also at the protein level. This result suggested that we should test our PITP and PI4K transgenic barley lines under NaCl stress. Ten transgenic lines from the homozygous T_3 generation with the wild type Golden Promise were tested at two developmental phases. Beside the control (i.e. no salt), 150mM NaCl treatment was applied during germination. We found differences (but not significant) between the control and the salt stress treatment in the shoot or in the root length, however none of the transgenic lines showed any better performance than the Golden Promise line (**Fig. S4/a-b**, respectively). In a second study the salinity tolerance of the PITP and PI4K barley lines were tested in hydroponic conditions. Again, no significant differences were detected between the transformant lines and the progenitor Golden Promise in the shoot length, shoot weight, root length, root weight, SPAD value or in the RWC after one week stress (**Fig. S5/a-f**, respectively).

Summarizing our results, we could not confirm the involvement of the PITP (or PI4K) signaling elements in salt stress. One of the obvious explanations is that two different experimental systems were used (different barley lines, salt concentration, developmental

stage of the plants tested). Clearly, more experiments should be performed to clarify this question.

Combined hypoxia with low temperature stress

With Golden Promise as a control line, five PITP and three PI4K transformant barley lines were used during a combined hypoxia and simultaneous cold stress. The applied stresses proved to be almost lethal to the wild type barley line, while some of the transformant lines suffered less damage (**Fig. 4**). The survival percentage of the PITP L4, PITP L15, PI4K L2 and PI4K L5 lines were nearly the same, but a slight increase was observed compared to the Golden Promise. In the case of PI4K L3, a notable increase (P=0.005) was detected in survival percentage compared to the wild type line, 10% of the seeds could germinate after the combined hypoxia and cold stress. Furthermore, the PI4K L3 line showed not only a significantly increased germination percentage, but this line showed a significantly increased (P=0.031) high vigour percentage as well (Table S4).

Cereals are obligate aerobes, therefore oxygen deficiency (hypoxia) or its total absence (anoxia) is a significant stress for them. Due to frequently occurring floods in autumn or spring, young seedlings often suffer hypoxic conditions in the field. As a consequence, the loss in yield could vary from 10 to more than 50%. Among the cereals, barley is very sensitive to hypoxia (Dennis et al. 2000). The importance of the increase in cytosolic calcium level in eukaryotic cells under oxygen deficiency is well-known. Elevated cytosolic Ca^{2+} levels were detected within the first minute of exposure to anoxia conditions in maize cells where Ca^{2+} influx originated mainly from the mitochondria (Subbaiah et al. 1998) as it could be inhibited with ruthenium red, a known inhibitor of intracellular membrane Ca^{2+}/H^{+}-antiporter (Subbaiah et al. 1994). These data support our assumptions, that the elevated hypoxic stress tolerance of our transgenic lines, which are overexpressing regulators known to be involved in Ca^{2+} signaling, is really due to the elevated level of intercellular Ca^{2+}. However, this hypothesis requires some more direct experimental proof.

Conclusions

A huge number of articles have already shown that the over-expression of transcription factors or effector genes could cause notable increase in different abiotic stress tolerances (reviewed by Mickelbart et al. 2015 and Lata et al. 2016). Many of these studies aimed to prove the involvement of the gene of interest in the given stress or response by over-expression, or much less frequently by silencing. Such studies further illustrate the value of plant transformation as an important method in gene function analyses over decades. In this study we examined the effect of the over-expression of *HvPITP* and *HvPI4K* genes in the model cereal plant, barley. The two studied genes code for the very first elements of the phosphoinositol signaling pathway. We aimed to test whether these early regulators had any role in abiotic stress tolerance. Our study also investigated whether an excess of an upstream signaling element would be sufficient to induce downstream cellular processes which finally lead to the increase of such a complex phenomenon as abiotic stress tolerance; or whether several other factors (i.e. other signaling pathways) are also required. To answer these questions we have developed over-expressing transgenic barley lines. However, since many transgenic lines showed developmental disorders in this study, we concluded that the over-expression of genes from the very beginning of the signal

transduction pathway result in more phenotypic ‘anomalies’ than the overexpression of more specific effector genes or even their transcription factors. So, from economic and agricultural point of view, it seems that the over-expression of an upstream element is not the best choice to improve abiotic stress tolerance in transgenic plant production. On the other hand, since we could show that the *HvPITP* and *HvPI4K* genes do indeed play a role in cold tolerance and in hypoxia tolerance, we concluded that over-expression of an early signaling element is a useful method for gene function analysis.

Abbreviations

ANOVA	analysis of variance
CBF	C-repeat binding factor
Chl	chlorophyll
Ct	threshold cycle
cv	convarietas
DAG	diacylglycerol
DNA	deoxyribonucleic acid
Fwd	forward (primer)
GP	*Hordeum vulgare* L. cv. ‘Golden Promise’
hpt	*hygromycin phosphotransferase* (marker gene)
IP_3	inositol 1, 4, 5-triphosphate
P	probability level
PCR	polymerase chain reaction
PI	phosphatidylinositol
PI4K	phosphatidylinositol 4-kinase
PI4P	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4, 5-bisphosphate
PITP	phosphatidylinositol transfer protein
PLC	phospholipase C
Rev	reverse (primer)
RWC	relative water content
SPAD	soil-plant analysis development
T_x	x^{th} transgenic generation
TGW	thousand grain weight

Two-letter prefix in front of gene names

Hv	*Hordeum vulgare* L.
Zm	*Zea mays* L.

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Author contributions

KG performed the Real-Time PCR experiments, the abiotic stress tests, carried out the data analyses and wrote the manuscript. ASz and KG genotyped the barley lines. MA measured the photosynthetic pigment content. ZsMT, IV and AS performed the plant transformation. AS carried out the *in vitro* works. BT initiated and managed partially this study. AV managed and supervised this study. WH, GG, AS and AV revised the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Fig. 1 Gene expression values of the *HvPITP* gene in PITP lines and PI4K lines (a-b, respectively) and *HvPI4K* gene in PITP lines and PI4K lines (c-d, respectively) from the segregating T_1 generation. Transcript levels were calculated with the $\Delta\Delta$Ct method using *cyclophilin* as the reference gene and the Golden Promise (GP) wild type line for the normalization. The values and the error bars that represent the standard deviation are originated from five independent plants and three technical replicates.

Fig. 2 Chlorophyll *a* + *b* content (panel a) and total carotenoids content (panel b) of the PITP L11, PI4K L4, PI4K L6 transgenic lines and the wild type Golden Promise (GP). The values indicated by different letters are significantly different at $p < 0.05$ level; 'undet' means undetectable values. Error bars represent the standard deviation calculated from five independent biological replicates.

Fig. 3 Survival percentage of transgenic barley lines and the wild type Golden Promise (GP) in the frost test at -6°C (panel a) and -3°C (panel b). Values indicated by asterisk are significantly different from the wild type Golden Promise at the $0.01 < P \leq 0.05$ (*) level.

Fig. 4 Survival percentage of five PITP and three PI4K transformant barley lines and the wild type Golden Promise (GP) in combined hypoxia and cold stress. Mean and standard deviation values were calculated from four replicates, which were originated from 25-25 seeds in every barley line. Values indicated by asterisks are significantly different from the wild type Golden Promise at the $P \leq 0.001$ (***), $0.001 < P \leq 0.01$ (**) and $0.01 < P \leq 0.05$ (*) levels.

Table 1 Phenotypic evaluation of the PITP and PI4K transgenic barley lines. The numbers in the table represent the average value and the standard deviation of seven plants from the homozygous T_2 generation in each line. Values indicated by asterisks represent significant increase (green cells) and significant decrease (red cells) from the wild type Golden Promise (GP) at $P \leq 0.001$ (***), $0.001 < P \leq 0.01$ (**) and $0.01 < P \leq 0.05$ (*) levels.

SUPPLEMENTARY DATA

Fig. S1 Expression vectors containing the *HvPITP* and *HvPI4K* genes (panel a, b, respectively) controlled by the constitutive *ubiquitin* promoter. The *hygromycin phosphotransferase* (*hpt*) selection marker gene is driven by the constitutive maize *35S* promoter. Vector maps were prepared using the Vector NTI® software. The amplicons were cloned into the pENTR/D−TOPO® based cloning, finally into the pBract214 binary recipient vector.

Fig. S2 Developmental disorders in transgenic barley at different developmental stages. Some transgenic plants showed developmental disorders in spikelet development during different generative phases (panel a-b). The white bars indicate 1.0 mm. Lack of internodes as an abnormal phenotype was seen in some overexpressing lines (see panel c). Some transgenic lines could not enter into the developmental phase Z30, i.e. 3-leaves stage and remained in the tillering phase (panel d). Chlorophyll deficiency in the PI4K L6 (panel e, middle plant) and the PITP L11 (panel e, right plant) transformant barley lines; while PITP L14 (panel e, left plant) represent the normal chlorophyll phenotype. Branched spike morphology in the PITP L4 plant (panel f).

Fig. S3 Gene expression values of *HvCBF4B*, *HvCBF9*, *HvCBF12*, *HvCBF14* (panel a-d, respectively) from the homozygous T_3 generation. Transcript levels were calculated with the $\Delta\Delta$Ct method using *cyclophilin* and Golden Promise (GP) wild type for normalization. The values and the error bars that represent the standard deviation originated from three independent plants and three technical replicates.

Fig. S4 Shoot length and root length (panel a-b, respectively) of six PITP and four PI4K transformant barley lines and the wild type Golden Promise (GP) during salinity stress. 150mM NaCl solution was used as salt treatment during the germination phase. Mean and standard deviation values were calculated from 3 independent biological replicates, which originated from 10-10 seedlings in every barley line.

Fig. S5 Shoot length, shoot weight, root length, root weight, SPAD value and relative water content (panel a-f, respectively) of six PITP and four PI4K transformant barley lines and the wild type Golden Promise (GP) during salinity stress. 150mM NaCl solution was used as salt treatment in the hydroponic test. Mean and standard deviation values were calculated from three independent biological replicates, which originated from 3-3 plants in every barley line.

Table S1 Modified half-strength Hoagland solution (pH 5.8) contained the following chemicals:

Table S2 Copy number values of the T_0 independent plants. The *hpt* marker gene was used as the assay target gene and Golden Promise wild type as a negative control.

Table S3 Branched spikes appearance in the transgenic PITP and PI4K lines. The values in the table were originated from seven plants in the homozygous T_2 generation in each line. Values indicated by asterisks are significantly different from the wild type Golden Promise (GP) at the $0.001 < P \leq 0.01$ (**) and $0.01 < P \leq 0.05$ (*) levels.

Table S4 High vigour percentage of five PITP and three PI4K transformant barley lines and the wild type Golden Promise (GP) in combined hypoxia and cold stress. Mean and standard

deviation values were calculated from four replicates, which originated from 25-25 seeds in every barley line. Values indicated by asterisks are significantly different from the wild type Golden Promise at the $0.01 < P \leq 0.05$ (*) level.

FIG 1

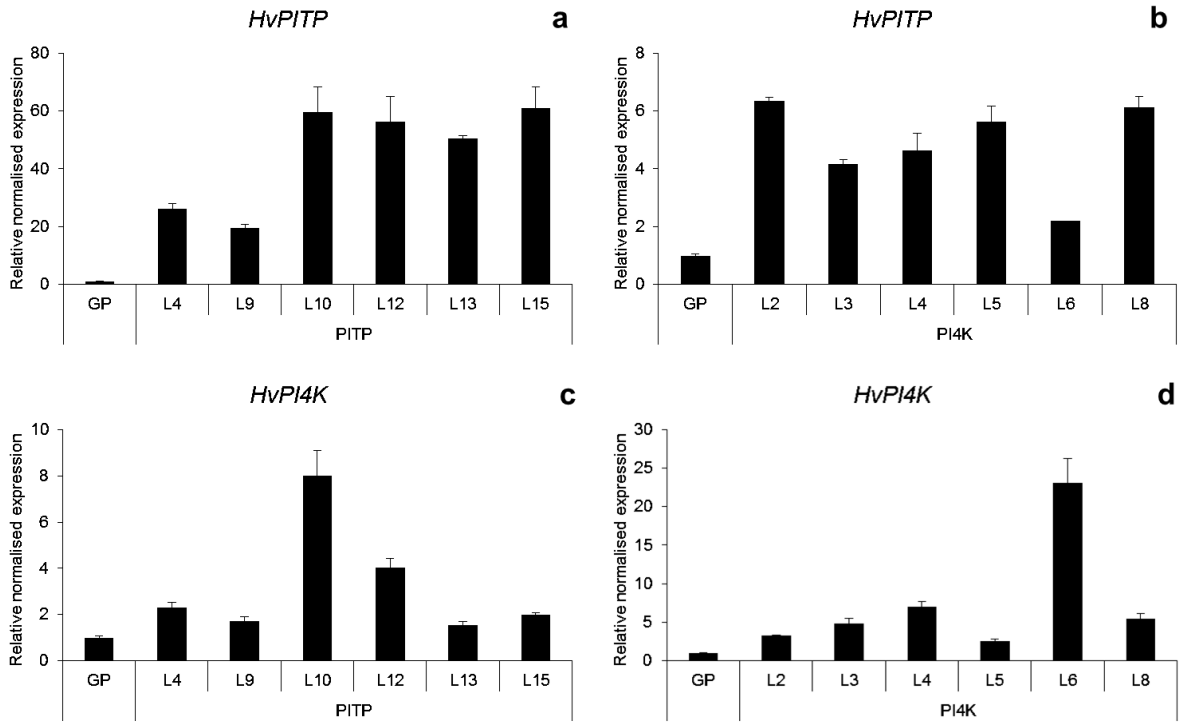


FIG 2

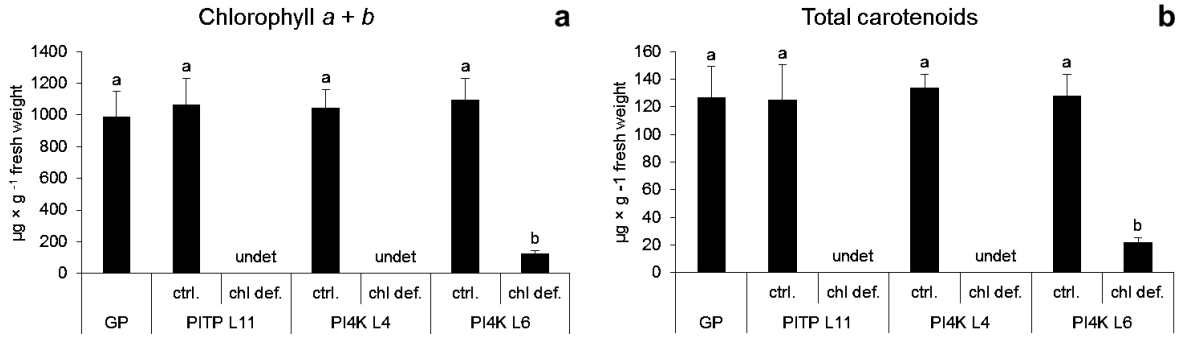


FIG 3

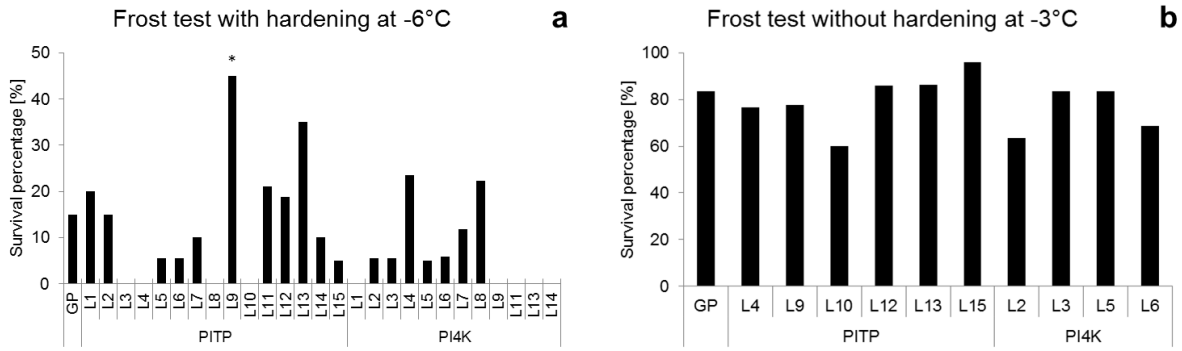


FIG 4

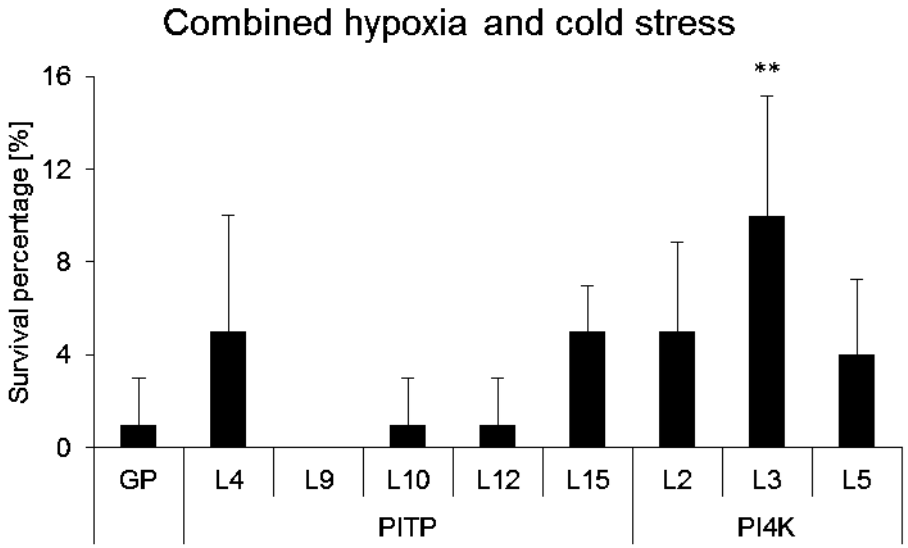
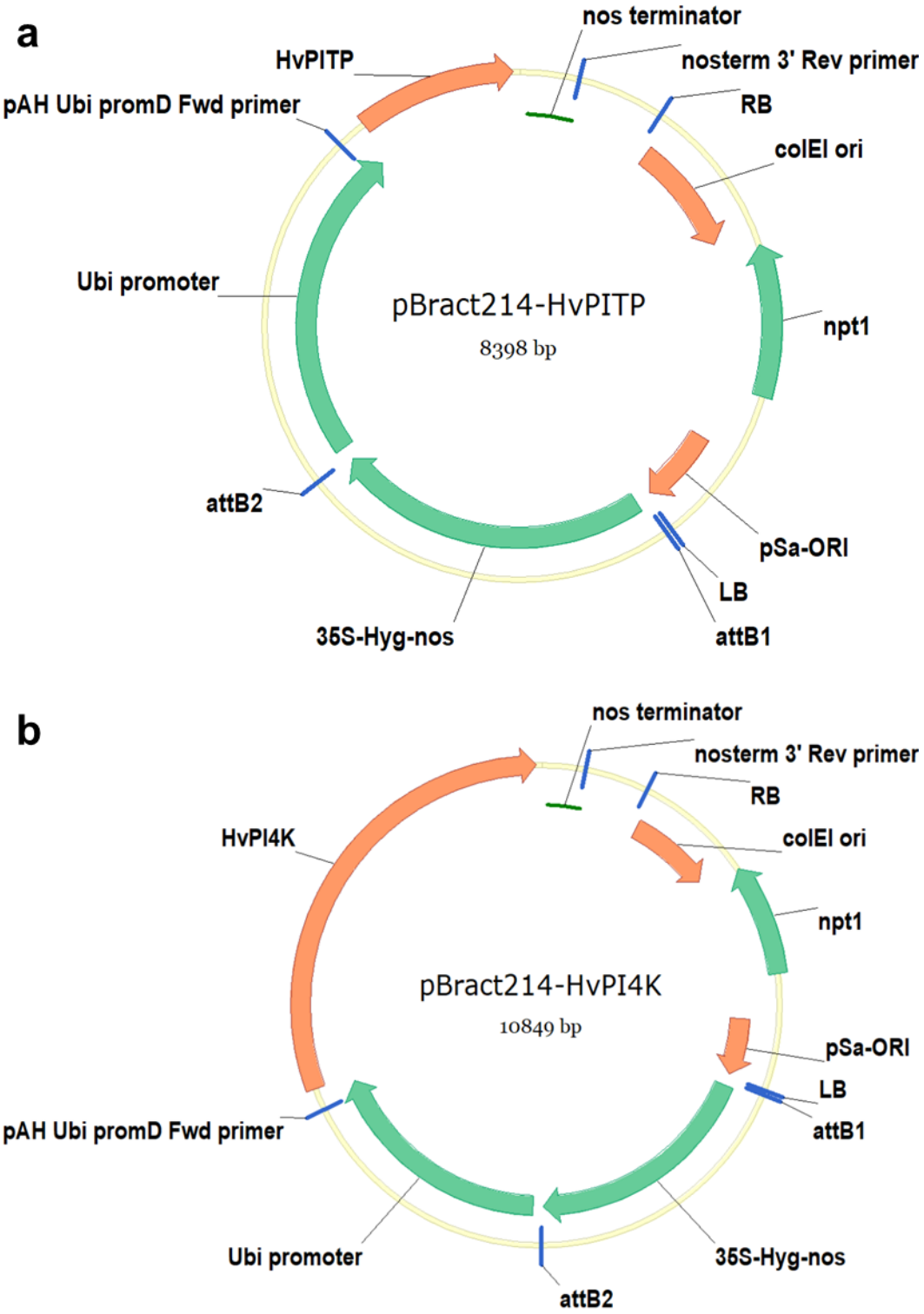

Combined hypoxia and cold stress
Survival percentage [%]
16
12
8
4
0
**
GP
L4
L9
L10
L12
L15
L2
L3
L5
PITP
PI4K

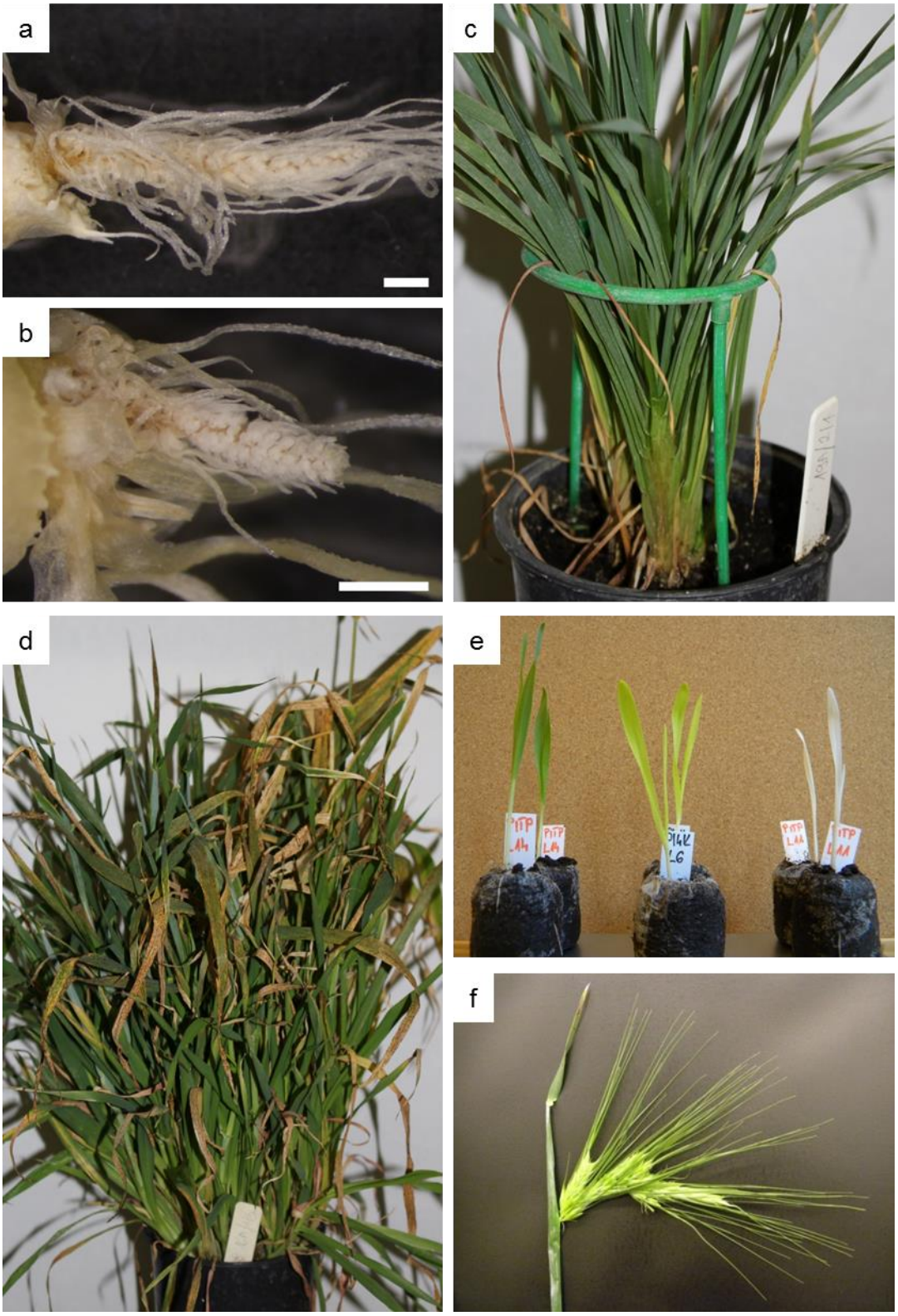
Table 1 Phenotypic evaluation of the PITP and PI4K transgenic barley lines. The numbers in the table represent the average value and the standard deviation of seven plants from the homozygous T_2 generation in each line. Values indicated by asterisks represent significant increase (green cells) and significant decrease (red cells) from the wild type Golden Promise (GP) at $P \leq 0.001$ (***), $0.001 < P \leq 0.01$ (**) and $0.01 < P \leq 0.05$ (*) levels.

Lines	GP	PITP						PI4K			
		L4	L9	L10	L12	L13	L15	L2	L3	L5	L6
Chlorophyll content (SPAD value)	46.6 ±4.9	49.7 ±4.9	47.9 ±4.3	50.5 ±3.7	46.8 ±3.4	44.0 ±4.7	50.2 ±5.4	44.0 ±5.9	52.1* ±6.5	45.9 ±4.7	45.1 ±5.7
Plant height [cm]	45.4 ±2.5	44.4 ±1.7	46.0 ±2.5	46.0 ±1.5	46.1 ±2.4	42.0* ±2.6	48.1 ±2.7	43.6 ±3.5	45.1 ±2.5	45.0 ±2.9	42.0* ±2.0
Heading date (days from planting)	56.7 ±1.8	56.1 ±1.5	56.7 ±2.1	56.0 ±1.8	55.3 ±2.1	57.7 ±1.0	55.3 ±1.4	55.4 ±1.6	55.3 ±1.0	54.7* ±1.4	57.6 ±1.3
Flowering date (days from planting)	59.1 ±1.6	58.3 ±1.0	58.9 ±2.0	58.7 ±1.8	58.4 ±2.3	61.3* ±1.5	58.7 ±1.4	58.1 ±1.3	57.9 ±1.1	58.6 ±1.0	59.9 ±1.5
Dry weight of shoot biomass [g]	14.5 ±1.9	14.3 ±2.5	13.4 ±2.5	17.3* ±2.5	13.4 ±2.0	14.3 ±2.6	11.5* ±0.7	11.6* ±2.5	12.4 ±1.4	13.1 ±2.1	13.1 ±1.5
Spike number per plant (pc)	10.0 ±2.8	8.3 ±1.5	9.1 ±2.5	12.3 ±1.7	10.1 ±2.5	13.3* ±4.1	7.6 ±1.1	10.0 ±2.0	9.6 ±2.1	14.4** ±3.7	14.9*** ±2.5
Length of head spike [cm]	9.3 ±0.8	9.1 ±0.5	9.0 ±0.4	9.6 ±0.9	9.1 ±1.2	8.2* ±1.0	9.4 ±0.6	8.8 ±0.8	8.4 ±0.9	8.4 ±0.7	7.4*** ±1.2
Grain weight of head spike [g]	0.99 ±0.2	0.91 ±0.2	0.89 ±0.1	0.89 ±0.3	0.85 ±0.2	0.46*** ±0.3	0.77 ±0.1	0.70* ±0.3	0.90 ±0.2	0.64** ±0.1	0.44*** ±0.2
Grain number of head spike (pc)	28.4 ±2.0	25.6 ±4.6	24.6 ±2.9	25.6 ±6.6	24.0 ±4.7	18.0 ±11.0	24.1 ±2.0	22.4 ±6.6	26.9 ±2.7	18.3** ±3.4	16.9* ±5.2
Grain number per plant (pc)	164.0 ±17.4	131.7 ±46.6	130.6 ±15.2	160.3 ±42.3	128.0 ±29.6	114.4** ±42.4	113.6** ±16.7	141.3 ±50.4	147.3 ±21.7	122.3* ±17.6	107.1** ±53.1
Grain weight per plant [g]	4.96 ±0.52	4.37 ±1.58	4.40 ±0.49	4.79 ±1.46	3.95 ±1.06	2.45** ±0.95	3.34** ±0.40	3.66 ±1.30	4.31 ±0.80	3.74* ±0.49	2.59 ±1.27
Thousand grain weight [g]	30.4 ±3.4	33.2 ±3.6	33.8 ±2.3	29.5 ±3.9	30.8 ±3.6	21.2*** ±3.0	29.7 ±3.8	26.4* ±4.1	29.3 ±3.1	30.7 ±2.6	24.4** ±2.9

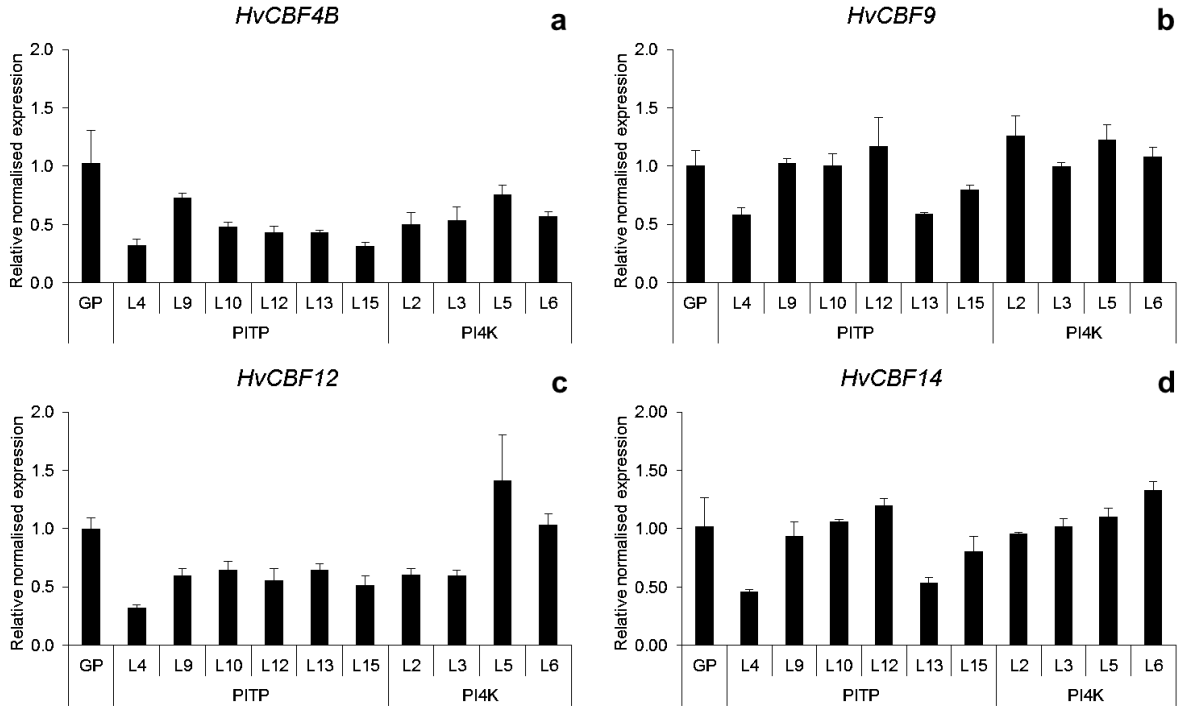
SUPLEMENTAL FIG 1



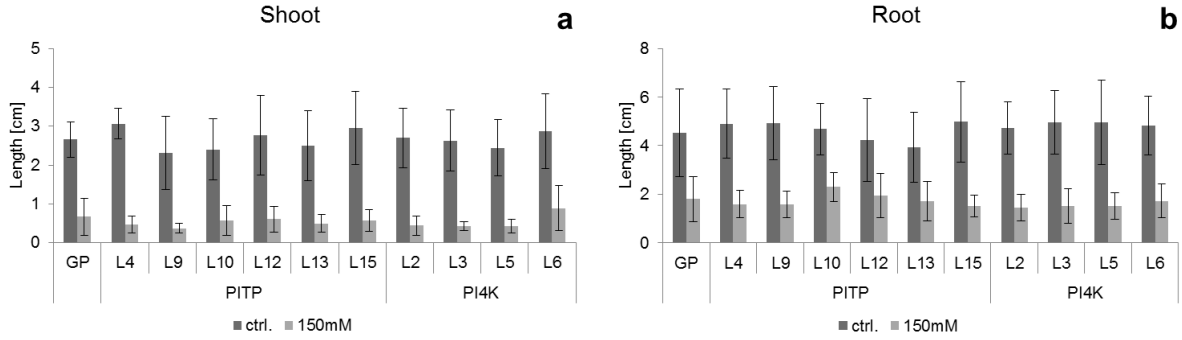
SUPLEMENTAL FIG 2



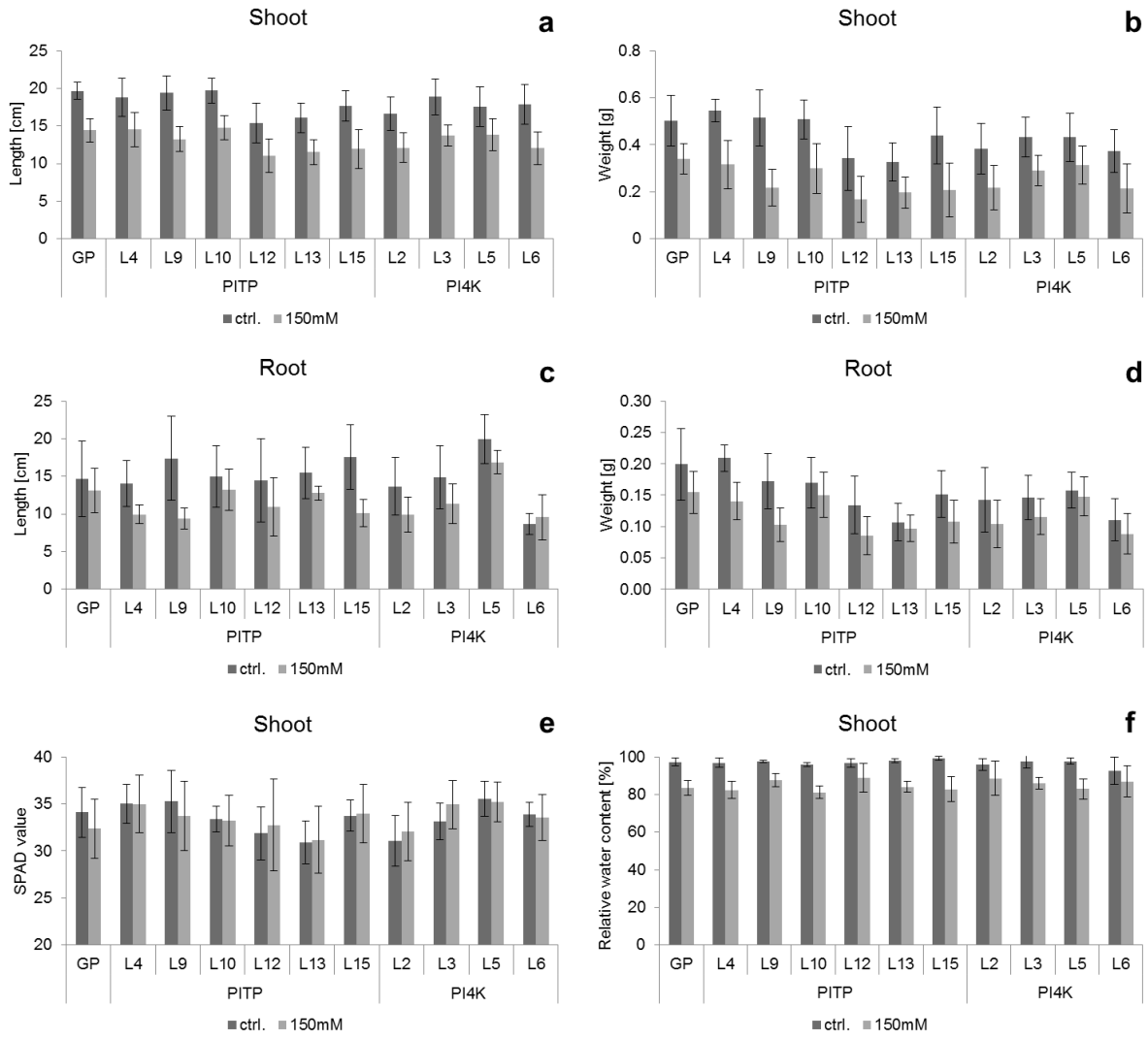
SUPLEMENTAL FIG 3



SUPPLEMENTAL FIG 4



SUPPLEMENTAL FIG 5



SUPPLEMENTAL TABLE 1

Table S1 Modified half-strength Hoagland solution (pH 5.8) contained the following chemicals:

Macronutrient solution	
KNO_3	2.50 mM
$Ca(NO_3)_2 \times 4\ H_2O$	2.50 mM
$MgSO_4 \times 7\ H_2O$	1.00 mM
KH_2PO_4	0.50 mM

Micronutrient solution	
H_3BO_3	5.78 µM
$MnCl_2 \times 4\ H_2O$	2.29 µM
$ZnSO_4 \times 7\ H_2O$	0.10 µM
$Na_2MoO_4 \times 2\ H_2O$	0.06 µM
$CuSO_4 \times 5\ H_2O$	0.04 µM

Fe-EDTA solution	
$C_{10}H_{12}N_2NaFeO_8$	0.11 mM

SUPPLEMENTAL TABLE 2

Table S2 Copy number values of the T_0 independent plants. The *hpt* marker gene was used as the assay target gene and Golden Promise wild type as a negative control.

Lines		Copy number
PITP	L1	1
	L2	1
	L3	1
	L4	1
	L5	6
	L6	1
	L7	2
	L8	2
	L9	2
	L10	1
	L11	1
	L12	1
	L13	1
	L14	1
	L15	1

Lines		Copy number
PI4K	L1	1
	L2	1
	L3	2
	L4	1
	L5	1
	L6	1
	L7	1
	L8	1
	L9	2
	L10	2
	L11	1
	L12	2
	L13	2
Golden Promise		0

SUPPLEMENTAL TABLE 3

Table S3 Branched spikes appearance in the transgenic PITP and PI4K lines. The values in the table were originated from seven plants in the homozygous T_2 generation in each line. Values indicated by asterisks are significantly different from the wild type Golden Promise (GP) at the $0.001 < P \leq 0.01$ (**) and $0.01 < P \leq 0.05$ (*) levels.

Lines	GP	PITP						PI4K			
		L4	L9	L10	L12	L13	L15	L2	L3	L5	L6
Total number of spikes	70	58	64	86	71	93	53	70	67	101	104
Branched spikes (%)	2.9	3.4	6.3	10.5	4.2	16.1**	0.0	12.9*	3.0	0.0	7.7

SUPPLEMENTAL TABLE 4

Table S4 High vigour percentage of five PITP and three PI4K transformant barley lines and the wild type Golden Promise (GP) in combined hypoxia and cold stress. Mean and standard deviation values were calculated from four replicates, which originated from 25-25 seeds in every barley line. Values indicated by asterisks are significantly different from the wild type Golden Promise at the $0.01 < P \leq 0.05$ (*) level.

Lines	GP	PITP					PI4K		
		L4	L9	L10	L12	L15	L2	L3	L5
Mean	1.00	4.00	0.00	1.00	1.00	5.00	5.00	7.00*	3.00
St. dev.	2.00	3.27	0.00	2.00	2.00	2.00	3.83	3.83	2.00