New Biotechnology • Volume 33, Number 5 • September 2016

ELSEVIER

Whole transcriptome analysis of transgenic barley with altered cytokinin homeostasis and increased tolerance to drought stress

Petr Vojta[1,2], Filip Kokáš[1], Alexandra Husičková[3], Jiří Grúz[4], Veronique Bergougnoux[1], Cintia F. Marchetti[1], Eva Jiskrová[1], Eliška Ježilová[3], Václav Mik[4], Yoshihisa Ikeda[1] and Petr Galuszka[1]

[1] Department of Molecular Biology, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University in Olomouc, Šlechtitelů 27, 783 71 Olomouc, Czech Republic
[2] Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University in Olomouc, Hněvotínská 1333/5, 779 00 Olomouc, Czech Republic
[3] Department of Biophysics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University in Olomouc, Šlechtitelů 27, 783 71 Olomouc, Czech Republic
[4] Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University in Olomouc, Šlechtitelů 27, 783 71 Olomouc, Czech Republic

Cytokinin plant hormones have been shown to play an important role in plant response to abiotic stresses. Herein, we expand upon the findings of Pospíšilová et al. [30] regarding preparation of novel transgenic barley lines overexpressing *cytokinin dehydrogenase 1* gene from Arabidopsis under the control of mild root-specific promotor of maize β-glycosidase. These lines showed drought-tolerant phenotype mainly due to alteration of root architecture and stronger lignification of root tissue. A detailed transcriptomic analysis of roots of transgenic plants subjected to revitalization after drought stress revealed attenuated response through the HvHK3 cytokinin receptor and up-regulation of two transcription factors implicated in stress responses and abscisic acid sensitivity. Increased expression of several genes involved in the phenylpropanoid pathway as well as of genes encoding *arogenate dehydratase/lyase* participating in phenylalanine synthesis was found in roots during revitalization. Although more precursors of lignin synthesis were present in roots after drought stress, final lignin accumulation did not change compared to that in plants grown under optimal conditions. Changes in transcriptome indicated a higher auxin turnover in transgenic roots. The same analysis in leaves revealed that genes encoding putative enzymes responsible for production of jasmonates and other volatile compounds were up-regulated. Although transgenic barley leaves showed lower chlorophyll content and down-regulation of genes encoding proteins involved in photosynthesis than did wild-type plants when cultivated under optimal conditions, they did show a tendency to return to initial photochemical activities faster than did wild-type leaves when re-watered after severe drought stress. In contrast to optimal conditions, comparative transcriptomic analysis of revitalized leaves displayed up-regulation of genes encoding enzymes and proteins involved in photosynthesis, and especially those encoded by the chloroplast genome. Taken together, our results indicate that the partial cytokinin insensitivity induced in barley overexpressing cytokinin dehydrogenase contributes to tolerance to drought stress.

Corresponding author: Galuszka, P. (petr.galuszka@upol.cz)

http://dx.doi.org/10.1016/j.nbt.2016.01.010

Introduction

Cytokinins (CKs) are plant hormones which together with auxins mainly influence plant morphology. Their role in other physiological processes, such as senescence and nutrient remobilization, is very well described [1]. Evidence, mostly from studies of Arabidopsis (*Arabidopsis thaliana*), suggests also an important role of CKs in the regulation of responses to environmental stresses [2]. CK-deficient Arabidopsis plants exhibited a strong stress-tolerant phenotype associated with increased cell membrane integrity and abscisic acid (ABA) hypersensitivity [3]. ABA's stress-related role consists mainly in induction of stomatal closure to prevent water losses under conditions of water limitation. Moreover, loss-of-function mutants of CK receptors and proteins involved in the CK-signaling pathway have been shown to be strongly tolerant to drought and salt stress because they up-regulated many stress-inducible genes [4,5]. Similarly, rice seedlings with knock-down proteins of the CK transduction pathway have been observed to be tolerant of osmotic stress but hypersensitive to salt stress [6]. Activated CK receptors negatively control osmotic stress responses and thus confirm that reduced CK status is a prerequisite for better drought tolerance.

On the other hand, increased drought tolerance or avoidance by stress-induced CK accumulation has been proven in several plant species [7–12]. A transgenic approach has exploited expression of the CK biosynthetic *isopentenyl transferase* (*IPT*) gene under a stress- and maturation-inducible promoter. Under drought-stress conditions, the transgenic plants maintained high photosynthetic activity in contrast to control plants due to the direct effect of CKs on delaying leaf senescence. The acquired drought tolerance was also due to CKs' effect on maintenance of nitrate acquisition from soil [13]. Thus, stress-induced CK synthesis in these transgenic plants promoted sink strengthening through the maintenance and coordination of N and C assimilation during water stress.

In abiotic stress responses, CKs can act in orchestration with other phytohormones. Auxin's role in drought tolerance has been demonstrated when increased activity of auxin conjugating enzyme, which reduces auxin maxima in leaves, led to the accumulation of late-embryogenesis abundant proteins responsible for the switch from plant growth to stress adaptation [14]. Auxin is able to induce the expression of genes encoding enzymes participating in biosynthesis of such stress-related hormones as ethylene [15]; vice versa, ethylene promotes local auxin biosynthesis and consequently reduces root cell elongation [16]. As CKs are known to affect production of both auxin and ethylene, coordinated regulation of hormonal biosynthetic pathways could play a crucial role in plants' adaptation to abiotic stresses [17]. Plants with stress-induced CK production showed up-regulation of brassinosteroid synthesis and signaling genes [11,18]. Brassinosteroids act synergistically with another group of plant hormones, gibberellins (GAs), due to shared components in their signaling pathways [19]. Transgenic Arabidopsis seedlings constitutively overexpressing GA-responsive genes exhibited improved tolerance to various abiotic stresses; stress tolerance was accompanied by biosynthesis of salicylic acid [20], another plant hormone mainly implicated in stress responses.

Plants exposed to drought stress show an alteration of CK content. Hormonal analysis of wild-type (WT) maize leaves subjected to drought showed a gradual decline in CK and GA contents during stress [21]. A comprehensive study on maize seedlings exposed to salt and osmotic stress also demonstrated rapid decline in some CK forms due to enforced CK catabolism. During acclimatization, however, accelerated CK metabolism led to a moderate increase in active CK forms [22]. Higher accumulation of all CK forms was also determined in tobacco exposed to severe drought stress [23]. Hence, accumulation of active CKs among other processes might contribute to the mechanisms by which plants overcome stress status and avoid growth inhibition. Regarding stress signaling, CKs do not, due to the slow response of their biosynthetic genes to stress induction, have a direct function similar to ABA, which directly affects stomatal closure [22].

Maintenance of high photosynthetic capacity is an important prerequisite for preserving crop yield under adverse environmental conditions. Although increasing CK content by senescence-regulated expression of a CK biosynthetic gene is an efficient tool for prolonging leaf photosynthetic activity [24], engineered wheat plants with senescence-regulated CK production showed no differences in yield-related parameters [25]. Shoot growth inhibition and promotion of root growth have been regarded as advantageous for crop stability under stressful conditions and constitute an integral part of plant stress tolerance [26,27]. Accordingly, plants with reduced shoot-to-root ratios as a consequence of CK deficiency showed greater tolerance to or avoidance of drought stress [28,29]. Hence, down- and up-regulation of CK levels *in planta* can have a synergistically positive effect on enhanced tolerance to water deficit: in the first case, through alteration of plant morphology to a root architecture that is better adapted to withstand water deprivation, and, in the second case, through activation of photosynthetic processes and source–sink relations.

Recently, we prepared several barley transgenic lines overexpressing a cytokinin catabolic enzyme–cytokinin dehydrogenase 1 (CKX) from Arabidopsis targeted to various subcellular compartments. Transgenic barley exhibited greater tolerance to or avoidance of drought stress that most probably was due to higher lignification and changes in root morphology [30]. While focusing primarily on post-stress revitalization, the in-depth transcriptomic analysis of our transgenic barley lines aimed to clarify and describe in detail all processes that enable CK-deficient barley plants to cope better with drought.

Material and methods

Plant material and cultivation

Transgenic and WT plants of the spring barley cultivar Golden Promise were grown in an environmental chamber with a photoperiod of 15°C/16 hours light and 12°C/8 hours darkness. The light source was a combination of mercury tungsten lamps and sodium lamps providing an intensity of 300 μmol m^{-2} s^{-1}. Plants were cultivated in a 2:1 mixture of soil and perlite (Perlit Ltd., Czech Republic). Soil composition was 1:1 professional substrate (peat type) for growing plants (Rašelina Soběslav, Czech Republic) and a muck-type arable soil from the Olomouc Region (Czech Republic).

Application of drought stress

For transcriptomic analysis of the root system exposed to drought stress, plants were grown hydroponically in a modified Hoagland solution [31] within an environmental chamber under the same

conditions as given above. The experiment was performed in the following arrangement: 2/3 of each vessel was filled with two transgenic genotypes and 1/3 with WT plants. In total, 27 plants from each genotype were cultivated together in three vessels. Three plants were pooled per biological replicate. Drought stress was induced on plants 4 weeks old by pouring the solution out of the growth vessel after the plants had been temporarily removed. Plants were then returned to the vessel, where they were further kept for another 24 hours, after which the solution was poured back into the vessel. The entire root system was collected after 24 hours of stress and after 14 days of revitalization. The youngest fully expanded leaves were collected for analyzing chlorophyll content every 2nd or 3rd day.

In order to perform transcriptome analysis of the upper part, barley plants were cultivated in shallow trays (30 cm × 20 cm with a depth of 5 cm) filled with soil and watered on a daily basis. Drought stress was applied to 4-week-old plants by keeping them without watering for 4 days, after which regularly watering on a daily basis was resumed. Photosynthetic parameters and relative water content [32] were determined on the youngest fully expanded leaves. Samples were collected 12 hours after the last watering, the 4th day of the stress application, 12 hours after re-watering, and after 14 days of revitalization.

Real-time PCR analysis

Isolation of total RNA was performed using an RNAqueous Kit (Life Technologies, USA). Isolated RNA was then treated using a TURBO DNA-free Kit (Life Technologies) and purified using magnetic beads (Agencourt RNA-CLEAN XP, Beckman Coulter, USA). cDNA was obtained using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Lithuania). Real-time PCR was set up with cDNA as template in total volume of 10 μL containing POWER SYBR Green PCR Master Mix (Life Technologies) and 300 nM of each primer. Reactions were run in the StepOnePlus™ Real-Time PCR System using the default program (Applied Biosystems, USA). Primers were designed using Primer Express 3.0 software (Supplemental Table S1).

The number of transcripts per ng of isolated total RNA was detected based on calibration curves made with genes cloned into the transformation vector. The relative quantification of most abundant endogenous genes involved in CK metabolism was made using the ΔΔCt method [33] with barley cyclophilin, actin, and elongation factor 1 (AK354091.1, AK248432.1, AK361008.1) analyzed as reference genes and then evaluated statistically using DataAssist v3.0 Software (Life Technologies). Each measurement consisted of four biological and two technical replicates.

RNA-seq analysis

Working with 2.5 μg of total RNA from each sample, extracted as described above, Illumina® TruSeq® Stranded mRNA Sample Preparation Kit (Illumina, USA) was used for cDNA library preparation. Library concentration was assessed using a Kapa Library Quantification Kit (Kapa Biosystems, USA) and all libraries were pooled to a final 8 pM concentration for cluster generation and sequencing. The clusters were generated using an Illumina® TruSeq® SR Cluster Kit v3 cBot HS and sequenced on a HiSeq SR Flow Cell v3 with a HiSeq 2500 Sequencing System. Two independent libraries were prepared for each genotype at each time point from two biological replicates (3 pooled plants in each).

The reads generated by sequencing were mapped to the reference genome of *Hordeum vulgare* v.25 (Ensembl, UK) using the TopHat2 v.2.0.12 splice-read mapper [34] with default parameters. The reads mapped to the transcripts annotated in the reference genome were quantified by using HTSeq v.0.6.0 [35] with respect to the stranded library. The tests for differential gene expression were performed using the DESeq2 package [36] implemented in R (R Development Core Team, 2008). The technical replicates were first analyzed as two independent experiments, which yielded similar results (see Figure 4 in Ref [37]). Thereafter, technical replicates were merged into one technical replicate to obtain higher coverage of the reference transcriptome. Gene ontology (GO) annotation of the reference genome was improved using the Blast2GO v.3.0 program [38], nt database (b2g_Jan15), the ncbi-blast+ v.2.2.28 program [39], as well as the UniProKB (http://www.uniprot.org/, 02.2015) and PGSB (http://pgsb.helmholtz-muenchen.de/plant/, 31.07.2014) databases (see Figure 1 in Ref [37]). This additional GO annotation helped to increase the number of GO annotated genes to 17,885 (from a total of 26,074 predicted genes in barley).

Quantification of lignin and its precursor

Lignin quantification was carried out in protein-free cell wall fractions using the acetyl bromide method [40]. Cinnamic and benzoic acids were determined by an LC–MS method described previously [41]. Monolignols and aromatic amino acids were analyzed with a UHPLC-QTOF-MS system (Synapt G2-Si, Waters, UK) operating in positive ion mode. Coumaryl, coniferyl and sinapyl alcohols were quantified by detecting product ions of the corresponding $[M-H_2O^+H]^+$ ions (i.e., $133 > 105.08$, $163 > 131.06$, and $193 > 105.08$, respectively) while using $^{13}C_6$-isovanillic acid ($157 > 114.05$) as an internal standard. The relative levels of aromatic amino acids were estimated from peak area acquired in resolution MS mode. All compounds were identified and quantified based on authentic standards.

Measurement of chlorophyll content

Chlorophyll content was determined using a chlorophyll content meter (CCM-300, Opti-Sciences, Hudson, NH, USA) in plants 7–42 days old that were grown hydroponically under drought and control conditions. Measurement for each line consisted of at least 15 biological replicates and two technical replicates. Each transgenic genotype was grown together with the same number of WT barley plants in the same plastic vessel.

Chlorophyll fluorescence imaging

Chlorophyll fluorescence was monitored on the abaxial side of the youngest fully developed attached leaves using an imaging system (FluorCam 700 MF, Photon Systems Instruments, Czech Republic). All measurements were performed on at least four replicates. To measure fluorescence signal, short (microseconds in length) measuring flashes of red light placed 20 ms apart were applied and the signals detected during the measuring flash and just prior to the measuring flash were subtracted. The overall integral light intensity of the measuring flashes was low enough not to close the photosystem II reaction centers but still able to measure fluorescence. The minimum chlorophyll fluorescence yield (F_0) was determined after 40 min of dark adaptation by application of

the measuring flashes only. A saturating pulse of 1.6 s (white light, 2500 μmol m^{-2} s^{-1}) was applied to determine the maximum chlorophyll fluorescence yield in a dark-adapted state (F_M). The leaves were then exposed to red actinic light (200 μmol m^{-2} s^{-1}) for 8 min. After 5 s, the actinic light was accompanied by a series of saturating pulses placed 30 s apart to estimate the maximum fluorescence yield during light adaptation (F'_M). The non-photochemical quenching of chlorophyll fluorescence was calculated as $(F_M-F'_M)/F'_M$ and the coefficient of photochemical quenching (q_P) as $(F'_M-F_t)/(F'_M-F'_0)$, where F_t is the fluorescence yield measured just prior to application of the saturating pulse and F'_0 is the minimal fluorescence for a light-adapted state calculated as $F_0/((F_V/F_M)+(F_0/F'_M))$ [42].

Gas exchange parameters

Gas exchange parameters were measured on the youngest fully developed, attached leaves using an open gas-exchange system (Li-6400, LI-COR Biosciences Inc., Lincoln, NE, USA). Six to eight plants of each transgenic line were measured for each treatment (control, stress, and re-watering). The measurement was started after 3 min of adaptation to chamber conditions (380 μmol CO_2 mol^{-1}, 70% relative humidity, 16°C). The rate of CO_2 assimilation (A, mmol (CO_2) m^{-2} s^{-1}), stomatal conductance (g_s, mol (H_2O) m^{-2} s^{-1}), and transpiration rate (mol (H_2O) m^{-2} s^{-1}) were measured every 30 s over 5 min. Intrinsic water-use efficiency (mmol (CO_2) mol^{-1} (H_2O)) was calculated as the ratio of averaged values of A and g_s.

Water potential

The water potential was estimated on four leaves of each treatment and for each line using a Model 600 pressure chamber instrument from PMS Instrument Company (Albany, OR, USA).

Statistical analysis

Two-sample *t*-tests and ANOVA (Tukey and Bonferroni tests) at significance level 0.05 were performed using OriginPro 8.5.1 (OriginLab Corporation, Northampton, MA, USA). Some of the *t*-tests were carried out with STATISTICA 12 (StatSoft CR, Czech Republic).

Results and discussion

Two genotypes of *AtCKX1*-overexpressing transgenic barley, which were not affected in their yield parameters, were selected for detailed transcriptomic analysis: one targeting expressed recombinant CKX protein to vacuoles (*vAtCKX1*) and the other with predicted cytosolic localization (*cAtCKX1*). The genotype expressing a secreted CKX to apoplast (*aAtCKX1*) was negatively affected in its yield parameters and was used only for the analysis of photosynthetic parameters. To study differences in transcriptomes, 28 single-read sequencing libraries were prepared and sequenced on an Illumina Hi-Seq 2500 system. The basic features of all sequenced libraries are summarized in Supplemental Table S2.

Photosynthetic parameters of transgenic plants under optimal conditions and drought stress

Heterologous expression of *AtCKX1* negatively affected chlorophyll content, which was estimated in the developmental stage during which the highest expression of the transgene was detected [30]. In comparison to WT leaves of plants cultivated hydroponically, 25%, 11%, and 6% decreases were measured in 4- to 5-week-old leaves of *vAtCKX1*, *aAtCKX1*, and *cAtCKX1* plants, respectively (Fig. 1a). All transgenic genotypes maintained chlorophyll content around WT levels when exposed to two periods of drought stress and re-watering, and this was in contrast to their reduced levels observed under optimal conditions (Fig. 1b).

To examine changes in the photosynthetic apparatus, the chlorophyll fluorescence induction and gas exchange of leaves were examined in the plants cultivated in soil. Drought stress, detected as bending of the leaves, was accompanied by decreases in g_s and, consequently, decreases in transpiration rate and CO_2 assimilation (Table 1). Lower accessibility of CO_2 due to closed stomata led to lower usage of light excitations for photochemical reactions (i.e., a decrease in photochemical quenching and an induced increase in the regulated thermal dissipation of absorbed light) and a rise in the non-photochemical quenching of chlorophyll fluorescence (Table 1). Increases in non-photochemical quenching of chlorophyll fluorescence of light-adapted leaves is a known consequence of drought stress [43,44]. Such a response reflects the usage of absorbed excitation energy to regulate heat dissipation and serves as a protective mechanism against damage to photosystem II. As a consequence, drought stress induces a decrease in photochemical quenching [45] and reflects fewer photosystem II reaction centers being open for photosynthetic electron transport. Interestingly, both of these changes caused by water deficiency were least marked in the *cAtCKX1* and *aAtCKX1* genotypes despite the fact that they had lower g_s (Table 1).

The most notable changes were observed after 24 hours of re-watering: when plants had once again been watered, all measured parameters had a tendency to return to their initial values and the return of photochemical activities in all three transgenic lines was more noticeable in comparison to those for the WT plants (Table 1).

Gas exchange parameters did not differ between genotypes prior to stress treatment. Drought stress did, however, induce a more significant decrease in g_s in the transgenic *cAtCKX1* and *aAtCKX1* genotypes compared to WT. The strong decrease in g_s protects plants from unendurable loss of water through transpiration; on the other hand, it results in lower accessibility of CO_2 for photosynthesis. It is noteworthy that although the *cAtCKX1* plants still had significantly reduced g_s after 24 hours of re-watering, their rate of CO_2 assimilation had by that time completely recovered to its initial value. Our results indicate that *cAtCKX1* barley was the most effective in using water for photosynthesis (Table 1). While the water potential of WT plants after 24 hours of re-watering remained almost unchanged in comparison to stressed plants, all transgenic genotypes had significant increases in this value (Table 1). Transgenic plants with altered root morphology and stronger lignification therefore have greater ability to redistribute the necessary amount of water to the aerial parts during recovery from drought stress.

Effect of cytokinin deficiency on the aerial part of vAtCKX1 plants under optimal conditions

The mild expression of *AtCKX1* under the control of β-glycosidase promoter had a positive effect on root system development

(a)

Chlorophyll content in mg m^{-2}

Plant age in days	*vAtCKX1*	WT	*cAtCKX1*	WT	*aAtCKX1*	WT
7	460 ± 98	436 ± 57	483 ± 70	485 ± 64	450 ± 34	485 ± 64
9	475 ± 62	454 ± 77	N.D. ± N.D.	518 ± 57	532 ± 37	518 ± 57
11	415 ± 56	425 ± 56	N.D. ± N.D.	496 ± 55	496 ± 51	496 ± 55
14	481 ± 27	492 ± 28	643 ± 59	597 ± 66	492 ± 47	555 ± 43
16	560 ± 24	551 ± 26	529 ± 66	553 ± 36	476 ± 74	535 ± 33
18	627 ± 39	618 ± 38	529 ± 66	553 ± 36	462 ± 21	459 ± 38
21	687 ± 29	651 ± 61	N.D. ± N.D.	N.D. ± N.D.	597 ± 49	592 ± 45
23	682 ± 39	680 ± 50	567 ± 28	576 ± 31	505 ± 26	534 ± 45
25	544 ± 58	581 ± 44	666 ± 68	664 ± 38	603 ± 42	589 ± 39
28	461 ± 83	555 ± 46	689 ± 50	712 ± 54	608 ± 34	628 ± 34
30	451 ± 47	599 ± 62	675 ± 41	716 ± 35	547 ± 37	582 ± 44
35	389 ± 55	528 ± 61	783 ± 32	791 ± 33	476 ± 62	532 ± 43
39	502 ± 63	577 ± 71	773 ± 65	721 ± 37	550 ± 52	591 ± 68
42	536 ± 68	552 ± 74	736 ± 59	691 ± 61	575 ± 59	581 ± 71

(b)

Plant age in days	*vAtCKX1*	WT	*cAtCKX1*	WT	*aAtCKX1*	WT
	2 hours long drought		2 hours long drought		2 hours long drought	
7	404 ± 96	395 ± 80	N.D. ± N.D.	N.D. ± N.D.	467 ± 30	435 ± 58
11	450 ± 31	430 ± 82	N.D. ± N.D.	N.D. ± N.D.	517 ± 44	483 ± 64
14	437 ± 33	442 ± 30	648 ± 67	550 ± 47	504 ± 40	472 ± 34
16	540 ± 46	562 ± 40	493 ± 81	494 ± 60	473 ± 53	422 ± 66
18	619 ± 46	587 ± 71	494 ± 81	494 ± 60	425 ± 62	413 ± 48
21	646 ± 37	664 ± 48	N.D. ± N.D.	N.D. ± N.D.	489 ± 71	508 ± 62
23	669 ± 38	687 ± 34	569 ± 69	517 ± 41	459 ± 28	453 ± 40
25	698 ± 34	696 ± 37	581 ± 47	572 ± 31	N.D. ± N.D.	N.D. ± N.D.
27	766 ± 38	713 ± 38	686 ± 49	648 ± 36	500 ± 39	488 ± 41
28	24 hours long drought		24 hours long drought		24 hours long drought	
29	Revitalization		Revitalization		Revitalization	
30	N.D. ± N.D.	N.D. ± N.D.	N.D. ± N.D.	N.D. ± N.D.	582 ± 60	680 ± 42
35	754 ± 41	722 ± 44	726 ± 78	741 ± 57	615 ± 48	663 ± 60
39	786 ± 61	751 ± 74	672 ± 57	660 ± 64	663 ± 64	675 ± 53
42	756 ± 74	801 ± 68	647 ± 90	686 ± 62	674 ± 47	721 ± 48

FIGURE 1

Chlorophyll content in mg m^{-2} for WT, *vAtCKX1*, *cAtCKX1*, and *aAtCKX1* barley leaves. Plants were cultivated hydroponically (a) under optimal conditions (highlighted results indicate significant decreases in transgenic plants at chlorophyll level), and (b) under conditions of mild stress applied to 7-day-old seedlings and subsequent severe stress applied to 4-week-old plants with subsequent re-watering.

whereas the aerial part was not substantially affected [30]. The height of *vAtCKX1* plants was slightly reduced (Fig. 2a,d), while *cAtCKX1* plants exhibited no visible changes in their aerial part during the first 4 weeks of development. Differential expression examination revealed that approximately 400 genes were significantly affected in the 6-week-old aerial part in contrast to more than 2400 genes affected in the roots of hydroponically cultivated *vAtCKX1* plants [30]. In order to understand those mechanisms only regulated by the altered hormonal status during leaf development, we performed an in-depth transcriptomic analysis of *vAtCKX1* plants of the same age but cultivated in the soil. In contrast to hydroponically cultivated plants, approximately four times more genes were found to be affected by altered CK content. Of the total 26,067 annotated genes, 988 and 609 genes were significantly down- or up-regulated, respectively (adjusted *P*-value ≤ 0.01; Supplemental Table S3). GO terms at level 6 of the most significantly affected genes from both sequencing experiments were compared and those 15 most affected and which overlap in the two environments are summarized in Table 2.

The four most negatively affected processes in leaves of *vAtCKX1* plants were linked to photosynthesis (Table 2). The fluorescence photosynthetic parameters, suggesting a decrease in energy transfer to the photosystem II core complexes (Table 1), together with lower chlorophyll content in non-stressed leaves (Fig. 1a), indicated that the photosynthetic apparatus and photosynthesis were slightly affected in transgenic plants. On the transcriptomic level, the effect was much more pronounced in those plants cultivated in the shallow soil (Table 2).

TABLE 1

Transgenic and wild type (WT) barley plants grown in shallow soil tested for ability to recover after 4 days of drought stress. Plants were stressed by drought in the 4th week of growth. Physiological parameters were determined directly prior to stress, on the 3rd day of the stress period, and 24 hours after the stress period. Quenching characteristics of chlorophyll fluorescence determined after drought stress and during revitalization were calculated as percentages of their values prior to stress

	WT	*vAtCKX1*	*cAtCKX1*	*aAtCK1*
Prior to stress				
CO_2 assimilation (mmol (CO_2) m^{-2} s^{-1})	2.00 ± 0.70	2.60 ± 0.70	1.60 ± 0.90	2.10 ± 0.70
Stomatal conductance (mol (H_2O) m^{-2} s^{-1})	0.06 ± 0.03	0.08 ± 0.02	0.05 ± 0.02	0.04 ± 0.01
Transpiration rate (mol (H_2O) m^{-2} s^{-1})	0.30 ± 0.13	0.38 ± 0.09	0.24 ± 0.10	0.20 ± 0.05
Non-photochemical quenching	0.72 ± 0.12	0.85 ± 0.10	0.99 ± 0.08*	0.87 ± 0.13
Photochemical quenching	0.41 ± 0.06	0.38 ± 0.04	0.32 ± 0.03*	0.36 ± 0.05
Drought stress				
CO_2 assimilation (mmol (CO_2) m^{-2} s^{-1})	1.30 ± 0.94	1.19 ± 1.13	−1.33 ± 0.77*	−1.77 ± 0.75*
Stomatal conductance (mol (H_2O) m^{-2} s^{-1})	0.036 ± 0.026	0.031 ± 0.019	0.005 ± 0.003*	0.005 ± 0.003*
Transpiration rate (mol (H_2O) m^{-2} s^{-1})	0.18 ± 0.13	0.16 ± 0.09	0.03 ± 0.02*	0.03 ± 0.01*
Non-photochemical quenching (%)	137 ± 23	171 ± 21	121 ± 16	102 ± 24
Photochemical quenching (%)	71 ± 12	57 ± 12	82 ± 15	76 ± 11
Water potential (MPa)	−1.11 ± 0.09	−1.25 ± 0.15	−1.69 ± 0.11*	−1.73 ± 0.04*
Re-watering				
CO_2 assimilation (mmol (CO_2) m^{-2} s^{-1})	2.21 ± 0.47	1.43 ± 0.53*	1.31 ± 0.87*	1.03 ± 0.99*
Stomatal conductance (mol (H_2O) m^{-2} s^{-1})	0.09 ± 0.03	0.07 ± 0.02	0.04 ± 0.01*	0.05 ± 0.02*
Transpiration rate (mol (H_2O) m^{-2} s^{-1})	0.43 ± 0.11	0.36 ± 0.08	0.19 ± 0.07*	0.23 ± 0.09*
Non-photochemical quenching (%)	126 ± 18	101 ± 20	95 ± 16*	93 ± 5*
Photochemical quenching (%)	86 ± 9	104 ± 12*	108 ± 11*	103 ± 6*
Water potential (MPa)	−1.04 ± 0.21	−0.59 ± 0.02*	−0.59 ± 0.04*	−0.46 ± 0.07*
Relative water content (%)	78.38 ± 7.64	94.97 ± 1.02*	97.82 ± 2.06*	91.84 ± 5.05*

* Significant difference between WT and transgenic tissue according to unpaired Student's *t*-test at $P \leq 0.05$.

Three of four putative genes coding for prephenate/arogenate dehydratase, an enzyme participating in the final steps of the aromatic amino acid pathway that produces tyrosine and phenylalanine [46], were up-regulated in the leaves of *vAtCKX1* plants. Phenylalanine is the primary substrate for the phenylpropanoid pathway that gives rise to lignin, flavonoids, and anthocyanins. Accordingly, the most up-regulated GO terms were GO:0009963 'Positive regulation of flavonoid biosynthetic process' and GO:0009718 'Anthocyanin-containing compound biosynthetic process.' Enforced production of phenylalanine thus might serve as a pool for the larger amount of flavonoids and anthocyanins in transgenic leaves, where these compounds participate in protection mechanisms against various stresses. However, four of eight genes encoding phenylalanine ammonia lyases, whose activity is considered a key switch between the phenylpropanoid pathway and primary aromatic amino acid metabolism [47], were significantly down-regulated. Nevertheless, all four genes, *MLOC_4684*, *MLOC_62322*, *MLOC_79728* and *MLOC_477*, were found to be up-regulated (by 3.6-, 3.0-, 2.7-, and 1.9-fold, respectively) in the roots of *vAtCKX1* plants [30]. Hence, and inasmuch as the aromatic amino acid metabolism was not affected in *vAtCKX1* transgenic roots, surplus phenylalanine might be translocated from leaves to roots, where it can supply enhanced lignin deposition.

The third most enriched process in *vAtCKX1* leaves was linked to the activity of lipoxygenases, which enzymes participate in the release of volatile compounds, including jasmonates (JAs), from intracellular lipids [48]. These compounds are usually released during plant defense against various pathogens. As the result is based on two independent experiments in which two biological replicates were sequenced and compared to the respective WT plants, it is not very likely that the observed lipoxygenase activation was merely a response to an undetected biotic stressor. In addition to plant defenses, JAs participate in several developmental processes such as trichome formation and leaf senescence [49]. Interestingly, JA-dependent formation of trichomes is accompanied by production of such secondary compounds as flavonoids, anthocyanins, and terpenoids [49,50]. Although there is not enough evidence to indicate cross-talk between JAs and CKs, it is predicted that their interaction might be antagonistic inasmuch as JAs strongly inhibit the CK-induced callus growth [51]. Nevertheless, the interplay of both phytohormone groups probably depends not only on the CK:JA ratio but also on other hormones [52]. In total, 12 of 55 and 8 of 39 genes categorized as GO:0009753 'Response to jasmonic acid' and GO:0010026 'Trichome differentiation,' respectively, were found to be significantly upregulated in the roots of two independent *vAtCKX1* lines [30]. Hence, predicted JA production in the upper part of transgenic plants might affect mainly roots and their fine architecture. Volatile methyl JA can be translocated as a rapid chemical signal from shoot to root and function there as a gene expression inducer [53]. Nevertheless, enforced JA production directly in roots is also feasible inasmuch as GO:0016165 'Linoleate 13S-lipoxygenase activity' is among enriched terms in transgenic roots during revitalization (see below).

Whole transcriptome response of vAtCKX1 *plants during revitalization after drought stress*

In addition to optimal conditions, three other kinds of sequencing libraries were generated from the upper part of *vAtCKX1* and WT plants cultivated in the shallow soil: the first from plants exposed

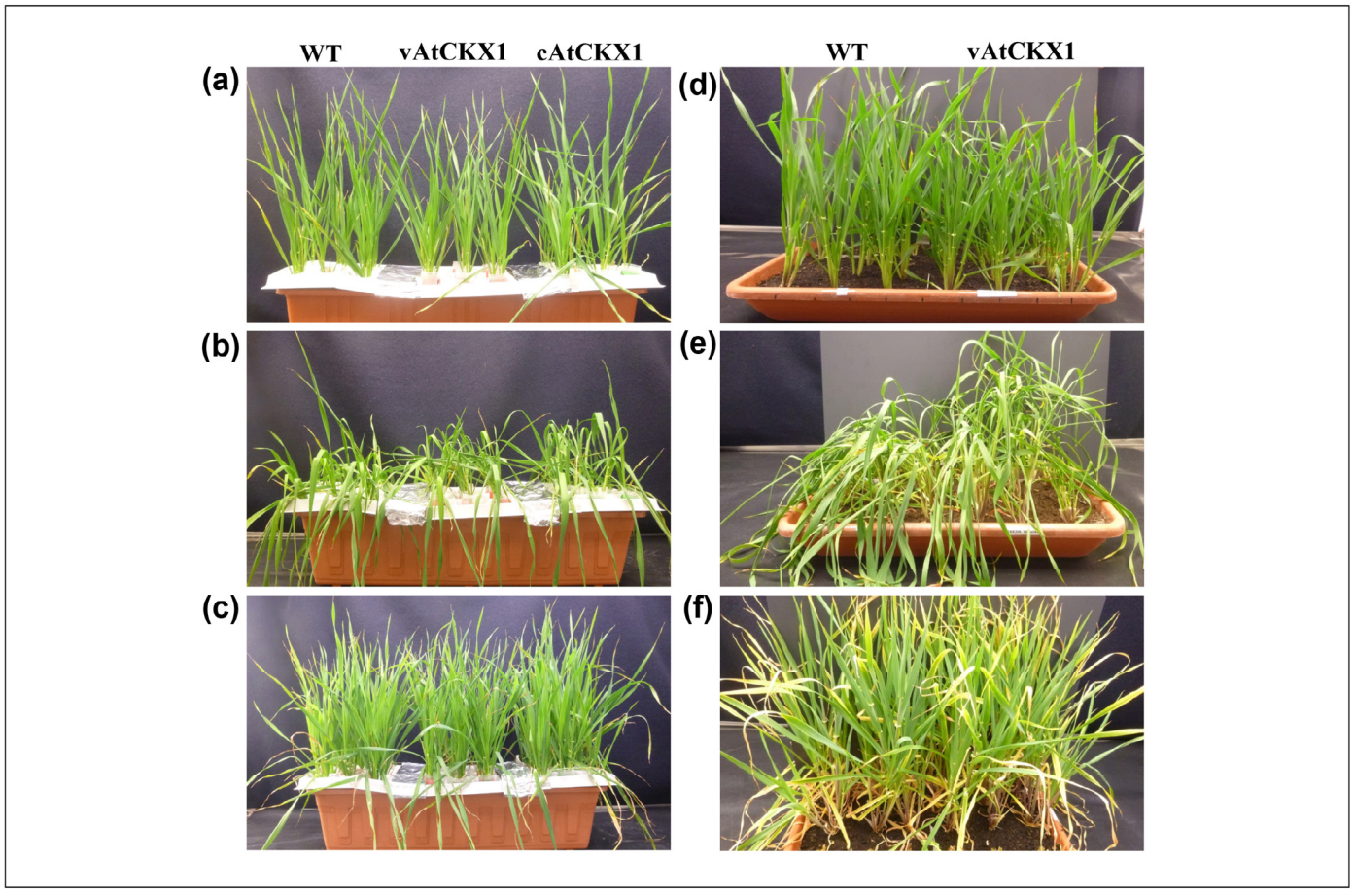


FIGURE 2

Photographs of transgenic and wild-type (WT) barley plants cultivated in hydroponic system (left) or shallow soil (right). (a,d) optimally watered 4-week-old plants, (b,e) plants suffering from severe drought stress, (c,f) regenerated plants 2 weeks after the application of drought stress.

to 4-day drought (Fig. 2e), the second from material 12 hours after re-watering, and the third from leaves having undergone 14-day revitalization (Fig. 2f). Unsurprisingly, only five genes were significantly altered (adjusted *P*-value ≤ 0.05) between stressed transgenic and WT leaves, thereby indicating that transcriptomes of both genotypes were strongly affected by the water deficit (Supplemental Table S3, see Supplemental Table S2 in Ref [37]). Twelve hours after re-watering, 10 and 9 genes were significantly up- and down-regulated, respectively, between *vAtCKX1* and WT leaves (Supplemental Table S3). Additionally, 5 of these 19 genes were not altered between the libraries made from optimally cultivated versus stressed leaves of either transgenic or WT leaves. Two putative *F-box-like proteins* (*MLOC_75620*, *MLOC_43997*), which have been shown to play an essential role in multiple phytohormone-signaling pathways [54], and one *receptor-like protein kinase* (*MLOC_17138*) were detected among them. The protein MLOC_17138 contains in addition to the kinase domain, a pfam01657 domain associated with a role in salt stress response and antifungal activity.

Transgenic plants overexpressing *AtCKX1* exhibit better growth parameters (e.g., biomass production and yield) when encountering drought stress [30]. To understand processes attributed to the beneficial growth of *vAtCKX1* plants, a comparative transcriptomic analysis was carried out examining transgenic versus WT leaves 2 weeks after revitalization from stress. Of the total 26,067 barley genes, 301 and 31 genes were significantly up- and down-regulated, respectively, in revitalized *vAtCKX1* leaves in contrast to WT (Supplemental Table S3). The enriched GO terms in up-regulated genes of *vAtCKX1* are summarized in Table 3A.

Products of many genes up-regulated by *vAtCKX1* participate as structural proteins or enzymes of the photosynthetic apparatus. Accordingly, the measured photosynthetic parameters of transgenic plants were better in the early stage of revitalization (Table 1), and chlorophyll content reached WT levels 2 weeks after re-watering (Fig. 1b), as compared to plants of the same age grown for their entire life span under optimal conditions. Interestingly, the most activated genes comprised those encoded by the barley chloroplast genome (indicated by the prefix EPlHVUG). In total, 14 of 112 translatable chloroplast genes were 2- to 3-fold up-regulated with high significance (adjusted *P*-value ≤ 0.05). Real-time PCR was performed on six selected genes to confirm the accuracy of the transcriptomic data (Supplemental Fig. S1). Chloroplasts are a known target of CK action. Indeed, exogenously applied CK is able directly to activate the expression of several chloroplast-encoded genes in detached barley leaves which accumulated also the stress hormone ABA [55]. Because it is not yet clear whether CK acts directly on chloroplast transcription, we can only speculate that the increase in chloroplast transcripts observed

TABLE 2

The most affected gene ontology (GO) terms in the upper part of *vAtCKX1* plants cultivated hydroponically or in soil compared to wild-type plants. Percentages are shown of differentially expressed genes (adjusted *P*-value $\leq$ 0.05) at GO level 6 and higher from total number of genes with the same GO number. MF, molecular function; BP, biological process. Genes affected in both culture conditions are in bold. Genes in several GO terms are not listed because the term parsed to several other child terms

GO number	Category	GO term	Total #	% of affected genes		Accession of affected genes in format *MLOC_XXXXX*
				Hydrop.	Soil	
Up-regulated						
GO:0004664	MF	Prephenate dehydratase activity	4	50.0	25.0	*23316, 65725, 56414*
GO:0008131	MF	Primary amine oxidase activity	6	16.7	50.0	***70980***, *4986, 17390*
GO:0016165	MF	Linoleate 13S-lipoxygenase activity	16	31.3	18.8	***64972***, *54031*, ***55029***, *71275, 51884, 69572*
GO:0005544	MF	Calcium-dependent phospholipid binding	10	20.0	20.0	*54932, 40592, 55134, 15770*
GO:0004834	MF	Tryptophan synthase activity	5	20.0	20.0	*59863, 61188*
GO:0009963	BP	Positive regulation of flavonoid biosynthetic process	9	11.1	22.2	*81070, 54366, 19988*
GO:0004034	MF	Aldose 1-epimerase activity	7	14.3	14.3	***5638***
GO:0005337	MF	Nucleoside transmembrane transporter activity	7	14.3	14.3	***55464***
GO:0009718	BP	Anthocyanin-containing compound biosynthetic process	11	18.2	9.1	*61512, 65788, 64248*
GO:0009407	BP	Toxin catabolic process	27	7.4	18.5	***17760***, *73593*, ***68101***, *57709, 72489*
GO:0047262	MF	Polygalacturonate 4-α-galacturonosyltransferase activity	8	12.5	12.5	*11661, 57229*
GO:0031418	MF	L-Ascorbic acid binding	13	15.4	7.7	***77814***, *64248*
GO:0004806	MF	Triglyceride lipase activity	41	9.8	12.2	***17298, 18031***, ***80878***, ***80586***, *58940*
GO:0006569	BP	Tryptophan catabolic process	14	14.3	7.1	*12847, 57323, 69262*
GO:0015996	BP	Chlorophyll catabolic process	33	3.3	15.2	*80455, 34851, 55009, 21175,* ***64277***
Down-regulated						
GO:0008937	MF	Ferredoxin-NAD(P) reductase activity	4	25.0	50.0	*7761, 53537, 40355*
GO:0010027	BP	Thylakoid membrane organization	106	2.8	61.3	***58382***; not listed
GO:0051667	BP	Establishment of plastid localization	63	4.8	55.6	Not listed
GO:0045548	MF	Phenylalanine ammonia lyase activity	7	28.6	28.6	*79728, 62322, 477, 4684*
GO:0009658	BP	Chloroplast organization	137	2.2	54.0	Not listed
GO:0019682	BP	Glyceraldehyde-3-phosphate metabolic process	202	1.5	51.5	Not listed
GO:0006720	BP	Isoprenoid metabolic process	237	1.3	46.0	Not listed
GO:0010310	BP	Regulation of hydrogen peroxide metabolic process	15	6.7	26.7	*33774, 1518, 15501, 65632, 1340*
GO:0030855	BP	Epithelial cell differentiation	7	14.3	14.3	*38181, 54366*
GO:0016054	BP	Organic acid catabolic process	153	3.9	14.4	Not listed
GO:0008544	BP	Epidermis development	9	11.1	11.1	*38181, 54366*
GO:0009699	BP	Phenylpropanoid biosynthetic process	22	9.1	9.1	*4684, 477, 57736, 79728*
GO:0070726	BP	Cell wall assembly	11	9.1	9.1	*52864, 67760*
GO:0032870	BP	Cellular response to hormone stimulus	205	8.8	5.7	Not listed
GO:0007166	BP	Cell surface receptor signaling pathway	30	3.3	10.0	*63541, 17680, 44275, 72162*

in revitalized *vAtCKX1* transgenic plants relays an accumulation of CK in leaves upon water stress. Our hypothesis is supported by the strong activation of endogenous *IPT* genes in *vAtCKX1* leaves at several developmental time points as a consequence of CK depletion [30]. Hence, increased local maxima of CKs, produced by IPT activity localized in chloroplasts, might trigger similar machinery as was described in CK-treated detached leaves to activate the chloroplast genome. The analysis of endogenous CK content in chloroplasts under these conditions would provide support for our hypothesis. It is noteworthy that none of the chloroplast-encoded genes were down-regulated in *vAtCKX1* plants cultivated under optimal conditions when as many nucleus-encoded genes participating in photosynthesis were down-regulated compared to those in WT plants (Table 2). Nevertheless, eight chloroplast-encoded genes were significantly up-regulated (Supplemental Fig. S1), indicating that the phenomenon is linked rather to CK imbalance than to activation by drought.

Among other interesting genes significantly up-regulated in revitalized *vAtCKX1* leaves were these coding for four putative aquaporins (*MLOC_56278*, *MLOC_71237*, *MLOC_552*, *MLOC_22808*), which are channel proteins facilitating the transport of water through plasma and intracellular membranes. The increased expression of several genes encoding barley aquaporins had already been observed in plants exposed to salinity stress [56]. Those authors had hypothesized that an increase in water channel activity would facilitate maintenance or recovery of growth during or after the stress period.

Two genes classified under the GO term 'phenylpropanoid catabolic process' encode putative laccases – aromatic compound:oxygen oxidoreductase (Table 3A), which might participate in lignin degradation or its polymerization. Nevertheless, in contrast to root tissue, significantly altered lignin content in the upper part of *vAtCKX1* plants was not determined in comparison to that in WT plants (Fig. 4a).

TABLE 3A

The most enriched GO terms in up-regulated genes (adjusted *P*-value ≤ 0.05) in the aerial part of *vAtCKX1* plants collected 2-weeks after re-watering. Percentages are shown of differentially expressed genes at GO level 6 and higher from total number of genes with the same GO number. MF, molecular function; BP, biological process; CC, cellular component.

GO number	Category	GO term	Total #	% of affected genes	Accession of affected genes in format *MLOC_XXXXX* or *EPlHVUG000000XXXX*
GO:0016984	MF	Ribulose-bisphosphate carboxylase activity	5	40.0	*EPlHVUG00000010074, MLOC_21811*
GO:0009765	BP	Photosynthesis, light harvesting	32	34.4	Not listed
GO:0030076	CC	Light-harvesting complex	8	25.0	*EPlHVUG00000010021, MLOC_57061*
GO:0009718	BP	Anthocyanin-containing compound biosynthetic process	10	20.0	*MLOC_5324, 19814*
GO:0016165	MF	Linoleate 13S-lipoxygenase activity	16	18.8	*MLOC_37378, 51884, 71948*
GO:0045259	CC	Proton-transporting ATP synthase complex	27	14.8	*EPlHVUG00000010007, 10016, 10047, MLOC_26730*
GO:0009767	BP	Photosynthetic electron transport chain	56	14.3	*EPlHVUG00000010010, 10072, 10065, 10026, 10021, MLOC_52515, 22512, 39436*
GO:0046271	BP	Phenylpropanoid catabolic process	20	10.0	*MLOC_15203, 61189*
GO:0052716	MF	Hydroquinone:oxygen oxidoreductase activity	20	10.0	*MLOC_15203, 61189*
GO:0009579	CC	Thylakoid	331	9.9	Not listed
GO:0016597	MF	Amino acid binding	31	9.7	*MLOC_62844, 19879, 80634*
GO:0004499	MF	*N,N*-Dimethylaniline monooxygenase activity	22	9.1	*MLOC_11897, 11896*
GO:0009637	BP	Response to blue light	48	8.3	*MLOC_43394, 22512, 11312, 52515*
GO:0055082	BP	Cellular chemical homeostasis	47	6.4	*MLOC_22808, 65878, 69460*
GO:0034754	BP	Cellular hormone metabolic process	38	5.3	*MLOC_6666, 73942*

Furthermore, genes significantly affected between non-stressed and revitalized leaves were evaluated separately for *vAtCKX1* and WT plants. Those from the transgenic plants that did not overlap with WT plants were further compared with genes differentially regulated between the two genotypes (i.e., the 301 up- and 31 down-regulated genes). Only seven up-regulated genes remained as being not developmentally dependent but genotype dependent. None of the down-regulated genes meet both criteria (Table 3B). Two genes coding for putative enzymes of the flavonoid biosynthesis pathway – chalcone isomerase and isoflavone 2′-hydroxylase – were found to be up-regulated in *vAtCKX1* plants. These two genes combine with two other genes found to be up-regulated in revitalized *vAtCKX1* leaves and participating in the regulation of flavonoid metabolism (*chalcone isomerase*: *MLOC_5324*; *zinc-finger (B-box) protein*: *MLOC_19814*), indicating an enforced production of isoflavonoids and anthocyanins. Recently, an unambiguous positive effect of flavonoid and anthocyanin production in improving tolerance to drought stress has been shown [57]. Due to their antioxidative activity, the over-accumulation of flavonoids mitigates the negative effect of reactive oxygen species released under stress conditions. Peroxiredoxin (MLOC_74367) belongs to a family of cysteine-dependent peroxidases which also participate in detoxification of plant cells by scavenging reactive oxygen species [58]. An orthologue of peroxiredoxin has been found among another 25 over-accumulated proteins in wheat seedlings of a cultivar that is drought-stress tolerant in comparison to a

TABLE 3B

Significantly up-regulated genes in *vAtCKX1* leaves 2 weeks after re-watering (adjusted *P*-value ≤ 0.05) which are not developmentally dependent and also significantly up-regulated between revitalized and non-stressed leaves of *vAtCKX1* genotype but not in wild-type plants (adjusted *P*-value ≤ 0.001); R2W, 2-week revitalization; NS, non-stressed

Gene number	Gene annotation	Mean expression (R2W)	Fold change	
			***vAtCKX1* (R2W) versus WT (R2W)**	***vAtCKX1* (R2W) versus *vAtCKX1* (NS)**
MLOC_8529	Nematode-resistance protein	1471	4.28	2.21
MLOC_14310	GDSL esterase/lipase	1163	2.51	2.62
MLOC_74636	tolB protein (WD40-like Beta Propeller)	185	2.38	3.60
MLOC_30661	Putative isoflavone 2′-hydroxylase	78	2.31	6.66
MLOC_70609	unknown protein located in chloroplast stroma	104	2.30	4.30
MLOC_74367	Peroxiredoxin (Thioredoxin-like fold)	2443	2.23	2.66
MLOC_80571	Chalcone isomerase	598	2.19	2.86

drought-sensitive one [59]. MLOC_14310 belongs to a large family of GDSL-type esterase/lipases with hydrolytic activity toward triacylglycerols. Members of this family are involved in plant development, morphogenesis, secondary metabolite synthesis, and defense responses, and some members are activated by JAs. The closest rice orthologue of MLOC_14310 (LOC_Os01g46080) was found to be activated by desiccation stress in rice leaves [60]. Moreover, pepper GDSL lipase caused higher susceptibility to pathogens but increased tolerance to osmotic stress when overexpressed in Arabidopsis [61].

Taken together, the differential expression study in *vAtCKX1* and WT leaves before and after the stress period reveal several genes whose increased expression initiated by the CK imbalance may lead to better drought tolerance and/or faster growth after rewatering.

Response of vAtCKX1 *and* cAtCKX1 *roots during stress and revitalization*

Due to the impossibility of collecting root tissues from soil without causing mechanical stress, transcriptome of the root system was studied from plants grown hydroponically. Twelve sequencing libraries were generated from *vAtCKX1*, *cAtCKX1*, and WT roots collected at two time points: during the severe drought stress (Fig. 2b) and 2 weeks after revitalization (Fig. 2c). Similarly as in the aerial part, stress induced a strong response at the transcriptome level (see Supplemental Table S1 in Ref [37]). Between transgenic plants and WT plants, only a few genes were deregulated during the stress (Supplemental Table S4). Just seven genes were significantly up-regulated in both *vAtCKX1* and *cAtCKX1* genotypes, including, for example, putative *nicotianamine synthase* (*MLOC_71596*) and *4-coumarate CoA ligase* (*MLOC_18901*) involved in lignification. Fifty-seven genes were significantly down-regulated. The most strongly down-regulated gene in both lines was a putative *F-box-like protein* (*MLOC_75620*; 12.6- and 5.3-fold), which was found also among the most strongly down-regulated genes in the early and late phases of leaf revitalization. The unambiguous and strong depletion of *MLOC_75620* transcripts in all transgenic samples indicates that this F-box protein might play a crucial role in regulating responses in CKX-overexpressing plants via cross-talk with other hormones. F-box proteins represent one of the largest superfamilies in plants, that is, involved in the process of ubiquitination and protein degradation. To date, only a limited number of F-box proteins have been functionally characterized. Most of them are involved in regulating hormone signaling pathways, where they degrade repressors or activators of auxin, GA, ethylene, and JA response [54].

Analysis of differentially expressed genes in revitalized 6-week-old roots (Supplemental Table S4) revealed that the gene encoding one of the cytokinin receptors (*HvHK3*; *MLOC_44452*) was significantly down-regulated in both transgenic genotypes (Fig. 3a,c). The gene was also down-regulated in 6-week-old *vAtCKX1* and *cAtCKX1* roots cultivated under optimal conditions (see Figure 3B in Ref [30]), thus leading to the conclusion that cessation of CK perception via HvHK3 is a developmental consequence rather than a response to stress. The addition of one *CKX* gene to the barley genome led to a hormonal imbalance which plants tend to buffer by regulation of endogenous CKX and IPTs, which are CK biosynthetic enzymes, in a very sensitive way [30]. Plant *IPT* genes are generally very weakly expressed, and the enzyme's activity is regulated by farnesylation [62]. Significant up-regulation of two abundant endogenous CKX enzymes – HvCKX4 and HvCKX5 – was observed in both independent experiments with 6-week-old *AtCKX1*-overexpressing plants, while other HvCKXs were down-regulated or unchanged in comparison to WT plants. Although it is difficult to estimate the final CK homeostasis, one might expect a local minimum in CK content that leads to cessation in *HvHK3* transcription and CK perception through this receptor.

CK receptors belong to a small group of histidine kinases. The Arabidopsis genome encompasses three real CK receptors binding CKs (AHK2, AHK3, and AHK4/CRE1) and three others implicated in CK transduction cascade and osmosensing without the ability directly to bind CKs (AHK1, CKI1, and CKI2) [4]. In contrast to other genes participating in CK signal transduction, phosphotransfer proteins, and response regulators (RR, Fig. 3d), CK receptors show lineage-specific expansion between dicot and monocot species that implies their specific and evolutionarily old function among all green plants [63]. Thus, in the barley genome, HvHK3 is an orthologue of AHK3, while the orthologue of AHK2 is missing and AHK4 has otherwise two duplicated orthologues (Fig. 3a). A similar representation of CK receptors was found in rice [63].

Interestingly, Arabidopsis knock-outs of AHK3 and AHK2 or double knock-out manifest strong drought-tolerance phenotype [4]. This is the first direct evidence demonstrating that CKs might be a negative regulator of the stress signaling pathway. Tran et al. explained the phenomenon by the up-regulation of many stress and ABA responsive genes in *ahk2/ahk3* double knock-out already under non-stress conditions. To check if the response of *AtCKX1*-overexpressing barley was similar, we blasted the barley orthologous genes closest to these significantly up-regulated genes in the Arabidopsis double knock-out (Supplemental Table S5). In total, 23 and 25 orthologues of the 40 genes presented in Tran et al. were also significantly up-regulated in *vAtCKX1* and *cAtCKX1* transgenic barley, respectively. Among them, we found several regulatory genes implicated in responses to stress, such as *MLOC_37104* (an orthologue of *ANAC055*), *NAC* (no apical meristem) transcription factor [64], and *MLOC_71611* (an orthologue of *AtMYC2*). Overexpression of both transcription factors in Arabidopsis led to drought-tolerance and increased sensitivity to ABA [65]. Both genes have been shown to be activated by JAs [66], the overproduction of which is expected in the upper part of *AtCKX1* transgenic plants. While it is interesting that both genes have relatively strong expression in barley and are more abundant in roots than in leaves, their action has been studied mainly in Arabidopsis leaves [64,65]. Hence, drought tolerance mediated via these two transcription factors might be universal for the entire plant body. Recently, rice plants overexpressing *OsNAC9*, the closest orthologue of *MLOC_37104*, under the control of a root-specific promoter have been shown to be more drought tolerant during vegetative development due to their enlarged root diameter and aerenchyma formation [67].

Altered homeostasis of CKs in *AtCKX1*-overexpressing barley, which influences root system architecture, might anticipate changes in auxin levels, transport, and perception. There exist several groups of auxin response genes in plant genomes that react sensitively to auxin imbalance. Auxin early response genes are divided into two categories – the Aux/IAA and SAUR (small auxin

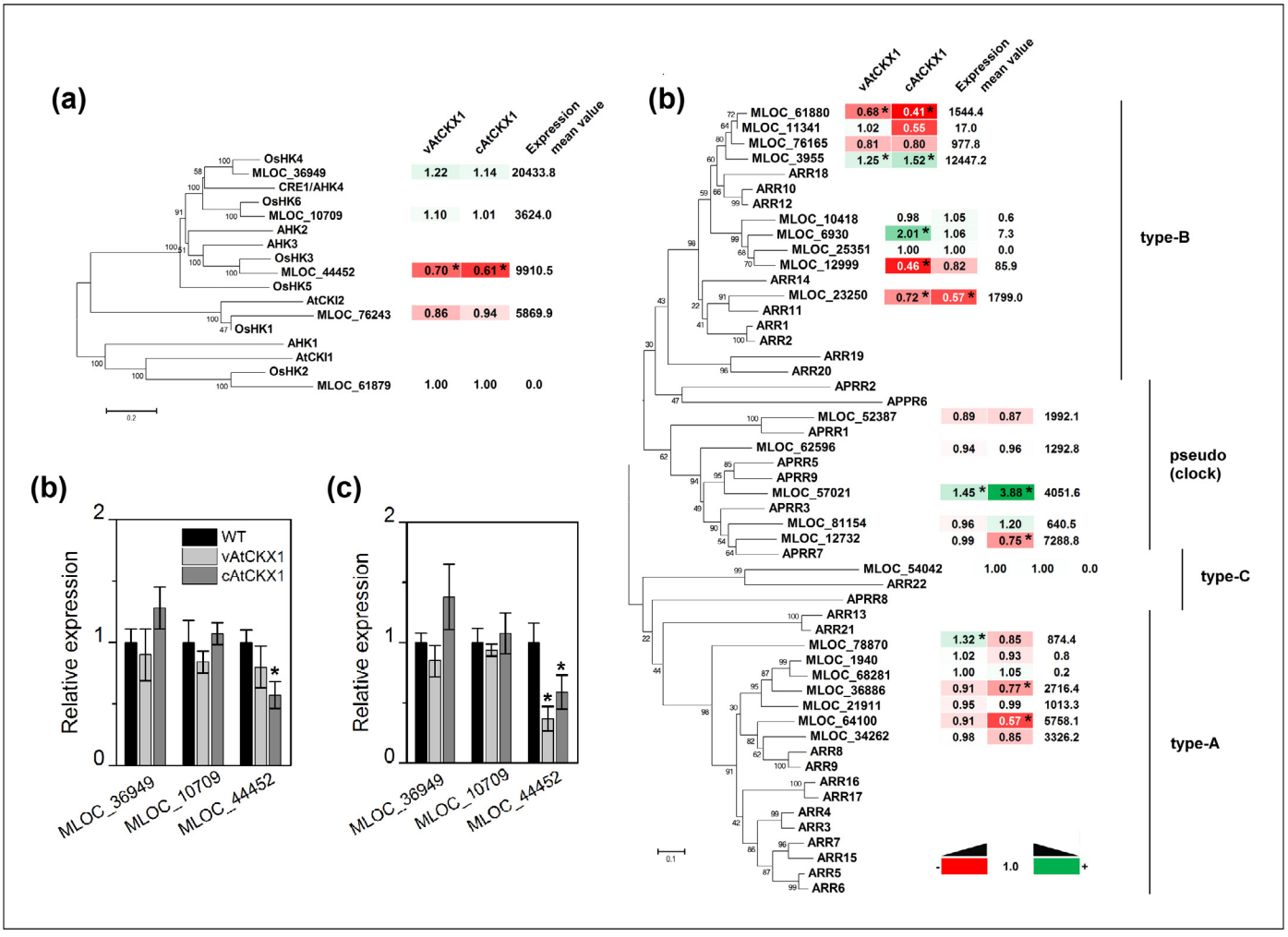


FIGURE 3

Cytokinin perception in *vAtCKX1* and *cAtCKX1* plants. (a) Phylogenetic relationship of barley, rice, and Arabidopsis histidine kinases implicated in CK perception. Expression relative to wild-type (WT) of all homologues found in the annotated barley genome are depicted in up- and down-regulation color code together with their mean expression values. Real-time PCR analysis of three CK receptors in roots cultivated (b) under optimal conditions or (c) 2 weeks after re-watering following the drought stress. (d) Phylogenetic relationship of all CK response regulators found in the barley genome and their Arabidopsis counterparts. Expression relative to WT plants is shown in the color code. Unrooted trees were generated using ClustalW by neighbor-joining method. *Significant difference between WT and transgenic tissue according to unpaired Student's *t*-test at $P \leq 0.05$.

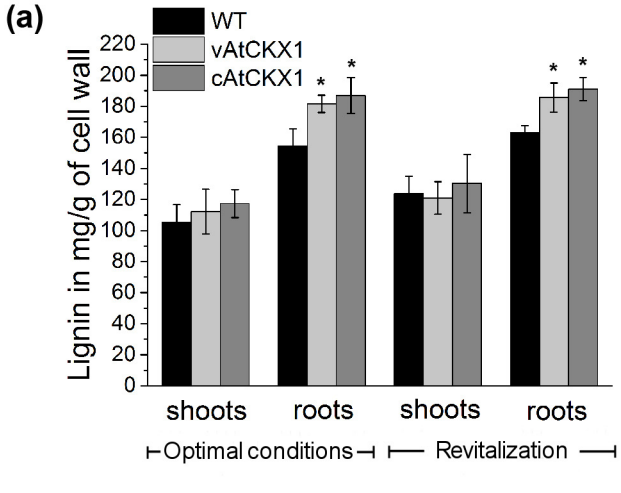
up RNA) – and all regulate plant physiology by modulating the interaction with auxin response elements of other transcription factors, such as a major group of auxin response factors (ARFs). Auxin action is influenced by its polar transport that is mediated by auxin efflux carriers (PINs). Its homeostasis is also regulated by *GH3* (*Gretchen Hagen3*) genes encoding mainly auxin–amino acid synthetases that form from surplus auxin its inactive conjugates [68]. AtCKX1/WT comparative data indicated that transgenic roots contain elevated levels of auxin, inasmuch as 3 of 12 predicted and expressed PIN transporters and 3 of 7 *GH3* genes were significantly up-regulated in *cAtCKX1* roots (Supplemental Table S6). Analysis of *vAtCKX1* roots showed a similar but slightly weaker tendency.

Many of the 48 significantly down-regulated genes in Arabidopsis *ahk2/ahk3* double knock-out with higher stress tolerance are auxin early responsive genes (*SAUR* and *IAA/AUX*). The dwarf phenotype of *ahk2/ahk3* plants was attributed to the observed down-regulation of auxin response [4]. Because *vAtCKX1* and *cAtCKX1* plants were not substantially affected in their aerial part but had positively altered root system morphology, a different way of auxin response might be expected. In contrast to *ARF* and *GH3* genes, several *SAUR* and *IAA/AUX* genes exhibited lower expression in transgenic plants than in WT plants. Nevertheless, no straightforward comparison can be made between the auxin response of Arabidopsis *ahk2/ahk3* mutant and *AtCKX1*-overexpressing barley (Supplemental Tables S5 and S6). Particularly noteworthy was that two type-A *AtRR* genes were found among the down-regulated genes of the *ahk2/ahk3* mutant [4]. Two of seven *HvRR* genes were significantly down-regulated in *cAtCKX1* transgenic barley, thus implicating reduced CK sensing due to decreased CK content and/or silenced HvHK3 receptor (Fig. 3d). Taken together, the comparison of our transcriptomic data with the previous work of Tran et al. [4] suggests that the general mechanism of drought-stress tolerance relates in part to CK insensitivity, which can be acquired by receptor knock-out or CKX overexpression in all species across the plant kingdom.

Up-regulation of genes in the phenylpropanoid biosynthesis pathway leads to higher lignification of AtCKX1 *transgenic roots exposed to drought stress*

Our pilot whole-transcriptome characterization of *vAtCKX1* transgenic roots revealed strong up-regulation of many genes encoding proteins involved in the phenylpropanoid biosynthesis pathway [30]. Lignin content was quantified by the acetyl bromide method in protein-free cell wall samples prepared separately from whole root and leaf mass of hydroponically cultivated plants. Both transgenic genotypes showed systematic increase in total lignin content in roots from the 2nd week after germination (data not shown). Approximately up to 20% higher lignin content was determined in roots of transgenic compared to WT plants cultivated under optimal conditions 6 weeks after germination, while lignin content in leaves was not significantly altered (Fig. 4a).

Analysis of lignin precursors in 4-week-old transgenic plants showed that *cAtCKX1* accumulated significantly greater amounts of cinnamates and monolignols than did WT tissue (Fig. 4b). Four-week-old roots of *vAtCKX1* genotype contained a larger amount of sinapyl alcohol, similarly as 2 weeks later, when grown under optimal conditions. The exposure to drought stress and the following revitalization resulted in more pronounced accumulation



(b)

	4-week-old (optimal conditions)			6-week-old (optimal conditions)			6-week-old (revitalization)		
aromatic amino acids									
	Phe	Tyr	Trp	Phe	Tyr	Trp	Phe	Tyr	Trp
vAtCKX1	**1.15**	**1.13**	**1.38**	**1.02**	**0.95**	**0.82**	**1.35**	**1.38**	**1.25**
cAtCKX1	**1.36***	**1.26***	**1.68***	n.d.	n.d.	n.d.	**1.16**	**1.15**	**1.26**
	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
benzoates									
	4-HBA	Salicylic	Vanillic	4-HBA	Salicylic	Vanillic	4-HBA	Salicylic	Vanillic
vAtCKX1	**0.88**	n.d.	**0.88**	**0.90**	**0.77**	**0.87**	**1.23***	n.d.	**1.21**
cAtCKX1	**1.25***	n.d.	**1.21**	n.d.	n.d.	n.d.	**1.00**	n.d.	**1.10**
[ng/g of FW]	54.5 ± 4.6		234.2 ± 85.5	55.8 ± 4.9	75.4 ± 19.7	78.6 ± 7.2	52.7 ± 7.0		159.7 ± 42.7
cinnamates									
	Coumaric	Ferulic	Caffeic	Coumaric	Ferulic	Caffeic	Coumaric	Ferulic	Caffeic
vAtCKX1	**0.94**	**1.07**	**1.05**	**1.55**	**1.32***	**1.60**	**1.67***	**1.00**	**1.53***
cAtCKX1	**1.59***	**1.53***	**1.91***	n.d.	n.d.	n.d.	**1.11**	**0.92**	**1.63***
[ng/g of FW]	74.1 ± 16.0	51.6 ± 18.6	3.63 ± 0.10	59.6 ± 14.4	41.3 ± 4.2	9.28 ± 2.07	400.3 ± 99.7	61.4 ± 10.7	4.05 ± 0.11
monolignols									
	CoOH	FOH	SiOH	CoOH	FOH	SiOH	CoOH	FOH	SiOH
vAtCKX1	**0.90**	**1.03**	**1.86***	**1.16**	**1.10**	**2.05***	**2.31***	**1.22**	**1.80***
cAtCKX1	**2.54***	**1.54***	**1.64***	n.d.	n.d.	n.d.	**3.08***	**1.49***	**1.78***
[ng/g of FW]	1.82 ± 0.27	0.48 ± 0.15	1.72 ± 0.09	4.10 ± 1.11	0.85 ± 0.20	2.20 ± 0.41	1.81 ± 0.21	0.19 ± 0.05	2.04 ± 0.42

FIGURE 4

Lignin content and intermediates of the phenylpropanoid pathway in *vAtCKX1* and *cAtCKX1* transgenic barley. (a) Total lignin content of hydroponically cultivated plants under optimal conditions (6th week) and during 2-week revitalization (6th week); each value represents the mean of four biological replicates. (b) Quantification of aromatic amino acids, phenylpropanoid intermediates, and benzoic acids in roots of hydroponically cultivated plants (each in three biological replicates) collected during 4th and 6th weeks of growth under optimal conditions and 2nd week of revitalization after drought stress; n.q., not quantified due to lack of internal standard; n.d., not determined; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; 4-HBA, 4-hydroxybenzoic acid; CoOH, coumaryl alcohol; FOH, coniferyl alcohol; SiOH, sinapyl alcohol; *significant difference between WT and transgenic tissue according to unpaired Student's *t*-test at $P \leq 0.05$. (c) Real-time PCR analysis of four selected genes encoding enzymes involved in the phenylpropanoid pathway in transgenic barley roots 2 weeks after re-watering following the drought stress; *MLOC_29114*, *cinnamoyl-CoA reductase*; *MLOC_4684*, *phenylalanine ammonia lyase*; *MLOC_58764*, *4-coumarate:CoA ligase*; *MLOC_69871*, *cinnamate 4-hydroxy*lase.

of all quantified intermediates of the phenylpropanoid pathway (Fig. 4b) as well as in higher lignin content of the root cell wall fraction in both transgenic genotypes compared to WT plants. However, lignification in transgenic roots having passed through severe stress was not greater than in the transgenic roots cultivated under optimal conditions (Fig. 4a). Measurement confirmed that decreased CK sensitivity has a positive impact on root lignification but that the effect is not augmented by exposure to stress, even though more precursors are formed. Thus, the inability to produce more lignin after revitalization from stress may consist in limiting of specific peroxidases participating in monolignol polymerization. The most enriched GO terms in revitalized roots of both transgenic genotypes nicely corresponded to the observed increased amount of phenylpropanoid pathway intermediates and

TABLE 4

The most affected gene ontology (GO) terms in the roots of *vAtCKX1* and *cAtCKX1* plants cultivated hydroponically 2 weeks after re-watering compared to wild-type plants. Percentages are shown of differentially expressed genes (adjusted *P*-value $\leq$ 0.05) at GO level 6 and higher from total number of genes with the same GO number. MF, molecular function; BP, biological process. Genes affected in both genotypes are in bold. Genes in several GO terms are not listed because the term parsed to several other child terms

GO number	Category	GO term	Total #	% of affected genes		Accession of affected genes in format *MLOC_XXXXX*
				vAtCKX1	*cAtCKX1*	
Up-regulated						
GO:0008792	MF	Arginine decarboxylase activity	2	100.0	100.0	***58866***, ***39205***
GO:0047987	MF	Hydroperoxide dehydratase activity	2	100.0	100.0	***8106***, ***21933***
GO:0004664	MF	Prephenate dehydratase activity	4	75.0	100.0	*23316*, ***65725, 56414, 60716***
GO:0009916	MF	Alternative oxidase activity	4	75.0	50.0	***34173***, *82029*, ***53632***
GO:0045548	MF	Phenylalanine ammonia lyase activity	7	71.4	71.4	***79728***, ***62322***, ***4684, 19798***, ***67067***
GO:0003979	MF	UDP-glucose 6-dehydrogenase activity	3	66.7	100.0	***5287, 70967***, *63077*
GO:0005345	MF	Purine nucleobase transmembrane transporter activity	3	66.7	66.7	***66246***, ***32910***
GO:0004470	MF	Malic enzyme activity	6	50.0	83.3	***11548, 75667, 35785***, *64502, 51144*
GO:0009805	BP	Coumarin biosynthetic process	9	55.6	55.6	*66898, 20110, 17364, 19988, 52497*
GO:0009699	BP	Phenylpropanoid biosynthetic process	26	53.9	46.2	Not listed
GO:0016165	MF	Linoleate 13S-lipoxygenase activity	16	50.0	56.3	Not listed
GO:0052542	BP	Defense response by callose deposition	10	50.0	50.0	***57937***, ***51297***, ***42826***, ***17648***, ***59580***
GO:0006986	BP	Response to unfolded protein	25	40.0	40.0	Not listed
GO:0035967	BP	Cellular response to topologically incorrect protein	25	40.0	40.0	Not listed
GO:0042538	BP	Hyperosmotic salinity response	10	40.0	40.0	***69262***, ***52084***, ***42826***, ***2170***
GO:0004348	MF	Glucosylceramidase activity	5	40.0	40.0	***398***, *53162, 13522*
GO:0010942	BP	Positive regulation of cell death	5	40.0	40.0	***12681***, ***75133***
GO:0003885	MF	D-Arabinono-1,4-lactone oxidase activity	9	33.3	44.4	***31769***, ***34835***, ***68610***, *59091*
GO:0003978	MF	UDP-glucose 4-epimerase activity	6	33.3	33.3	***70713***, ***10406***
GO:0015020	MF	Glucuronosyltransferase activity	12	25.0	41.7	***4722***, *54026, 65730*, ***8254***, ***56928***
Down-regulated						
GO:0004650	MF	Polygalacturonase activity	32	21.9	12.5	*6444, 4738, 68357, 67885*, ***51158***, ***53562***, *75889, 13213, 72199*
GO:0001666	BP	Response to hypoxia	13	7.7	23.1	***36714***, *1340, 65221*
GO:0042886	BP	Amide transport	28	3.6	25.0	*22335, 59508, 20029, 71333, 10510*, ***56891***, *58935*
GO:0009735	BP	Response to cytokinin	13	7.7	15.4	***23250***, *58762*
GO:0008375	MF	Acetylglucosaminyltransferase activity	36	2.8	19.4	*38958*, ***37085***, *65593, 74430, 5087, 63430, 60533*
GO:0016307	MF	Phosphatidylinositol phosphate kinase activity	18	5.6	16.7	***81640***, *62872, 5875*
GO:0008509	MF	Anion transmembrane transporter activity	104	4.8	16.4	Not listed
GO:0042594	BP	Response to starvation	44	6.8	13.6	***56127***, ***7416***, ***44452***, *16652, 4685, 57969*
GO:0005667	CC	Transcription factor complex	30	3.3	16.7	***76757***, *67781, 7755, 36554, 62730*
GO:0045786	BP	Negative regulation of cell cycle	25	8.0	12.0	***57670***, ***65158***, *62665*
GO:0031669	BP	Cellular response to nutrient levels	45	6.7	13.3	***56127***, ***7416***, ***44452***, *16652, 4685, 57969*
GO:0042559	BP	Pteridine-containing compound biosynthetic process	15	6.7	13.3	***74131,*** *10075*
GO:0008643	BP	Carbohydrate transport	41	4.9	14.6	*65088, 13612, 17903, 280, 63767, 67524, 10342, 59161*
GO:0019901	MF	Protein kinase binding	36	5.6	13.9	*46471*, ***57670***, *66940, 51179*
GO:0010565	BP	Regulation of cellular ketone metabolic process	16	6.3	12.5	***36714***, *14398*
GO:0004672	MF	Protein kinase activity	1099	4.3	14.5	Not listed
GO:0000272	BP	Polysaccharide catabolic process	43	7.0	11.6	*40915, 54306, 59047, 49756, 4022*
GO:0009751	BP	Response to salicylic acid	54	5.6	13.0	*21464, 1340*, ***36714***, *14398*, ***43518***, *71936, 10787*
GO:0003950	MF	NAD+ ADP-ribosyltransferase activity	11	9.1	9.1	*66554, 72444*
GO:0009723	BP	Response to ethylene	35	8.6	8.6	*44452, 53881, 64636*

stronger lignification. Indeed, about half of the genes assigned as participating in the phenylpropanoid pathway were significantly up-regulated, as were 5 of 7 genes encoding phenylalanine ammonia lyases and all four genes encoding prephenate dehydratase. The up-regulation of the four selected genes was confirmed by real-time PCR (Fig. 4c). Lignin synthesis depends on precursors the same as those for benzoic acids. The levels of the three most abundant benzoic acids were reduced in the *vAtCKX1* roots cultivated under optimal conditions (Fig. 4b). Hence, a metabolic switch between lignin and benzoic acid production seems to be a downstream process of reduced CK status. Nevertheless, a more precise analysis of all possible products needs to be conducted in the future to elucidate all consequences of the observed dramatic regulation of genes involved in the phenylpropanoid pathway.

Among other up-regulated processes, we could identify also hydroperoxide dehydratase and linoleate 13S-lipoxygenase activity, which may stimulate the production of JAs or other volatile compounds, the biosynthesis of coumarin, and the response to hyperosmotic salinity (Table 4).

Conclusions

Here, we presented one of the first whole-transcriptome studies done on barley plants. Although a rough draft of the barley genome has been available for several years [69], its precise annotation and the classification of predicted genes into GO categories are still incomplete and in some aspects unsatisfying. For instance, the GO term 'response to cytokinin' counts only 13 putative genes and none of the type A-RR are included. Nevertheless, the

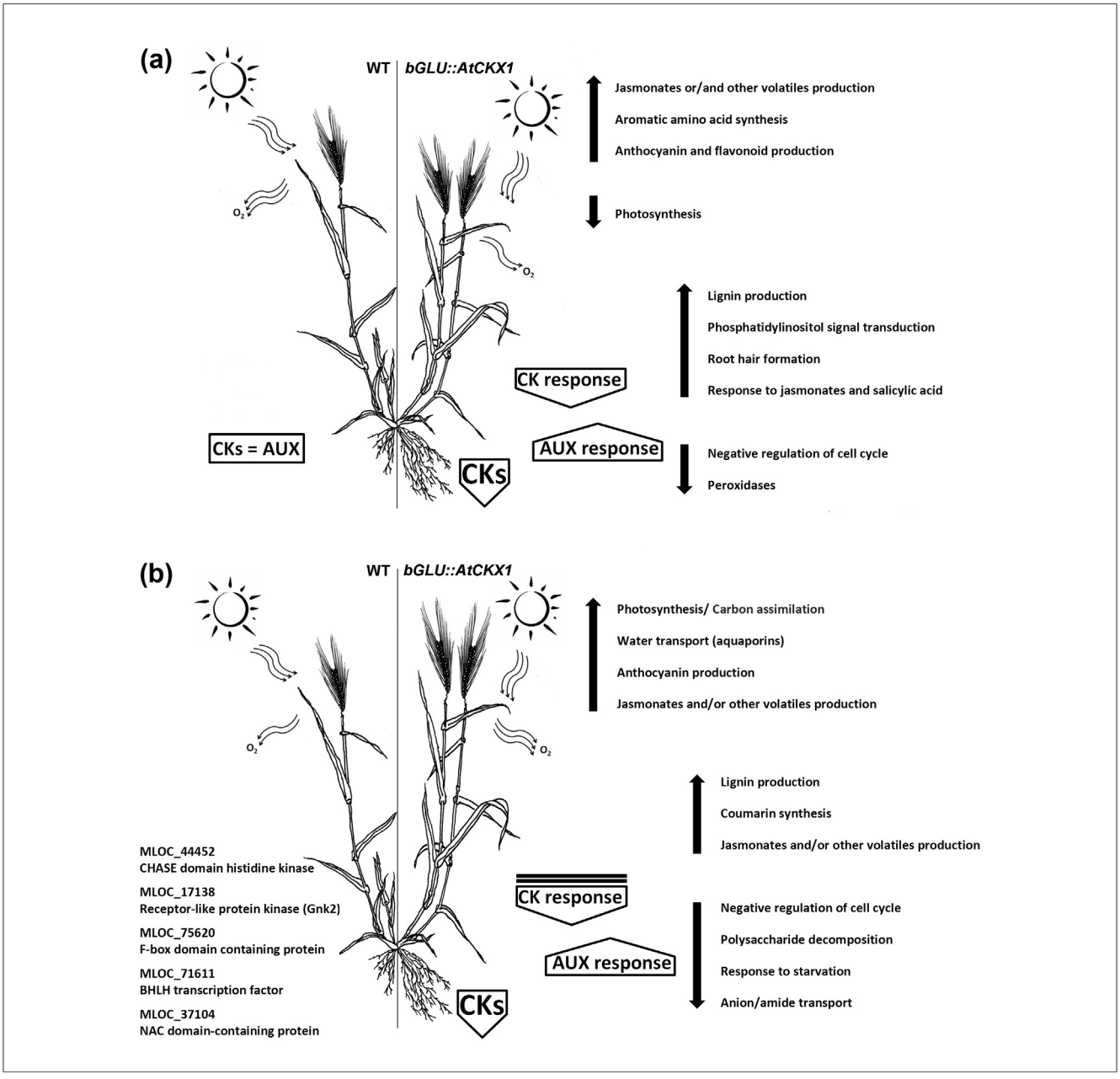


FIGURE 5

Conceptual diagram of main physiological responses in transgenic barley overexpressing *CKX* gene under the control of mild root-specific promotor (a) under optimal conditions or (b) during revitalization after drought stress. Predicted key regulatory genes are listed.

challenging RNA-seq method enabled us to comprehensively inspect all processes occurring in plant tissue with CK imbalance and suggest a new context especially toward other phytohormones. In addition to the fact that the described transgenic plants showed better drought avoidance due to modified root morphology, probably as a consequence of an altered cytokinin-to-auxin ratio, partial CK insensitivity due to down-regulation of HvHK3 receptor expression influenced also other physiological processes leading to drought tolerance (Fig. 5). Up-regulation of four *aquaporin* genes might have contributed to the fact that all transgenic genotypes were able to increase water potential faster than were WT plants. The process of leaf revitalization is accompanied by up-regulation of genes implicated in photosynthesis, and especially those encoded by the chloroplast genome. This aspect leads to faster regeneration of transgenic plants that is observed as higher biomass accumulation. Altered CK status noticeably accelerates secondary metabolism derived from phenylalanine and leads to accumulation of intermediates of the phenylpropanoid pathway in the roots. In this manner, more lignin is deposited in the root tissue and formation of such other compounds as anthocyanins and flavonoids can be expected. The comparative transcriptomic analyses disclosed several genes that might play a crucial role in the drought-tolerant phenotype of *AtCKX1*-overexpressing barley plants. In addition to two transcription factors of the MYB and NAC families shown to increase sensitivity to ABA, the expression of a putative F-box-like protein (MLOC_75620) was strongly depleted in all transgenic tissues. This protein might be involved in ubiquitination of repressors or activators of other phytohormone transduction pathways and thus mediate cross-talk of CKs with auxins, GAs, or JAs. Enforced production of JAs or other volatile compounds might be another process attributed to *AtCKX1*-overexpression phenotype inasmuch as linoleate 13S-lipoxygenase activity is among the most enriched GO terms in both transgenic leaves as well as roots after re-watering. In conclusion, introduction of one CK-degradation enzyme into the barley genome under the control of a mild promoter resulted in a CK-insensitive phenotype that activates processes enabling plants to regenerate better after a water deficit (Fig. 5).

Acknowledgements

The authors thank Hana Pospíšilová for providing plant material. This work was supported by the Czech Science Foundation (grant no. 14-12355S) as well as the Czech Ministry of Education, Youth and Sports through the National Programme for Sustainability I (project no. LO1204), and Mobility (project no. 7AMB15AR011) programs.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2016.01.010.

References

[1] Zalabák D, Pospíšilová H, Šmehilová M, Mrízová K, Frébort I, Galuszka P. Genetic engineering of cytokinin metabolism: prospective way to improve agricultural traits of crop plants. Biotechnol Adv 2013;31:97–117.
[2] Ha S, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Tran LSP. Cytokinins: metabolism and function in plant adaptation to environmental stresses. Trends Plant Sci 2012;17:172–9.
[3] Nishiyama R, Watanabe Y, Fujita Y, Le DT, Kojima M, Werner T, et al. Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. Plant Cell 2011;23:2169–83.
[4] Tran LSP, Urao T, Qin F, Maruyama K, Kakimoto T, Shinozaki K, et al. Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in Arabidopsis. Proc Natl Acad Sci U S A 2007;104:20623–28.
[5] Nishiyama R, Watanabe Y, Leyva-Gonzalez MA, Ha CV, Fujita Y, Tanaka M, et al. *Arabidopsis* AHP2, AHP3, and AHP5 histidine phosphotransfer proteins function as redundant negative regulators of drought stress response. Proc Natl Acad Sci U S A 2013;110:4840–5.
[6] Sun L, Zhang Q, Wu J, Zhang L, Jiao X, Zhang S, et al. Two rice authentic histidine phosphotransfer proteins, OsAHP1 and OsAHP2, mediate cytokinin signaling and stress responses in rice. Plant Phys 2014;165:335–45.
[7] Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, et al. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proc Natl Acad Sci U S A 2007;104:19631–36.
[8] Zhang P, Wang WQ, Zhang GL, Kaminek M, Dobrev P, Xu J, et al. Senescence-inducible expression of isopentenyl transferase extends leaf life, increases drought stress resistance and alters cytokinin metabolism in cassava. J Integr Plant Biol 2010;52:653–69.
[9] Ghanem ME, Albacete A, Smigocki AC, Frébort I, Pospíšilová H, Martínez-Andújar C, et al. Root-synthesized cytokinins improve shoot growth and fruit yield in salinized tomato (*Solanum lycopersicum* L.) plants. J Exp Bot 2011;62:125–40.
[10] Merewitz EB, Gianfagna T, Huang B. Photosynthesis, water use, and root viability under water stress as affected by expression of *SAG12-ipt* controlling cytokinin synthesis in *Agrostis stolonifera*. J Exp Bot 2011;62:383–95.
[11] Peleg Z, Reguera M, Tumimbang E, Walia H, Blumwald E. Cytokinin-mediated source/sink modifications improve drought tolerance and increase grain yield in rice under water-stress. Plant Biotechnol J 2011;9:747–58.
[12] Kuppu S, Mishra N, Hu R, Sun L, Zhu X, Shen G, et al. Water-deficit inducible expression of a cytokinin biosynthetic gene *IPT* improves drought tolerance in cotton. PLoS ONE 2013;8:e64190.
[13] Reguera M, Peleg Z, Abdel-Tawab YM, Tumimbang EB, Delatorre Ca, Blumwald E. Stress-induced cytokinin synthesis increases drought tolerance through the coordinated regulation of carbon and nitrogen assimilation in rice. Plant Physiol 2013;163:1609–22.
[14] Zhang SW, Li CH, Cao J, Zhang YC, Zhang SQ, Xia YF, et al. Altered architecture and enhanced drought tolerance in rice via the down-regulation of indole-3-acetic acid by *TLD1/OsGH3.13* activation. Plant Physiol 2009;151:1889–901.
[15] Tsuchisaka A, Theologis A. Unique and overlapping expression patterns among the Arabidopsis 1-amino-cyclopropane-1-carboxylate synthase gene family members. Plant Physiol 2004;136:2982–3000.
[16] Růžička K, Ljung K, Vanneste S, Podhorská R, Beeckman T, Friml J, et al. Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell 2007;19:2197–212.
[17] Peleg Z, Blumwald E. Hormone balance and abiotic stress tolerance in crop plants. Curr Opin Plant Biol 2011;14:290–5.
[18] Rivero RM, Gimeno J, Van Deynze A, Walia H, Blumwald E. Enhanced cytokinin synthesis in tobacco plants expressing P_{SARK}::*IPT* prevents the degradation of photosynthetic protein complexes during drought. Plant Cell Physiol 2010;51:1929–41.
[19] Wang L, Wang Z, Xu Y, Joo SH, Kim SK, Xue Z, et al. *OsGSR1* is involved in crosstalk between gibberellins and brassinosteroids in rice. Plant J 2009;57:498–510.
[20] Alonso-Ramírez A, Rodríguez D, Reyes D, Jiménez JA, Nicolás G, López-Climent M, et al. Evidence for a role of gibberellins in salicylic acid-modulated early plant responses to abiotic stress in Arabidopsis seeds. Plant Physiol 2009;150:1335–44.
[21] Wang C, Yang A, Yin H, Zhang J. Influence of water stress on endogenous hormone contents and cell damage of maize seedlings. J Integr Plant Biol 2008;50:427–34.
[22] Vyroubalová Š, Václavíková K, Turečková V, Novák O, Šmehilová M, Hluska T, et al. Characterization of new maize genes putatively involved in cytokinin metabolism and their expression during osmotic stress in relation to cytokinin levels. Plant Physiol 2009;151:433–47.
[23] Havlová M, Dobrev PI, Motyka V, Štorchová H, Libus J, Dobrá J, et al. The role of cytokinins in responses to water deficit in tobacco plants over-expressing *trans*-zeatin O-glucosyltransferase gene under *35S* or *SAG12* promoters. Plant Cell Environ 2008;31:341–53.
[24] Gan S, Amasino RM. Inhibition of leaf senescence by autoregulated production of cytokinin. Science 1995;270:1986–8.
[25] Sýkorová B, Kurešová G, Daskalova S, Trčková M, Hoyerová K, Raimanová I, et al. Senescence-induced ectopic expression of the *A. tumefaciens ipt* gene in wheat delays leaf senescence, increases cytokinin content, nitrate influx, and nitrate reductase activity, but does not affect grain yield. J Exp Bot 2008;59:377–87.

[26] Sharp RE, LeNoble ME. ABA, ethylene and the control of shoot and root growth under water stress. J Exp Bot 2002;53:33–7.
[27] Albacete AA, Martínez-Andújar C, Pérez-Alfocea F. Hormonal and metabolic regulation of source–sink relations under salinity and drought: from plant survival to crop yield stability. Biotechnol Adv 2014;32:12–30.
[28] Werner T, Nehnevajova E, Köllmer I, Novák O, Strnad M, Krämer U, et al. Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in *Arabidopsis* and tobacco. Plant Cell 2010; 22:3905–20.
[29] Macková H, Hronková M, Dobrá J, Turečková V, Novák O, Lubovská Z, et al. Enhanced drought and heat stress tolerance of tobacco plants with ectopically enhanced cytokinin oxidase/dehydrogenase gene expression. J Exp Bot 2013;64:2805–15.
[30] Pospíšilová H, Jiskrová E, Vojta P, Mrízová K, Kokáš P, Majeská Čudejková M, et al. Transgenic barley overexpressing a cytokinin dehydrogenase gene shows greater tolerance to drought stress. New Biotechnol 2016;33:692–705.
[31] Vlamis J, Williams DE. Ion competition in manganese uptake by barley plants. Plant Physiol 1962;37:650–5.
[32] Turner NC, Abbo S, Berger JD, Chaturvedi SK, French RJ, Ludwig C, et al. Osmotic adjustment in chickpea (*Cicer arietinum* L.) results in no yield benefit under terminal drought. J Exp Bot 2007;58:187–94.
[33] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 2001;25: 402–8.
[34] Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 2013;14:R36.
[35] Anders S, Pyl PT, Huber W. HTSeq – a Python framework to work with high-throughput sequencing data. Bioinformatics 2014;31:166–9.
[36] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2. Genome Biol 2014;15:550.
[37] Kokáš F, Vojta P, Galuszka P. Comparative transcriptome analysis of barley (*Hordeum vulgare*) exposed to drought and subsequent re-watering. Data Brief 2016 [submitted for publication].
[38] Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 2005;21:3674–6.
[39] Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture and applications. BMC Bioinform 2009;10:421.
[40] Moreira-Vilar FC, Siqueira-Soares RDC, Finger-Teixeira A, De Oliveira DM, Ferro AP, Da Rocha GJ, et al. The acetyl bromide method is faster, simpler and presents best recovery of lignin in different herbaceous tissues than klason and thioglycolic acid methods. PLoS ONE 2014;9:e110000.
[41] Gruz J, Novák O, Strnad M. Rapid analysis of phenolic acids in beverages by UPLC–MS/MS. Food Chem 2008;111:789–94.
[42] Oxborough K, Baker NR. Resolving chlorophyll *a* fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components – calculation of *qP* and Fv'/Fm' without measuring Fo'. Photosynth Res 1997;54:135–42.
[43] Hassan IA. Effects of water stress and high temperature on gas exchange and chlorophyll fluorescence in *Triticum aestivum* L.. Photosynthetica 2006;44: 312–5.
[44] Roostaei M, Mohammadi SA, Amri A, Majidi E, Nachit M, Haghparast R. Chlorophyll fluorescence parameters and drought tolerance in a mapping population of winter bread wheat in the highlands of Iran. Russ J Plant Physiol 2011;58:351–8.
[45] Robredo A, Pérez-López U, Miranda-Apodaca J, Lacuesta M, Mena-Petite A, Muñoz-Rueda A. Elevated CO_2 reduces the drought effect on nitrogen metabolism in barley plants during drought and subsequent recovery. Environ Exp Bot 2011;71:399–408.
[46] Rippert P, Matringe M. Molecular and biochemical characterization of an *Arabidopsis thaliana* arogenate dehydrogenase with two highly similar and active protein domains. Plant Mol Biol 2002;48:361–8.
[47] Cass CL, Peraldi A, Dowd PF, Mottiar Y, Santoro N, Karlen SD, et al. Effects of *PHENYLALANINE AMMONIA LYASE* (*PAL*) knockdown on cell wall composition, biomass digestibility, and biotic and abiotic stress responses in *Brachypodium*. J Exp Bot 2015;66:4317–35.
[48] Feussner I, Wasternack C. The lipoxygenase pathway. Annu Rev Plant Biol 2002;53:275–97.
[49] Wasternack C. Action of jasmonates in plant stress responses and development – applied aspects. Biotechnol Adv 2014;32:31–9.
[50] Tian D, Tooker J, Peiffer M, Chung SH, Felton GW. Role of trichomes in defense against herbivores: comparison of herbivore response to *woolly* and *hairless* trichome mutants in tomato (*Solanum lycopersicum*). Planta 2012;236:1053–66.
[51] Ueda J, Kato J. Identification of jasmonic acid and abscisic acid as senescence-promoting substances from *Cleyera ochnacea* DC. Agric Biol Chem 1982; 46:1975–6.
[52] O'Brien JA, Benková E. Cytokinin cross-talking during biotic and abiotic stress responses. Front Plant Sci 2013;4:451.
[53] Baldwin IT, Schemelz EA, Ohnmeiss TE. Wound-induced changes in root and shoot jasmonic acid pools correlate with induced nicotine synthesis in *Nicotiana sylvestris* spegazzini and comes. J Chem Ecol 1994;20:2139–57.
[54] Moon J, Parry G, Estelle M. The ubiquitin–proteasome pathway and plant development. Plant Cell 2004;16:3181–95.
[55] Zubo YO, Yamburenko MV, Selivankina SY, Shakirova FM, Avalbaev AM, Kudryakova NV, et al. Cytokinin stimulates chloroplast transcription in detached barley leaves. Plant Physiol 2008;148:1082–93.
[56] Wei W, Alexandersson E, Golldack D, Miller AJ, Kjellbom PO, Fricke W. HvPIP1;6, a barley (*Hordeum vulgare* L.) plasma membrane water channel particularly expressed in growing compared with non-growing leaf tissues. Plant Cell Physiol 2007;48:1132–47.
[57] Nakabayashi R, Yonekura-Sakakibara K, Urano K, Suzuki M, Yamada Y, Nishizawa T, et al. Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. Plant J 2014;77:367–79.
[58] Cha JY, Barman DN, Kim MG, Kim WY. Stress defense mechanisms of NADPH-dependent thioredoxin reductases (NTRs) in plants. Plant Signal Behav 2015;10:e1017698.
[59] Cheng Z, Dong K, Ge P, Bian Y, Dong L, Deng X, et al. Identification of leaf proteins differentially accumulated between wheat cultivars distinct in their levels of drought tolerance. PLoS ONE 2015;10:e0125302.
[60] Jiang Y, Chen R, Dong J, Xu Z, Gao X. Analysis of GDSL lipase (GLIP) family genes in rice (*Oryza sativa*). Plant Omics 2012;5:351–8.
[61] Hong JK, Choi HW, Hwang IS, Kim DS, Kim NH, Choi DS, et al. Function of a novel GDSL-type pepper lipase gene, *CaGLIP1*, in disease susceptibility and abiotic stress tolerance. Planta 2008;227:539–58.
[62] Galichet A, Hoyerová K, Kamínek M, Gruissem W. Farnesylation directs AtIPT3 subcellular localization and modulates cytokinin biosynthesis in Arabidopsis. Plant Physiol 2008;146:1155–64.
[63] Tsai YC, Weir NR, Hill K, Zhang W, Kim HJ, Shiu SH, et al. Characterization of genes involved in cytokinin signaling and metabolism from rice. Plant Physiol 2012;158:1666–84.
[64] Tran LSP, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, et al. Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the *early responsive to dehydration stress 1* promoter. Plant Cell 2004;16:2481–98.
[65] Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Society 2003;15:63–78.
[66] Bu Q, Jiang H, Li CB, Zhai Q, Zhang J, Wu X, et al. Role of the *Arabidopsis thaliana* NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. Cell Res 2008;18:756–67.
[67] Redillas MCFR, Jeong JS, Kim YS, Jung H, Bang SW, Choi YD, et al. The overexpression of *OsNAC9* alters the root architecture of rice plants enhancing drought resistance and grain yield under field conditions. Plant Biotechnol J 2012;10:792–805.
[68] McSteen P. Auxin and monocot development. Cold Spring Harb Perspect Biol 2010;2:a001479.
[69] Mayer KFX, Waugh R, Langridge P, Close TJ, Wise RP, et al. A physical, genetic and functional sequence assembly of the barley genome. Nature 2012; 491:711–6.