epigenomes

MDPI

Article

Increase of DNA Methylation at the *HvCKX2.1* Promoter by Terminal Drought Stress in Barley

Korana Surdonja [1], Kai Eggert [2], Mohammad-Reza Hajirezaei [2], Vokkaliga Thammegowda Harshavardhan [1,4], Christiane Seiler [1], Nicolaus von Wirén [2], Nese Sreenivasulu [1,3] and Markus Kuhlmann [1,*]

1 Research Group Abiotic Stress Genomics, Interdisciplinary Centre for Crop Plant Research (IZN), Hoher Weg 8, 06120 Halle (Saale), Germany; surdonja@ipk-gatersleben.de (K.S.); seiler@IPK-gatersleben.de (C.S.)

2 Department of Physiology and Cell Biology, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, OT Gatersleben, D-06466 Seeland, Germany; eggert@ipk-gatersleben.de (K.E.); mohammad@ipk-gatersleben.de (M.-R.H.); vonwiren@ipk-gatersleben.de (N.v.W.)

3 International Rice Research Institute (IRRI), Grain Quality and Nutrition Center, DAPO Box 7777, Metro Manila 1301, Philippines; n.sreenivasulu@irri.org

4 National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 10617, Taiwan; harsha@ntu.edu.tw

* Correspondence: kuhlmann@ipk-gatersleben.de; Tel.: +49-394825172; Fax: +49-394825758

Academic Editor: Etienne Bucher
Received: 25 April 2017; Accepted: 20 June 2017; Published: 27 June 2017

Abstract: Terminal drought stress during grain filling is the major abiotic factor that limits crop yield in barley. The mother plant acclimates to the environment and perceives signals that result in a change of the physiological state within the grain and therefore affect the seed development and germination of the next generation. Small regulatory RNAs have been described to be involved in plant drought stress response by suppressing the respective target genes. Based on their origin and function, these small RNAs are classified as micro RNAs (miRNA), short interfering RNAs (siRNA) or heterochromatic small interfering RNA (hc-siRNA). In addition, 24mer sized hc-siRNAs are associated with RNA directed DNA methylation (RdDM) and transcriptional gene silencing (TGS). The analysis of hc-siRNA by small RNA sequencing in barley caryopses after imposition of terminal drought stress allowed the identification of stress specific 24mers. Based on the sequence homology of the siRNAs to the promoter region of CYTOKININ-OXIDASE 2.1 (*HvCKX2.1*), this putative target gene was selected for further investigation. Terminal drought stress leads to an increased level of DNA methylation at the *HvCKX2.1* promoter and the seeds derived from drought stressed plants showed faster shoot emergence. Accumulation of cytokinin ribosides, which are the known substrates of cytokinin-oxidase, can explain the observed phenotype of faster shoot emergence from seeds of drought stressed mother plants. Analysis of transgenic plants with modulated levels of abscisic acid (ABA) in the grain confirmed the ABA/drought stress responsive *ProHvCKX2.1* methylation and correlation with shoot emergence speed.

Keywords: barley; DNA methylation RdDM; TGS; HvCKX2.1; heterochromatic small RNA; germination; terminal drought stress

1. Introduction

Among the food crops, barley is the fifth most important crop and ranks fourth among cereal crops in terms of production worldwide after maize, rice and wheat (FAO Statistical Yearbook 2009). The European Union contributes 25% (6.2 c) of the total 152 Mt of barley produced annually worldwide, and Germany ranks next to France in the European region with a total production of 1.2 Mt. Apart from

its use for feed and food, barley is widely used as a unique source of malt for beer and whisky production. Mediterranean regions represent a significant area of barley production, where it is mostly grown under rain fed conditions. These regions are characterized by a long hot and dry summer (coupled with an erratic rain fall pattern), alternated with cold, wet and relatively short winters [1]. Owing to these weather conditions, barley is often exposed to several stress conditions such as drought or high temperature, or a combination of both, often coinciding with the grain filling period. Increasing climatic disturbances worldwide and the forecast for more frequent and intense drought occurrences, especially in the arid and semi-arid regions of the world, poses major concerns for the production of barley as well [2,3]. Drought imposed after anthesis and fertilization will primarily affect seed size and quality [4,5], and is therefore an important abiotic component influencing barley grain traits. These traits contribute to the malting performance of barley.

Although the majority of information on epigenetic mechanisms in plants is derived from the dicotyledonous model plant *Arabidopsis thaliana*, the basic mechanisms can be generalized also for monocotyledonous plant species [6], supported by the presence of many of the involved enzymes. The early developmental stages after fertilization are the most sensitive ones for establishing epigenetic modifications mediated by small RNAs [7]. Here, DNA methylation is erased and newly mediated by the presence of small RNAs in a mechanism referred to as RNA directed DNA methylation (RdDM [8]). Small RNAs with regulatory function are separated in three main categories: microRNAs (miRNAs), small interfering RNAs (siRNAs) and heterochromatic-small RNAs (hc-siRNAs). miRNAs are transcribed from miRNA-genes as pre-miRNAs, processed by DICER-function and incorporated into ARGONAUTE-proteins. miRNAs are aiming their respective target transcript via sequence homology and suppress the target gene function by inhibition of translation or transcript degradation. The role of miRNAs in plant development is an emerging point of investigation. The involvement of miRNAs as key regulators of flowering time (miR159, miR172, miR156 and miR171), hormone signaling (miR159, miR160, miR167, miR164 and miR393), or shoot and root development (miR164), was reviewed by [9]. During early seedling development, the regulation mediated by the presence of miR165, miR166, miR164 and miR319 is of special importance for germination and developmental phase transitions [9,10]. In barley, miR172 was identified as a regulator of floret closing [11] and the density of grains [12]. Recently, the function of several miRNAs in response to abiotic stimuli, especially as drought stress response, was also further elucidated [13].

As the class of siRNAs is more inhomogeneous with respect to their processing and function, the investigation of the involvement of particular siRNAs is more complex. The involvement of siRNAs during plant development has been described for at least two mechanisms [14]. The first one involves posttranscriptional gene silencing (PTGS) via the presence of 21-nt long small RNAs [15]. Furthermore, 21mer siRNAs are the products of PTGS and represent hallmarks for genes suppressed via this mechanism. The second mechanism involves 24-nt heterochromatic small RNAs and addresses gene regulation mediated by RNA directed DNA methylation [16,17]. Particular 24-nt long siRNAs may have an important impact on seed development. Particularly in the female gametophyte and the developing seeds, the abundance of 24mers is much higher than in all of other plant tissues [7]. This indicates the importance of these regulators in the transition between generations and for seed development.

Here, we show the methylation of the *HvCKX2.1* promoter region mediated by the presence of drought stress specific heterochromatic small RNAs. Drought stress specific small RNAs were identified by RNA sequencing and their presence was correlated with increased DNA methylation. The germinated seedlings of drought stressed barley showed a reduced expression of *HvCKX2.1* compared to control seedlings. Furthermore, an increased accumulation of cytokinin (CK) during germination and an earlier shoot appearance was observed in seeds derived from drought stressed mother plants.

2. Results

2.1. Identification of Terminal Drought Stress Specific Small RNAs in Barley Caryopses

During female gametophyte development in the central cell and seed development in the endosperm, an accumulation of functional heterochromatic small RNAs (24mers) was described in plants [7]. The presence of heterochromatic small RNA in this phase is of importance for proper maintenance of the heterochromatic state in the respective homologous genomic loci. The effect of abiotic stress such as terminal drought stress might have severe impact in the seed development and subsequent plant performance in the next generation.

In order to analyze this effect of terminal drought stress on the population of small RNAs in barley, small RNA sequencing was performed on small RNAs extracted from caryopses of a barley line DH21 [18] with high yield stability under terminal drought stress. Terminal drought stress was applied by stopping the watering four days after anthesis. Caryopses were collected eight days after anthesis (Figure 1A). As described earlier [19], terminal drought stress results in decreased yield (Figure 1B) by affecting the seed number and filling efficiency (thousand kernel weight (TKW), Figure 1B).

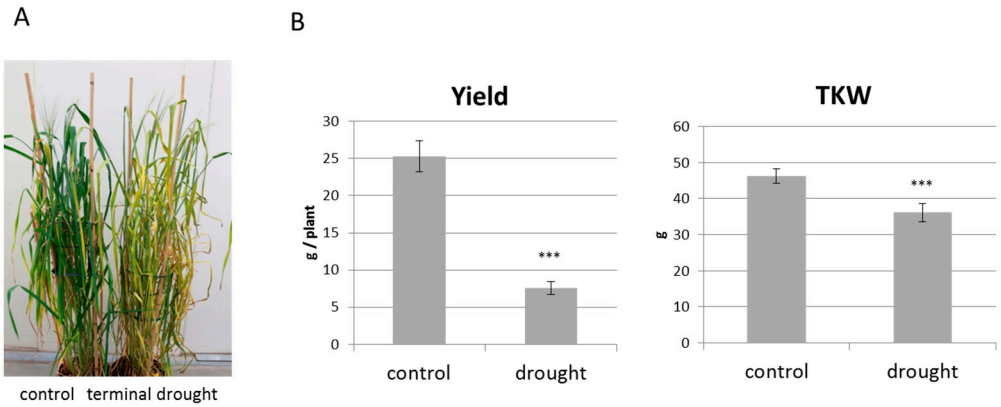

Figure 1. Phenotypic features of barley subjected to terminal drought stress. (**A**) DH21 barley line, control and drought stressed mother plant. Drought stress was imposed four days after flowering. (**B**) grain yield and thousand kernel weight (TKW) of DH21 at control and terminal drought stress condition. Yield and TKW are significantly reduced in drought stressed plants. N (control) = 6, N (drought stress) = 4 plants. Significant differences estimated by *t*-test and indicated by *** ($p < 0.001$).

After TrueQuant correction of the Illumina raw sequences [20], data were processed by GenXPro (GenXPro Frankfurt, Germany). The mean, standard deviation and coefficient of variation were calculated by GenXPro in house scripts. The dataset of detected reads is deposited at e!DAL [21] using the e!DAL (IPK, Gatersleben, Germany) system [22]. The frequency distribution of derived reads showed a clear overrepresentation (41–46% of all reads) of 24mer long sequences, as already described by Law and Zhai [23,24] for reproductive tissue. The read list of small RNAs was normalized (reads per million, RPM), filtered for 24mer size, mapped against the recently published barley genome [25] and analyzed for differential presence of individual sequences. A list was generated with reads present in both replicates after drought stress treatment and absent in both replicates under control conditions. The reads with highest abundance were chosen for further analysis. The detected sequence mapped to Morex_contig_38742 located on Chr. 3, 99654033-658338 containing the coding sequence for *HvCKX2.1* (MLOC_53923), a cytokinin oxidase/dehydrogenase (CKX; EC.1.5.99.12) schematically depicted in Figure 2A. The encoded enzyme is involved in degradation of cytokinin by cleaving the N^6-substituted side chain to produce adenine and unsaturated aldehyde 3-methyl-2-butenal [26]. *HvCKX2.1* was initially described by Mameaux et al. [27] and belongs to a gene family including thirteen identified members [28]. The detected sequence of the 24mer sized small RNA is homologous to the *HvCKX2.1* promoter region and 1451 base pairs (bp) upstream of the start ATG. It also maps

to chromosome 2: 549058859-885, and with >95% identity to 118 further positions in the barley genome. A second small RNA read could be detected in the list of drought stress specific small RNAs on position 32, homologous to the same region 1380 bp upstream of the *HvCKX2.1* start ATG. This sequence maps with 100% identity to 5 loci and >95% identity to 137 loci in the barley genome. The relative position of the small RNAs is shown in Figure 2A (detailed in Figure S1). The region 840–1850 bp upstream of the start ATG includes sequences of heterochromatic origin with high identity to retroelements and long terminal repeats (LTRs) from the COPIA (orange box, Figure 2A) and GYPSY (yellow box, Figure 2A) retroelement family (Gypsy database 2.0 [29]).

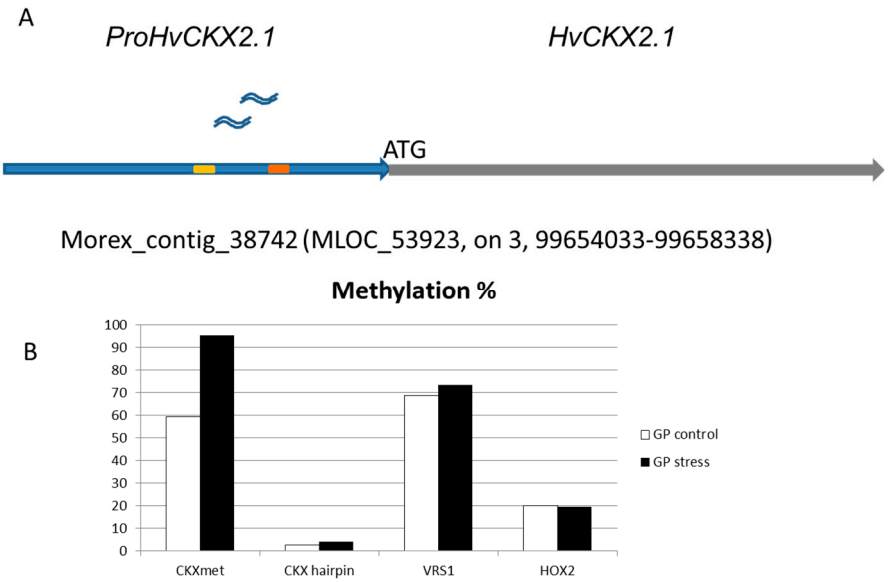

Figure 2. Identification of heterochromatic small RNA with homology to *CKX2.1* promoter. (**A**) relative positions of two small RNAs mapping to Morex_contig38742, 99654033-99658338 on chromosome 3, which contains HvCKX2.1. Two drought stress specific 24mers (blue) are homologous to 1451 bp and 1380 bp upstream of coding start site (ATG), respectively. Orange box: homology to COPIA, yellow box: homology to GYPSY retroelement. (**B**) methylation status quantified by methylation sensitive restriction enzyme digestion (MSRE) qPCR of control and drought stressed derived seeds at three days after imbibition. CKXmet region corresponds to −967 bp to −1237 bp upstream of the start ATG. No methylation was detected at −263 bp to −560 bp (CKX hairpin). ProHvHOX2 and ProHvVRS1 were used as reference regions.

Using the Plant Promoter Analysis Navigator PlantPAN 2.0 software (Tainan 701, Taiwan), several promoter motifs with potential regulatory function could be detected (Figure S2) in this region (967 to 1237 bp upstream of start ATG). Among the identified motifs were binding elements for Myb-, AP2-, bZIP and bHLH transcription factors. No small RNAs homologous to the coding region of *HvCKX2.1* or miR-target sites were detected in the analyzed dataset using the RegRNA 2.0 software based on the known miRNAs from *Hordeum vulgare* [30].

2.2. Methylation Status of ProHvCKX2.1

As the presence of 24mer small RNAs is a hallmark for RNA directed DNA methylation [31], the methylation status of the region identified was analyzed by methylation sensitive restriction digestion and quantified by quantitative real-time PCR after methylation sensitive restriction enzyme digestion (MSRE qPCR). To determine the level of DNA methylation, 5-day-old barley seedlings were analyzed individually for each condition. After extraction of genomic DNA, MSRE digestion was performed with the methylation sensitive restriction enzyme *Hpa*II, and two regions in the *ProCKX2.1* were tested

by qPCR (PCR2 and PCR3, Figure S2). Non-methylated *ProHvHOX2* was used as reference region for completeness of cleavage, and *ProHvVRS1* (80% relative methylation) as positive methylated control.

The analysis indicated substantial methylation (~60%) in the promoter region of the *HvCKX2.1* gene (Figure 2B). The region selected for analysis spanned 270 bp and included six *Hpa*II recognition sites (spanning the region from −967 bp to −1237 bp upstream of the start ATG) and was adjacent to the loci homologous to the detected small RNA. It includes sequence similarity with an LTR fragment known from the Blueberry Red Ringspot Virus (BRRV, GyDB [29]). No methylation was detectable in the 3′ promoter region −263 bp to −560 bp upstream of start ATG including three *Hpa*II recognition sites. In the seedlings derived from plants grown under terminal drought stress conditions, the level of methylation was increased by 30% to more than 90% relative methylation.

2.3. Seedlings Derived from Drought Stress Plants Exhibit Reduced Presence of HvCKX2.1 mRNA

A hallmark of transcriptional gene silencing mediated by RdDM is the presence of 24mer sized small RNAs homologous to the promoter sequence and 5′-methyl-cytosines in the promoter region. As these prerequisites could be confirmed, the question arises whether the change in methylation affects the transcription. To address this question, barley seedlings were tested for the presence of *HvCKX2.1* mRNA and enzyme function associated with cytokinin degradation.

In *Hordeum vulgare*, thirteen members of the CKX gene family could be identified based on sequence similarity [28,32]. Four members of the gene family are reported to be transcriptionally active in developing kernels 14 days after pollination (HvCKX1, 4, 9 and 11). Posttranscriptional silencing of HvCKX1 [33] and HvCKX2 [34] induced by an RNAi transgene resulted in increased yield. Constitutive overexpression of *ZmCKX1* and *HvCKX9* in barley was reported to increase root growth and tiller formation in barley [35]. Expression data for *HvCKX2.1* (MLOC_53923) derived from the publicly available database [36] based on RNA sequencing experiments [37], indicate transcriptional activity of the gene in developing caryopses and no transcript in four day old germinating seedlings. In other tissues and late developmental phases, the expression of *HvCKX2.1* is very low, also described by [35]. To investigate the gene-specific expression, the detectable mRNA of *HvCKX2.1* was quantified by real-time qPCR relative to *ACT* mRNA, 12 h and 24 h after imbibition of the seeds (Figure 3A). For proof of cDNA quality, a primer pair was chosen spanning an intronic sequence of 98 bp, and three independent biological replicates were analyzed in three technical replicates each. Melting curve analysis revealed unique amplicons, excluding contamination by genomic DNA. Supporting the expression data derived from the expression atlas, a considerable amount of *HvCKX2.1* mRNA could be detected in the early developmental stage 12 h after imbibition, while 24 h after imbibition, the level of *HvCKX2.1* mRNA was reduced to the detection limit of the method. In the seedlings derived from drought stressed mother plants, the level of expression 12 h after imbibition decreased by tenfold.

From its structure, the encoded protein contains a flavin adenine dinucleotide FAD-binding domain in the N-terminal half and a cytokinin binding domain in the C-terminal half. Both domains are prerequisites for a functional plant cytokinin dehydrogenase [38]. As described by Sakakibara [39], CKX acts in the degradation of cytokinin by cleaving the N^6-substituted side chain to produce adenine and unsaturated aldehyde 3-methyl-2-butenal [26]. The proposed substrates of CKX2.1 are the physiological active cytokinins isopentenyladenin (ip) and trans-zeatine (tz) [39].

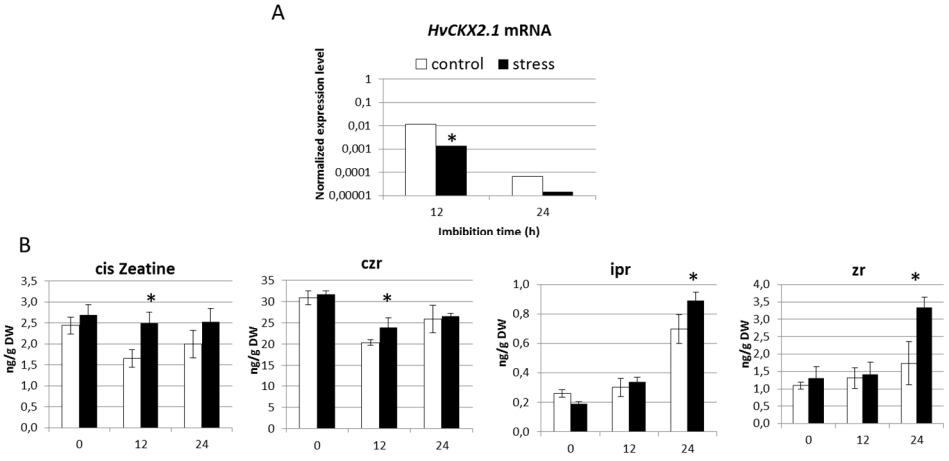

Figure 3. CKX2.1 mRNA and cytokinin abundance during germination. (**A**) abundance of *HvCKX2.1* mRNA relative to *ACTIN* mRNA in 12 h and 24 h imbibed seeds of barley cv. Golden Promise. Data represent the means and standard deviation (SD) values of three independent biological replicates. (**B**) cytokinin content during germination. Fifty milligrams of barley seedlings were pooled and taken for measurement. Data show the content of cis zeatine (active form of cytokinins), the riboside forms cis zeatine riboside (czr), isopentenyladenine riboside (ipr) and the transport form zeatine riboside (zr). N = 3 with standard deviation, * indicates significant difference (*t*-test, $p < 0.005$), 0, 12 and 24 indicate imbibition time (h).

The substrates originate from the methylerythriol (MEP) pathway, and for quantification their precursor isopentenylriboside (ipr $\geq$ ip) and cis- and trans-zeatine ribosides (cZr $\geq$ cZ, tZR $\geq$ tZ) were detected by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). For the analysis, dry seeds and imbibed seeds 12 h and 24 h after start of imbibition were used. Sampling was performed in four independent biological replicates from control and drought stressed mother plants. A significant accumulation of the phyto-active compounds cZ, cZr, ipr and Zr was detectable in the drought stressed samples after 12 h or 24 h (Figure 3B). No significant difference was observed in the accumulation of dihydrozeatin riboside (DZR), which is generated as a branch of the MEP pathway but is not a substrate of CKX degradation. These results revealed an accumulation of CK in the seedlings of drought stressed mother plants due to a reduced degradation mediated by *HvCKX2.1*.

2.4. Terminal Drought Stress Enhances the Shoot Emergence Rate of Seeds

The contribution of abscisic acid (ABA) and gibberellic acid (GA) to seed germination have been intensively investigated and the corresponding mechanisms described [40]. In the ABA response, the involvement of epigenetic regulators was already proposed. The function of cytokinin in this context is as yet unclear. Up to now, it was found that cytokinin has an antagonistic function to ABA and contributes to the regulation of germination [41].

To investigate the involvement of terminal drought stress and thereby ABA on the germination behavior, seeds derived from control and drought stressed plants were analyzed. To estimate the shoot emergence rate and speed, the seeds were treated according to International Seed Testing Association (ISTA) rules. The germination speed was estimated by appearance of roots and shoots as a percentage by visual inspection at 12, 24, 36, 48, 60, 72, 84, 96 h and seven days after placing the seeds on filter paper. The seeds derived from drought stressed mother plants showed a significant earlier shoot emergence at time points 12, 24 and 36 h compared to the control seeds (Figure 4A). Documentation of the plates is indicated in Figure S3.

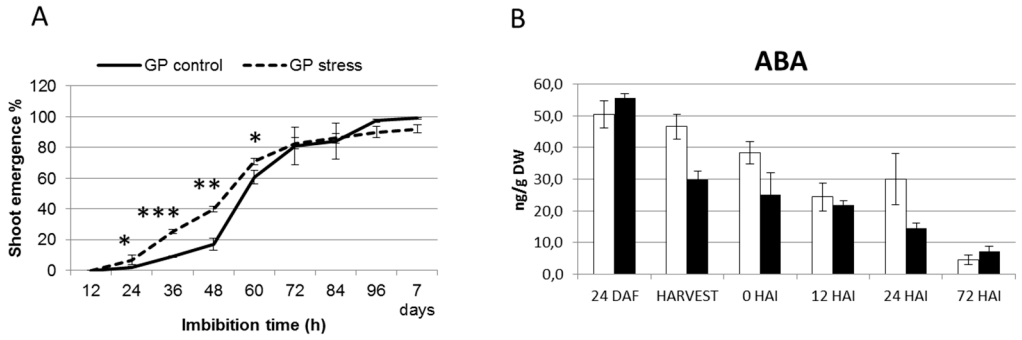

Figure 4. Shoot emergence and abscisic acid (ABA) content in seeds developed under terminal drought stress. (**A**) cumulative shoot emergence in percentage of Golden Promise seeds, $n = 100$ seeds/test in four replicates. Significant differences determined by Student's *t*-test are indicated by * $p < 0.5$, ** $p < 0.1$, *** $p < 0.05$. (**B**) ABA content in barley seedlings during germination estimated by mass spectroscopy. Fifty milligrams of barley seedlings were pooled and taken for each measurement. * indicates significant difference (*t*-test, $p < 0.005$), 0, 12, 24 and 72 indicate imbibition time (h). DAF days after flowering, HAI hours after imbibition. White bar: control, black bar: Seeds developed under terminal drought stress.

In order to address the contribution of ABA involved in the drought stress response, the level of this hormone was estimated by mass spectroscopy (MS) during the germination process. Here, samples were harvested from developing grains, at harvest, after storage and during germination, from plants subjected to terminal drought stress, respective control conditions (Figure 4B). In agreement with results reported earlier [19,42], ABA accumulated in response to terminal drought stress in barley grains, followed by an enhanced degradation during grain development and storage phase. The amount of ABA at harvest time is significantly lower in the drought stressed seeds compared to the control seeds. This reduced level of ABA is detectable through the entire germination phase in the drought stressed seedlings. In addition, 72 h after germination, no significant difference could be observed. As the levels of diphaseic acid (DPA, one degradation product of ABA) showed an inverse pattern (Supplementary Figure S4), we suggest that the observed pattern is derived from enhanced degradation rather than reduced synthesis of ABA.

2.5. Barley with Increased Levels of ABA During Grain Development Phase Show Faster Germination

To confirm our hypothesis that ABA as key regulator of the drought stress response mediates the effect of *HvCKX2.1* promoter methylation, transgenic barley plants referred to as LOHi [19] were tested. The transgenic barley plants were modulated for degradation of ABA during the phase of grain development [42]. In LOHi, an accumulation of ABA was measured in the flag leaf during anthesis. The used line contained a T-DNA including an RNA interference construct homologous to the ABA-hydroxylase gene 1 (*HvCYP707A1*, MLOC_4786, on chromosome 6:437771784-437774758) in barley, controlled by the late embryogenesis specific promoter *LEAB19*. This transgenic approach was chosen for two reasons: firstly, the transgenic modification led to altered ABA content in the grains mimicking drought stress. The modification is temporally and spatially restricted to the developing seed. Secondly, drought stress influenced the seed filling and thereby reduced the endosperm volume of the seed. As a consequence, several parameters were affected (e.g., seed size, starch and protein content) that might influence the initial step of water uptake during the imbibition phase.

The seeds derived from the transgenic LOHi plants grown under controlled greenhouse conditions did not show a significant change of the grain size or morphology (Figure 5A). In the transgenic LOHi lines, the level of ABA was mimicking the pattern of the drought stressed wild type. A lower level of ABA was detected during germination at 0 hours after imbibition (HAI), as a result of an enhanced degradation process (Figure 5B) after stress treatment during seed storage. The analysis of *CKX2.1* promoter methylation revealed an increase in the transgenic line (Figure 5C). At 0 HAI, the level of

cis-zeatin was significantly reduced in the LOHi seeds compared to the Golden Promise control seeds (Figure 5D). The germination analysis of the transgenic line revealed a significant increase in the shoot emergence rate in comparison with the untransformed cv. Golden Promise. The detected difference was comparable to the one estimated for seeds derived from drought stressed mother plants. In both cases, the strongest deviation from the control plants was detectable 36 h after imbibition.

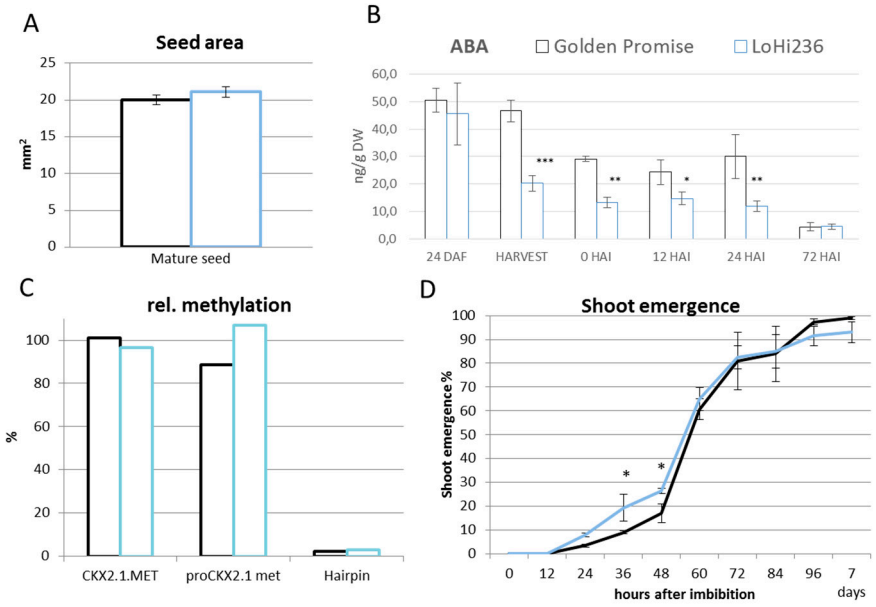

Figure 5. Analysis of seedlings with altered ABA homoeostasis during seed development. (**A**) comparison of the seed area (MARVIN) between Golden Promise and LOHi transgenics shows no difference; (**B**) ABA content in Golden Promise (wildtype, black) and LOHi (transgenic, blue) during germination estimated by mass spectroscopy. Fifty milligrams of barley seedlings were pooled and taken for each measurement. * indicates significant difference (*t*-test, * $p < 0.5$, ** $p < 0.1$, *** $p < 0.05$), 0 to 96 indicate imbibition time (h) + 7 days; (**C**) relative methylation status of the promoter region of *HvCKX2.1* estimated by MSRE qPCR (HpaII cleavage); (**D**) shoot emergence data of Golden Promise and LOHi236 (ProLEA19:ABA8-OH-1 RNAi).

3. Discussion

Recent studies suggest that ABA at its basal level plays an important role during grain filling events [4]. The change of ABA homeostasis in source (flag leaf) and sink (developing grains) tissues under post-anthesis drought stress in barley contributes to the drought resistance of the plant. It was concluded that substantially enhanced ABA levels in developing grains are due to strong activation of the ABA deconjugation pathway, and fine regulation of the ABA biosynthesis and degradation pathways [4,42]. This ABA mediated drought stress response strongly affects the physiological and phenotypical appearance of the grain. The influence of ABA on epigenetic regulation associated with DNA methylation and chromatin modification was described earlier [43–45]. In Arabidopsis, the heat and ABA dependent reactivation of transposable elements was shown [46]. The developmental phase from the female gametophyte until the developed endosperm of the grain is the most sensitive one for epigenetic imprinting mediated by heterochromatic small RNA [7]. 24mer sized heterochromatic RNAs act via RNA dependent DNA methylation, stabilize heterochromatic boundaries, and mediate transcriptional gene silencing if the targeted region affects promoters of functional genes [47–49]. High throughput RNA sequencing revealed the presence of small heterochromatic RNAs specific for terminal drought stress in the caryopsis in exactly this sensitive phase. For our analysis, we selected reads that were present under stress conditions and absent under control conditions. Among these 54 identified reads, we choose the one with the highest abundance under stress condition for

detailed analysis. The identified 24mer has homology to the promoter region of *HvCKX2.1*. As the barley genome sequence is not completely known and the gaps are particularly spanning repetitive elements, the origin of the 24mer could not be identified. The identified region of the promoter contained several features of heterochromatic DNA, namely LTR fragments and loci of viral origin. Using the MSRE qPCR, we could detect substantial methylation in the upstream region of the *HvCKX2.1* promoter. In the region homologous to the 24mers, a drought stress specific increase of methylation was detected. This indicates a spreading of methylation towards the regulatory promoter region of the gene. This drought stress specific increase of DNA methylation correlates with reduced *HvCKX2.1* expression at early germination and an increase of its substrate in germinating seedlings derived from plants grown under terminal drought stress conditions. The expression of *HvCKX2.1* might be affected by the amount of methylation in its promoter region. Thus, *HvCKX2.1* represents a natural target gene influenced by promoter DNA methylation regulated by the RdDM pathway. Although the drought stress is applied to the mother plant, and the physiological state is mediated as ABA response of the mother plant, the detectable effect can be sensed in the offspring. Therefore, it is tempting to call this effect transgenerational, although the developing grain was already present at the time of stress condition. Whether the increase in CK is the major signal leading to faster germination, or the faster ABA degradation is the main trigger, remains to be resolved. Interestingly, RNAi mediated silencing of *HvCKX2* [34] resulted in one transgenic line with a defect in germination that was not further investigated.

Using the transgenic approach, the ABA mediated effect on the offspring was investigated. The LOHi lines analysed showed a similar pattern of ABA homeoestasis during seed development as the drought stressed wild type plants [19]. The results obtained from this line phenocopied the increased germination speed compared to the drought stressed samples. In addition, on the hormonal level, the increased degradation of ABA was observed. In the same way, this line exhibited the increased methylation level in the *HvCKX2.1* promoter region. Thus, most likely, the presence of the heterochromatic small RNA is specifically dependent on an increase of ABA, as it is also detectable in the developing grain under terminal drought stress condition.

Interestingly, the offspring derived from the drought stressed mother plant showed a clear increase in germination speed. One main economic use of barley is the malt production. The malting process includes a 72 h long germination step of the grains. During this step, the germination speed is an important trait contributing to the loss of malt. Here, the drought stress mediated increased shoot emergence speed leads to higher loss during the malting process and reduces the malt quality. Our presented results provided first insights into the molecular bases of how terminal drought stress applied to the mother plant might affect germination speed in the next generation.

As additional and important observation, the increased degradation of ABA after drought stress was found. The increased degradation was leading to lower ABA levels already at the full maturity, when the grains are harvested. ABA is known to suppress germination and thereby shoot emergence in barley [50,51]. Our study showed that the elevated level of ABA caused by drought stress was degraded to a higher extent during the storage phase compared to non-stressed control plants. This enhanced degradation resulted in a lower level of ABA. A similar pattern was detected in the transgenic LOHi line. Due to the transgenic manipulation, the ABA homeostasis is disturbed during development, leading to a temporal accumulation of ABA [19]. This accumulation is followed by a faster degradation in the seed tissue. Correlating with this degradation, a faster shoot emergence was observed. Furthermore, an increased methylation of the CKX2.1 promoter was detected in the LOHi plants.

Based on our findings, we propose that the elevated ABA degradation also contributes to the faster shoot emergence rate observed. The interplay of ABA and GA, as well as the influence of CK on this trait, will be elucidated in future investigations.

Our analysis showed the importance of epigenetic modification influenced by abiotic factors. In our study, only HvCKX2.1 as target gene was analyzed in detail, as it showed the highest abundance

of associated small hc-RNAs. The other identified targets might also be of importance and contribute to plant developmental aspects that have not been addressed yet.

4. Material and Methods

4.1. Plant Cultivation and Used Barley Plants

Plants were grown on a substrate containing four parts of autoclaved compost, two parts of 'Rotes Substrat' (Klasmann-Deilmann GmbH, Geeste, Germany), 1.6 parts of sand and 0.8 parts of peat. Seeds were planted in either 54 or 96 well plastic trays and germinated in a climate chamber or a temperature controlled greenhouse for four weeks at 11 °C day and 7 °C night with 10 h light. After four weeks, seedlings were transferred to pots (diameter 16 cm) and allowed to mature in the greenhouse. Further growth conditions were divided into four phases: first phase at 14 °C day and 9 °C night with 12 h light for four weeks, second phase at 16 °C day and 9 °C night with 14 h light for two weeks, third phase at 20 °C day and 12 °C night with 16 h light for two weeks, and final fourth phase at 20 °C day and 14 °C night with 16 h light until harvest. Plants were fertilized with 'Plantacote plus' (AGLUKON GmbH, Düsseldorf, Germany) (15 g/pot) during the vegetative phase, and with liquid fertilizer 'Hakaphos Rot' (AGLUKON GmbH, Düsseldorf, Germany) (once a week, 2–4%) from the start of spike development. For the experimental analyses, wild-type barley (cv. Golden Promise), the advanced backcross BC3-DH line of elite cultivar Brenda and *Hordeum spontaneum* (DH21) [18] and homozygous transgenic plants (pLea:ABA8′-OH RNAi) were cultivated in a growth chamber (phytochamber) with a 16-h-light/8-h-dark cycle at 20 °C/15 °C, respectively. The transgenic barley line containing the ABA8´hydroxylase-RNAi construct controlled by barley Lea B19.3 promoter (LOHi) was described in [19]. Spikes were labelled at anthesis, drought stress was imposed, and these plants were maintained at 10% soil moisture level. Soil moisture was monitored using a moisture meter HH2 coupled with soil moisture sensor SM200 probes (Delta T Devices, Cambridge, UK). Another batch of plants from a given experiment was continuously watered and treated as unstressed control. Five plants each were grown independently and three technical replications were sampled.

The germination test was performed according to the International Rules for Seed Testing (ISTA). Each germination test consisted in 100 seeds that were germinated on 13 cm Petri dishes (25 seeds × 4 Petri dishes). The seeds were randomly selected and placed between two layers of filter paper and moistened with 9 mL of distilled H_2O. The germination test was carried out at 20 °C under 16 h light/8 h dark with four replications. Germination percentage was scored every 24 h. At 72 h after imbibition, shoot and root lengths were recorded. The seed was considered as germinated if root protrusion was visible (~2 mm).

4.2. Small RNA Sequencing

RNA was extracted by miRVana small RNA extraction kit (Ambion Life technologies, Carlsbad, California, USA), from caryopses harvested 8 days after anthesis. After quality control (Bioanalyzer, Agilent, Santa Clara, California, USA), small RNA libraries (two biological replicates for each condition) were processed as described in [52] for sncRNA libraries with TrueQuant technology for elimination of PCR bias. The fraction of 140–160 bp was size-selected by preparative polyacrylamide gel electrophoresis for Illumina HiSeq2000 sequencing of the small RNA fraction of 17–40 bp including the miRNA. To make the counts more comparable between the samples/smallRNA-Seq libraries, the data were normalized to tags per million (TPM) values. This can be done by using the following equation: "normalizedCount = count_Length/count_All_Length × 1,000,000" The remaining sequences were annotated by blastn : Version: blastn: 2.2.25+

1. "hairpin_and_mature_Hordeum_vulgare.fa"from miRBASE
2. "hairpin_and_mature.fa"from miRBASE
3. "nt.viridiplantae.fas"

4.3. Transcript Quantification

RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany). Quantitative real-time PCRs (qRT-PCR) were performed following [19], in order to assess transcript abundances in germinating seeds (three replicate RNA extractions per biological sample, and three technical replications per RNA extract). All primer sequences are given in Table S2, and their positions are indicated in Figure S2.

4.4. DNA Methylation Analysis

DNA methylation analysis by Methylation sensitive Restriction digest (MSRE)-qPCR was performed on an 7900 HT Fast Real-Time PCR System (Applied Biosystems Foster City, CA, USA) using Power SYBR Green Master Mix (Life Technologies Ltd., Carlsbad, CA, USA) as described by [53], with data analysis using the ΔΔCt method performed as described by [54]. Primer sequences (Metabion, Steinkirchen, Germany) are listed in Supplementary Table S2, and primer positions indicated in Supplementary Figure S2.

4.5. Plant Hormone (PH) Analysis -Abscisic Acid, Cytokinin, Auxin and Salicylic Acid Extraction

The extraction was carried out from 50 mg of freeze dried tissues with 1 mL extraction solvent (acetone:formic acid:water = 14:1:5) as described by Kojima and Seo [55,56] with minor modifications. The homogenate was sonicated and shaken for 1 h at 4° C. After centrifugation at 14,000 rpm for 10 min, the supernatant was transferred to a new tube. The extraction steps were repeated two additional times from the same pellet. The collected supernatants were combined and evaporated by vacuum centrifuge to the desired volume. The internal standard in MeOH (OlChemIm Ltd, Olomouc, Czech Republic) was added to the sample. The phytohormone standards and internal labeled hormone standards were purchased from OlChemIm Ltd, Olomouc, Czech Republic (Table S1). To remove interfering compounds, the extract was first passed through the Oasis HLB cartridge 30 mg (Waters, Milford, MA, USA) equilibrated with 0.1% formic acid in water. The eluate in 0.1% formic acid with MeOH was evaporated and then reconstructed with 0.1% formic acid in water. The hormone containing fraction was passed through an Oasis MCX cartridge 30 mg (Waters Milford, MA, USA), where the acidic and neutral hormones were eluted with 0.1% FA in MeOH. Basic phytohormones were eluted first with 5% ammonium in 60% MeOH and then with 5% ammonium in 60% acetonitrile. Each fraction was evaporated to dryness and later resolved with 0.5% FA in 50% MeOH and transferred to a HPLC vial.

For quantification of phytohormones by LC-ESI-MS/MS, 10 µL samples were injected into an LC system (ACQUITY Ultra-performance LC, Milford, MA, USA) coupled with Xevo TQ MS mass spectrometer from Waters, Milford, MA, USA. The sample analytes (CK) were separated on a CORTECS UPLC®C18+ 1.6 µm, 2.1 mm × 100 mm (Waters, Milford, MA, USA) coupled with a VanGuard pre-column (Waters, Milford, MA, USA) CORTECS C18+ 1.6µm, 2.1 mm × 5 mm. The column temperature was set to 40 °C. LC and MS method are described in detail in the supporting information.

4.6. Accession Numbers

HvCKX2.1: MLOC_53923, MOREX_Contig_38742, located on Chr. 3: 99654033-658338.

Supplementary Materials: The following are available online at www.mdpi.com/2075-4655/1/2/9/s1. Table S1: Standards used for PH and the detailed method, Table S2: Primers used in this study. Figure S1: Sequence and homology of identified 24mers to *ProCKX2.1*; Figure S2: Gene and promotor structure of *HvCKX2.1*; Figure S3: Representative picture of performed germination assay; Figure S4: DPA (diphaseic acid) content during germination in stressed and control seeds/plants frombarley *Hordeum vulgare* cv. Golden Promise.

Acknowledgments: We thank Mandy Püffeld, Jana Lorenz and Gabriele Einert for excellent technical assistance. The junior research group Abiotic Stress Genomics was funded by IZN (Interdisciplinary Center for Crop Plant Research, Halle (Saale), Germany). K.S. and C.S. were funded by BMBF (INNOGRAIN MALT). V.T.H. was funded by BMBF (GABI-GRAIN: Integrative genomics approach for exploring seed quality and yield under terminal drought; FKZ 0315041A and FKZ 0315041C). M.K. is funded by the WCH Science Campus Halle (IDRIB project). We thank Rhonda Meyer for critical reading of the manuscript.

Author Contributions: M.K., N.S., C.S. and N.v.W. conceived and designed the experiments; K.S. K.E., T.H., M.K. and M.-R.H. performed the experiments; M.K. and C.S., K. S., K.E., M.-R.H. analyzed the data; and M.K., C.S. and K.S. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Loss, S.P.; Siddique, K.H.M. Morphological and physiological traits associated with wheat yield increases in mediterranean environments. *Adv. Agron.* **1994**, *52*, 229–276.
2. Fereres, E.; Orgaz, F.; Gonzalez-Dugo, V. Reflections on food security under water scarcity. *J. Exp. Bot.* **2011**, *62*, 4079–4086. [CrossRef] [PubMed]
3. Overpeck, J.; Udall, B. Dry times ahead. *Science* **2010**, *328*, 1642–1643. [CrossRef] [PubMed]
4. Govind, G.; Seiler, C.; Wobus, U.; Sreenivasulu, N. Importance of aba homeostasis under terminal drought stress in regulating grain filling events. *Plant Signal Behav.* **2011**, *6*, 1228–1231. [CrossRef] [PubMed]
5. Sreenivasulu, N.; Harshavardhan, V.T.; Govind, G.; Seiler, C.; Kohli, A. Contrapuntal role of aba: Does it mediate stress tolerance or plant growth retardation under long-term drought stress? *Gene* **2012**, *506*, 265–273. [CrossRef] [PubMed]
6. Radchuk, V.V.; Sreenivasulu, N.; Radchuk, R.I.; Wobus, U.; Weschke, W. The methylation cycle and its possible functions in barley endosperm development. *Plant Mol. Biol.* **2005**, *59*, 289–307. [CrossRef] [PubMed]
7. Kohler, C.; Wolff, P.; Spillane, C. Epigenetic mechanisms underlying genomic imprinting in plants. *Annu. Rev. Plant Biol.* **2012**, *63*, 331–352. [CrossRef] [PubMed]
8. Matzke, M.A.; Kanno, T.; Matzke, A.J. Rna-directed DNA methylation: The evolution of a complex epigenetic pathway in flowering plants. *Annu. Rev. Plant Biol.* **2015**, *66*, 243–267. [CrossRef] [PubMed]
9. Wang, Q.L.; Li, Z.H. The functions of micrornas in plants. *Front. Biosci.* **2007**, *12*, 3975–3982. [PubMed]
10. Rubio-Somoza, I.; Weigel, D. Microrna networks and developmental plasticity in plants. *Trends Plant Sci.* **2011**, *16*, 258–264. [CrossRef] [PubMed]
11. Wang, N.; Ning, S.; Wu, J.; Tagiri, A.; Komatsuda, T. An epiallele at cly1 affects the expression of floret closing (cleistogamy) in barley. *Genetics* **2015**, *199*, 95–104. [CrossRef] [PubMed]
12. Houston, K.; McKim, S.M.; Comadran, J.; Bonar, N.; Druka, I.; Uzrek, N.; Cirillo, E.; Guzy-Wrobelska, J.; Collins, N.C.; Halpin, C.; et al. Variation in the interaction between alleles of HvAPETALA2 and microRNA172 determines the density of grains on the barley inflorescence. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 16675–16680. [CrossRef] [PubMed]
13. Ferdous, J.; Hussain, S.S.; Shi, B.J. Role of micrornas in plant drought tolerance. *Plant Biotechnol. J* **2015**, *13*, 293–305. [CrossRef] [PubMed]
14. Martinez de Alba, A.E.; Elvira-Matelot, E.; Vaucheret, H. Gene silencing in plants: A diversity of pathways. *Biochim. Biophys. Acta* **2013**, *1829*, 1300–1308. [CrossRef] [PubMed]
15. Mallory, A.; Vaucheret, H. Form, function, and regulation of argonaute proteins. *Plant Cell* **2010**, *22*, 3879–3889. [CrossRef] [PubMed]
16. He, X.-J.; Chen, T.; Zhu, J.-K. Regulation and function of DNA methylation in plants and animals. *Cell Res.* **2011**, *21*, 442–465. [CrossRef] [PubMed]
17. Matzke, M.; Kanno, T.; Huettel, B.; Daxinger, L.; Matzke, A.J.M. Targets of rna-directed DNA methylation. *Curr. Opin. Plant Biol.* **2007**, *10*, 512–519. [CrossRef] [PubMed]
18. Kalladan, R.; Worch, S.; Rolletschek, H.; Harshavardhan, V.T.; Kuntze, L.; Seiler, C.; Sreenivasulu, N.; Roder, M.S. Identification of quantitative trait loci contributing to yield and seed quality parameters under terminal drought in barley advanced backcross lines. *Mol. Breed.* **2013**, *32*, 71–90. [CrossRef]
19. Seiler, C.; Harshavardhan, V.T.; Reddy, P.S.; Hensel, G.; Kumlehn, J.; Eschen-Lippold, L.; Rajesh, K.; Korzun, V.; Wobus, U.; Lee, J.; et al. Abscisic acid flux alterations result in differential aba signalling responses and impact assimilation efficiency in barley under terminal drought stress. *Plant Physiol.* **2014**. [CrossRef] [PubMed]
20. Zawada, A.M.; Rogacev, K.S.; Muller, S.; Rotter, B.; Winter, P.; Fliser, D.; Heine, G.H. Massive analysis of cDNA ends (mace) and miRNA expression profiling identifies proatherogenic pathways in chronic kidney disease. *Epigenetics* **2014**, *9*, 161–172. [CrossRef] [PubMed]

21. Kuhlmann, M. DNA methylation of the CKX2.1 promotor by terminal drought stress in barley. *e!DAL* **2017**. [CrossRef]
22. Arend, D.; Junker, A.; Scholz, U.; Schuler, D.; Wylie, J.; Lange, M. PGP repository: A plant phenomics and genomics data publication infrastructure. *Database (Oxford)* **2016**, *2016*.
23. Law, J.A.; Jacobsen, S.E. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat. Rev. Genet.* **2010**, *11*, 204–220. [PubMed]
24. Zhai, J.; Liu, J.; Liu, B.; Li, P.; Meyers, B.C.; Chen, X.; Cao, X. Small RNA-directed epigenetic natural variation in arabidopsis thaliana. *PLoS Genet.* **2008**, *4*, e1000056.
25. Mascher, M.; Muehlbauer, G.J.; Rokhsar, D.S.; Chapman, J.; Schmutz, J.; Barry, K.; Munoz-Amatriain, M.; Close, T.J.; Wise, R.P.; Schulman, A.H.; et al. Anchoring and ordering NGS contig assemblies by population sequencing (POPSEQ). *Plant J.* **2013**, *76*, 718–727. [PubMed]
26. McGaw, B.A.; Horgan, R. Cytokinin oxidase from *Zea mays* kernels *Andvinca rosea* crown-gall tissue. *Planta* **1983**, *159*, 30–37. [PubMed]
27. Mameaux, S.; Cockram, J.; Thiel, T.; Steuernagel, B.; Stein, N.; Taudien, S.; Jack, P.; Werner, P.; Gray, J.C.; Greenland, A.J.; et al. Molecular, phylogenetic and comparative genomic analysis of the cytokinin oxidase/dehydrogenase gene family in the *Poaceae*. *Plant Biotechnol. J.* **2012**, *10*, 67–82. [PubMed]
28. Zalewski, W.; Gasparis, S.; Boczkowska, M.; Rajchel, I.K.; Kala, M.; Orczyk, W.; Nadolska-Orczyk, A. Expression patterns of *HvCKX* genes indicate their role in growth and reproductive development of barley. *PLoS ONE* **2014**, *9*, e115729.
29. Llorens, C.; Futami, R.; Covelli, L.; Dominguez-Escriba, L.; Viu, J.M.; Tamarit, D.; Aguilar-Rodriguez, J.; Vicente-Ripolles, M.; Fuster, G.; Bernet, G.P.; et al. The GYPSY database (GYDB) of mobile genetic elements: Release 2.0. *Nucleic Acids Res.* **2011**, *39*, D70–D74. [PubMed]
30. Chang, T.H.; Huang, H.Y.; Hsu, J.B.K.; Weng, S.L.; Horng, J.T.; Huang, H.D. An enhanced computational platform for investigating the roles of regulatory rna and for identifying functional RNA motifs. *BMC Bioinform.* **2013**, *14*.
31. Zhang, H.; He, X.; Zhu, J.K. RNA-directed DNA methylation in plants: Where to start? *RNA Biol.* **2013**, *10*, 1593–1596. [CrossRef] [PubMed]
32. Galuszka, P.; Frebortova, J.; Werner, T.; Yamada, M.; Strnad, M.; Schmulling, T.; Frebort, I. Cytokinin oxidase/dehydrogenase genes in barley and wheat: Cloning and heterologous expression. *Eur. J. Biochem.* **2004**, *271*, 3990–4002. [CrossRef] [PubMed]
33. Zalewski, W.; Galuszka, P.; Gasparis, S.; Orczyk, W.; Nadolska-Orczyk, A. Silencing of the *HvCKX1* gene decreases the cytokinin oxidase/dehydrogenase level in barley and leads to higher plant productivity. *J. Exp. Bot.* **2010**, *61*, 1839–1851. [CrossRef] [PubMed]
34. Zalewski, W.; Orczyk, W.; Gasparis, S.; Nadolska-Orczyk, A. *HvCKX2* gene silencing by biolistic or agrobacterium-mediated transformation in barley leads to different phenotypes. *BMC Plant Biol.* **2012**, *12*, 206. [CrossRef] [PubMed]
35. Mrizova, K.; Jiskrova, E.; Vyroubalova, S.; Novak, O.; Ohnoutkova, L.; Pospisilova, H.; Frebort, I.; Harwood, W.A.; Galuszka, P. Overexpression of cytokinin dehydrogenase genes in barley (*Hordeum vulgare* cv. Golden promise) fundamentally affects morphology and fertility. *PLoS ONE* **2013**, *8*, e79029. [CrossRef] [PubMed]
36. Petryszak, R.; Keays, M.; Tang, Y.A.; Fonseca, N.A.; Barrera, E.; Burdett, T.; Fullgrabe, A.; Fuentes, A.M.; Jupp, S.; Koskinen, S.; et al. Expression atlas update–an integrated database of gene and protein expression in humans, animals and plants. *Nucleic Acids Res.* **2016**, *44*, D746–D752. [CrossRef] [PubMed]
37. Mayer, K.F.X.; Waugh, R.; Langridge, P.; Close, T.J.; Wise, R.P.; Graner, A.; Matsumoto, T.; Sato, K.; Schulman, A.; Muehlbauer, G.J.; et al. A physical, genetic and functional sequence assembly of the barley genome. *Nature* **2012**, *491*, 711–716. [PubMed]
38. Malito, E.; Coda, A.; Bilyeu, K.D.; Fraaije, M.W.; Mattevi, A. Structures of Michaelis and product complexes of plant cytokinin dehydrogenase: Implications for flavoenzyme catalysis. *J. Mol. Biol.* **2004**, *341*, 1237–1249. [CrossRef] [PubMed]
39. Sakakibara, H. Cytokinins: Activity, biosynthesis, and translocation. *Annu. Rev. Plant Biol.* **2006**, *57*, 431–449. [CrossRef] [PubMed]
40. Graeber, K.; Nakabayashi, K.; Miatton, E.; Leubner-Metzger, G.; Soppe, W.J. Molecular mechanisms of seed dormancy. *Plant Cell Environ.* **2012**, *35*, 1769–1786. [CrossRef] [PubMed]

41. Wang, Y.; Li, L.; Ye, T.; Zhao, S.; Liu, Z.; Feng, Y.Q.; Wu, Y. Cytokinin antagonizes ABA suppression to seed germination of arabidopsis by downregulating abi5 expression. *Plant J.* **2011**, *68*, 249–261. [CrossRef] [PubMed]
42. Seiler, C.; Harshavardhan, V.T.; Rajesh, K.; Reddy, P.S.; Strickert, M.; Rolletschek, H.; Scholz, U.; Wobus, U.; Sreenivasulu, N. ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought-stress conditions. *J. Exp. Bot.* **2011**, *62*, 2615–2632. [CrossRef] [PubMed]
43. Mehrotra, R.; Bhalothia, P.; Bansal, P.; Basantani, M.K.; Bharti, V.; Mehrotra, S. Abscisic acid and abiotic stress tolerance—Different tiers of regulation. *J. Plant Physiol.* **2014**, *171*, 486–496. [CrossRef] [PubMed]
44. Demetriou, K.; Kapazoglou, A.; Tondelli, A.; Francia, E.; Stanca, M.A.; Bladenopoulos, K.; Tsaftaris, A.S. Epigenetic chromatin modifiers in barley: I. Cloning, mapping and expression analysis of the plant specific HD2 family of histone deacetylases from barley, during seed development and after hormonal treatment. *Physiol. Plant.* **2009**, *136*, 358–368. [CrossRef] [PubMed]
45. Kapazoglou, A.; Tondelli, A.; Papaefthimiou, D.; Ampatzidou, H.; Francia, E.; Stanca, M.A.; Bladenopoulos, K.; Tsaftaris, A.S. Epigenetic chromatin modifiers in barley: IV. The study of barley polycomb group (*PCG*) genes during seed development and in response to external ABA. *BMC Plant Biol.* **2010**, *10*, 73. [CrossRef] [PubMed]
46. Ito, H.; Kim, J.M.; Matsunaga, W.; Saze, H.; Matsui, A.; Endo, T.A.; Harukawa, Y.; Takagi, H.; Yaegashi, H.; Masuta, Y.; et al. A stress-activated transposon in arabidopsis induces transgenerational abscisic acid insensitivity. *Sci. Rep.* **2016**, *6*, 23181. [CrossRef] [PubMed]
47. Lewsey, M.G.; Hardcastle, T.J.; Melnyk, C.W.; Molnar, A.; Valli, A.; Urich, M.A.; Nery, J.R.; Baulcombe, D.C.; Ecker, J.R. Mobile small rnas regulate genome-wide DNA methylation. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E801–E810. [CrossRef] [PubMed]
48. Li, Q.; Gent, J.I.; Zynda, G.; Song, J.; Makarevitch, I.; Hirsch, C.D.; Hirsch, C.N.; Dawe, R.K.; Madzima, T.F.; McGinnis, K.M.; et al. RNA-directed DNA methylation enforces boundaries between heterochromatin and euchromatin in the maize genome. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 14728–14733. [CrossRef] [PubMed]
49. Movahedi, A.; Sun, W.; Zhang, J.; Wu, X.; Mousavi, M.; Mohammadi, K.; Yin, T.; Zhuge, Q. RNA-directed DNA methylation in plants. *Plant Cell Rep.* **2015**, *34*, 1857–1862. [CrossRef] [PubMed]
50. Huang, Y.; Cai, S.; Ye, L.; Hu, H.; Li, C.; Zhang, G. The effects of GA and ABA treatments on metabolite profile of germinating barley. *Food Chem.* **2016**, *192*, 928–933. [CrossRef] [PubMed]
51. Leymarie, J.; Robayo-Romero, M.E.; Gendreau, E.; Benech-Arnold, R.L.; Corbineau, F. Involvement of ABA in induction of secondary dormancy in barley (*Hordeum vulgare* L.) seeds. *Plant Cell Physiol.* **2008**, *49*, 1830–1838. [CrossRef] [PubMed]
52. Bokszczanin, K.L.; Krezdorn, N.; Fragkostefanakis, S.; Muller, S.; Rycak, L.; Chen, Y.; Hoffmeier, K.; Kreutz, J.; Paupiere, M.J.; Chaturvedi, P.; et al. Identification of novel small ncRNAs in pollen of tomato. *BMC Genom.* **2015**, *16*, 714. [CrossRef] [PubMed]
53. Finke, A.; Kuhlmann, M.; Mette, M.F. Idn2 has a role downstream of sirna formation in rna-directed DNA methylation. *Epigenetics* **2012**, *7*, 950–960. [CrossRef] [PubMed]
54. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45. [CrossRef] [PubMed]
55. Kojima, M.; Kamada-Nobusada, T.; Komatsu, H.; Takei, K.; Kuroha, T.; Mizutani, M.; Ashikari, M.; Ueguchi-Tanaka, M.; Matsuoka, M.; Suzuki, K.; et al. Highly sensitive and high-throughput analysis of plant hormones using MS-probe modification and liquid chromatography-tandem mass spectrometry: An application for hormone profiling in *Oryza sativa*. *Plant Cell Physiol.* **2009**, *50*, 1201–1214. [CrossRef] [PubMed]
56. Seo, M.; Jikumaru, Y.; Kamiya, Y. Profiling of hormones and related metabolites in seed dormancy and germination studies. *Methods Mol. Biol.* **2011**, *773*, 99–111. [PubMed]