Abscisic acid flux alterations result in differential ABA signalling responses and impact assimilation efficiency in barley under terminal drought stress

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ABA dose dependent orchestration of ABA signalling genes

ABSTRACT

ABA is a central player in plants response to drought stress. How variable levels of ABA under short-term versus long-term drought stress impact assimilation and growth in crops is unclear. We addressed this through comparative analysis, using two elite breeding lines of barley that show senescence or stay-green phenotype under terminal drought stress and by making use of transgenic barley lines that express AtNCED (Arabidopsis thaliana 9-cis-epoxycarotenoid dioxygenase) coding sequence or an RNAi sequence of ABA8'-OH (ABA 8'-hydroxylase) under the control of a drought-inducible barley promoter. The high levels of ABA and its catabolites in the senescing breeding line under long-term stress were detrimental for assimilate productivity whereas these levels were not perturbed in the stay-green type that performed better. In transgenic barley, drought-inducible AtNCED expression afforded temporal control in ABA levels such that the ABA levels rose sooner than in wild-type (WT) plants but also subsided, unlike as in WT, to near-basal levels upon prolonged stress treatment due to downregulation of endogenous HvNCED genes. The interdiction of ABA catabolism with RNAi approach of ABA8'-OH had lower ABA flux during the entire period of stress. These transgenic plants performed better than the WT under stress to maintain a favourable instantaneous water use efficiency and better assimilation. Gene expression analysis, protein structural modeling, and protein-protein interaction analyses of the members of the PYR/PYL/RCAR, PP2C, SnRK2, and ABI5/ABF family identified specific members that could potentially impact ABA metabolism and stress adaptation in barley.

Key words: Abscisic acid homeostasis, ABA signalling, seed filling, stay-green and senescing, terminal drought
INTRODUCTION

Drought compromises grain yield in cereals, especially when the stress occurs during post-anthesis (Boyer and Westgate, 2004; Sreenivasulu et al., 2007). In some crops, selection for and use of “stay green” types have been promoted as a means for combating drought stress susceptibility (Thomas and Howarth, 2000). However, the molecular basis of stay-green phenotype in barley has not been explored in detail. As the flag leaf is the principal source organ, sustaining its photosynthetic activity under post-anthesis drought stress is considered a strategy to mitigate the yield penalty. Water use efficiency (WUE) is the amount of biomass (carbon) accumulated per unit of water, hence an important trait under water-limited conditions (Condon and Richards, 1992; Rebetzke et al., 2002; Richards et al., 2002). Breeding for WUE is considered important for developing drought-tolerant crops (Blum, 1996; Richards, 1996; Richards et al., 2002). Genetic loci that control transpiration efficiency have been identified (Teulat et al., 2002; Hall et al., 2005; Juenger et al., 2005). In Arabidopsis, ERECTA as well as HARDY (encoding an AP2/ERF-like transcription factor) genes influence transpiration efficiency (Masle et al., 2005; Karaba et al., 2007).

Abscisic acid (ABA) synthesis is a universal response of plants to drought, and this triggers major reprogramming of the transcriptome, stomatal closure and restraint on transpirational water loss (Christmann et al., 2007; Cutler et al., 2010; Raghavendra et al., 2010). But this adaptation for survival inevitably reduces photosynthesis, grain filling and grain yield. There is very little information on how WUE and other physiological parameters are influenced in genotypes that differ in ABA homeostasis. There have been several studies on altering ABA levels by overexpression of the ABA biosynthesis or catabolism genes using constitutive promoters. For example, transgenic overexpression of NCED genes in tomato, Arabidopsis, bean and cowpea lead to enhanced ABA content in leaves or whole plants and reduced transpiration (Thompson et al., 2000; Iuchi et al., 2001; Qin and Zeevaart, 2002; Aswath et al., 2005). However, ubiquitous expression causes growth retardation. Improved drought tolerance was observed when transgenic tobacco and Arabidopsis plants were subjected to stress by withholding irrigation (Iuchi et al., 2001; Qin and Zeevaart, 2002). However, the impact on yield was not reported. Overexpression of the genes for ABA catabolic enzymes, CYP707A or ABA8’OH, decreased the ABA levels and caused an increase in the phaseic acid (PA) content (Millar et al., 2006; Umezawa et al., 2006; Yang and Zeevaart, 2006; Ji et al., 2011). When ABA8’OH was down-regulated in barley by an RNAi approach, the ABA
content increased (Gubler et al., 2008), but there is no information on the performance of these transgenics under stress conditions.

There is now a wealth of information on ABA signalling components. PYR/PYL/RCAR proteins function as soluble ABA receptors (Ma et al., 2009; Park et al., 2009), and act in concert with type 2C protein phosphatase (PP2C)-SnRK2 protein kinase complex (Umezawa et al., 2009; Vlad et al., 2009; Umezawa et al., 2010). Phosphorylated SnRK2 appears to be required for the activation of the ABA-induced transcriptional cascade. In the simplest model, binding of PYR/PYL/RCAR to ABA promotes interaction with PP2C, thereby inhibiting the phosphatase activity of PP2C; as a result, phosphorylated SnRK2s can activate the relevant transcription factors (TFs) (Kline et al., 2010). An increase in endogenous ABA resulting from environmental and/or developmental cues would then lead to the sequestration of PP2C and resultant activation of SnRK2s and their downstream AREB/ABF bZIP proteins and anion channels (Cutler et al., 2010; Hubbard et al., 2010; Klingler et al., 2010; Umezawa et al., 2010). The complete pathway from perception by PYR/PYL/RCARs to the activation of AREB/ABFs has been validated by transient expression experiments in Arabidopsis mesophyll protoplasts (Fujii et al., 2009). PYR/PYL/RCAR protein family members have been identified in rice, maize, sorghum, soybean, grapevine, citrus, and tomato (Klingler et al., 2010; Kim et al., 2011; Sun et al., 2011; Boneh et al., 2012; Romero et al., 2012). However, there is only sparse information on transcriptional regulation of diverse members of ABA receptors, especially in response to different levels of ABA (McCourt and Creelman, 2008; Umezawa et al. 2012).

How different levels of ABA impact ABA perception via the family of receptors and affect WUE in crop plants remain to be systematically explored. In this study, we addressed ABA homeostasis and WUE in barley. We employed a pair of breeding lines having a contrasting phenotypic response to drought. One line senesces while the other line shows a stay-green phenotype. Additionally, we investigated transgenic lines that we generated wherein ABA metabolism was altered by promoting biosynthesis or by diminishing catabolism under the control of drought-inducible promoter. These investigations afforded a hitherto unavailable account of ABA dosage-dependent responses in the ABA signalosome vis-à-vis WUE over short-term and long-term drought conditions. The information on a subset of ABA receptors will also be useful toward engineering cereal crops adapted to climate change.
RESULTS

Putative ABA signalling related genes in barley

A set of putative ABA signalling genes in barley was defined on the basis of the sequence homology to Arabidopsis genes. Rice orthologues were included to provide a monocotyledonae reference. The Arabidopsis PYR/PYL/RCAR family has 14 members; of these 13 act to inhibit the function of PP2Cs that act as negative regulators of ABA response. In barley, nine orthologues were identified (HvPYR/PYL1 through 9), of which eight were present as full length sequences (Table 1). PYR/PYL/RCAR proteins have a Bet v I domain that provides a scaffold for binding hydrophobic ligands. All Bet v I proteins have been grouped within the large START domain superfamily (Iyer et al., 2001; Radauer et al., 2008). The residues required for ABA binding are located within domains that form two loops around the ABA molecule. The one having a consensus sequence SGLPA is referred to as the “proline gate”, and the other with the sequence GG(E/D)HRL is known as “leucine latch” (Melcher et al., 2009; Santiago et al., 2009a). Both structures are generally well-conserved in the set of HvPYR/PYL proteins (Supplemental Fig. S1A), although within the proline gate, an S residue is replaced by T in HvPYR/PYL1 and an A by G in HvPYR/PYL9; within the HvPYR/PYL2 leucine latch, GGEHRL appears as DGNHPL. The phylogenetic analysis shown in Fig. 1A suggested that HvPYR/PYL1 and HvPYR/PYL2 are both members of the same subfamily 1 as Arabidopsis AtPYL7 through 10, and HvPYR/PYL5, 6 and 8 are similar to Arabidopsis subfamily 3 (AtPYR1, AtPYL1 through 3). HvPYR/PYL3, HvPYR/PYL4, HvPYR/PYL7 and HvPYR/PYL9 cluster with three rice proteins to form a separate clade. No HvPYR/PYL proteins could be assigned to Arabidopsis subfamily 2 comprising AtPYL4 through 6 and AtPYL11 through 13.

Six PP2C orthologues were identified (HvPP2C1 through 6) (Fig. 1B). Eleven conserved motifs have been associated with PP2C proteins (Bork et al., 1996). All these motifs are present in the deduced barley proteins, with the exception of HvPP2C3 containing only motifs 1 to 5, and HvPP2C6 lacking motifs 1, 3 and 9. (Supplemental Fig. S1B). These two proteins also lack the conserved tryptophan (W) residue, which is inserted between the PYR/PYL/RCAR proline gate and leucine latch regions (Supplemental Fig. S1B).

The Arabidopsis SnRK2 family consists of ten genes (Boudsocq et al., 2004). Of these, AtSnRK2.2, 2.3 and 2.6 (all belonging to subfamily III) are directly involved in ABA signal
perception. The barley orthologues comprised nine full length sequences. One of these, designated as *HvPKABA1/SnRK2.1*, has been characterized previously (Yamauchi et al., 2002). We designated the additional ones as *HvSnRK2.2* through 2.9. These formed three phylogenetic clusters (Fig. 1C): SnRK2.2, 2.3 and 2.9 (subfamily 1); SnRK2.4, 2.5, and 2.6 (subfamily 3); PKABA1/SnRK2.1, SnRK2.7 and 2.8 (subfamily 2). ABA-dependent activation of the kinase and its interaction with ABI1 requires an Asp-rich domain (Domain II) in the C-terminus (Belin et al., 2006; Yoshida et al., 2006). Domain II was found in the C-terminal region in PKABA1/SnRK2.1, SnRK2.4, SnRK2.5 and SnRK2.6 and SnRK2.8 (Supplemental Fig. S1C).

Members of the ABI5/ABF sub-family of bZIP transcription factors mediate ABA-induced transcription by interacting with ABA-response promoter elements (ABREs). Barley ABI5, ABF1, ABF2 and ABF3 have already been described as members of the bZIP transcription factor family and we identified one additional partial clone (named HvABF4, Fig. 1D). HvABI5 forms a subgroup with AtABF2 and AtABF3 in phylogenetic comparisons while HvABF1 seems to be orthologous to AtABI5 (Fig. 1D).

**The “stay-green” and “senescing” types of barley show differential photosynthetic efficiency and WUE under terminal drought**

Senescence is a normal and eventual response of plants experiencing terminal drought. Screening 16 elite barley breeding lines for terminal drought tolerance identified a line that showed stay-green (LP103) phenotype. This stay-green line displayed a reasonable level of drought tolerance and produced a superior grain weight (Fig. 2 and Supplemental Fig. S2). The other lines senesced and gave lower yields. Among them LP110 was chosen as a representative of senescing lines. Withholding water for three weeks induced senescence in the leaves and spikes of line LP110 (Fig. 2B). In contrast, LP103 plants remained green for longer duration before senescence ensued under post-anthesis drought (Fig. 2A). Stomatal conductance, transpiration and assimilation were markedly inhibited by drought in the senescing line, but assimilation was maintained at a higher level in the stay-green line than in the senescing line under stress (Fig. 2E-H). The WUE in senescing plants but not in stay-green ones was reduced by one week of drought stress exposure (Fig. 2H). The maintenance of WUE allowed the drought stressed stay-green plants to produce larger grains in contrast to the senescing ones (Figs. 2D). This result was reproducible over two cropping seasons. The
thousand grain weight (TGW) in the stay-green type, unlike the senescing genotype, was not compromised in drought conditions (Fig. 2D).

**Altered ABA flux and expression dynamics of ABA perception and sensor genes in stay-green and senescing lines**

ABA accumulated to higher levels (15-fold) in the flag leaf of drought-stressed senescing line than in the stay-green line. The difference was most pronounced in the early part of the grain filling period when the plants had not been exposed to four days of drought stress (Fig. 2J). By 12 days after stress (DAS) the ABA level in the flag leaf of the senescing line began to fall, but still remained at a higher level than in the control plants that had been watered regularly. The behaviour of the stay-green plants was different, throughout the period of stress (4 and 12 DAS) the flag leaf ABA content had increased only about three-fold over the level in irrigated plants. This level remained steady throughout the stress period (Fig. 2J). Both the assimilation rate and WUE were sustained better in stay-green line at 4 and 8 DAS (Fig. 2E and H). The flag leaf tissue in drought-stressed senescing plants at 8 DAS contained more ABA catabolites [dihydrophaseic acid (DPA), 2.7-fold] and an inactive form of ABA [ABA-glucose ester (ABA-GE), 4.5-fold] (Fig. 2I). The stay-green line did not show this effect. Thus, ABA metabolism in the senescing genotype was highly sensitive to drought stress, while it was relatively unperturbed in the stay-green type with well balanced ABA homeostasis.

Transcription of genes associated with ABA synthesis, degradation and de-conjugation was quantified using qRT-PCR (Supplemental Fig. S3). In congruence with the increase in the ABA content, *HvNCED2* transcript abundance in the flag leaf of the senescing line was six-fold higher in stressed plants than in irrigated control plants at 8 DAS. By 12 DAS, however, *NCED2* transcription had diminished, while *HvBG8* expression had up-regulated, suggesting that increased ABA flux might operate through deconjugation events (Fig. 2I and Supplemental Fig. S3). In contrast, ABA homeostasis in the stay-green line was maintained via the moderate induction of *HvNCED2* and *HvBG8*.

The transcription profiles of ABA receptor/signalling genes also showed contrasting regulation in stay-green and senescing plants. At 8 DAS, only *HvPYR/PYL5 and 7* were upregulated in drought stressed flag leaf of the stay-green line. During this stage, *HvPYR/PYL3, 4, 5, 6 and 8* were all down-regulated in drought stressed flag leaf of the
sensing line (Fig. 2K and Supplemental Fig. S4). Recall that ABA catabolite levels in plants under these conditions were elevated in stressed senescing plants but not in stay-green plants (Fig. 2I). Notably, five of the six genes (*HvPYR/PYL3, 4, 5, 6 and 8*) that we monitored were up-regulated in drought stressed flag leaf at 12 DAS in senescing line plants while none of them showed this change in the stay-green type (Supplemental Fig. S4). This suggests that the two lines differ in their capacity to bind ABA and/or in their sensitivity to ABA. Despite the difference in the ABA levels in the flag leaf, most of the *PP2C* and *SnRK2* genes were markedly induced by stress in both stay-green and senescing plants (Supplemental Fig. S4).

**Engineering ABA biosynthesis and catabolism pathways to alter ABA flux under post-anthesis drought**

Two transgenic approaches were undertaken to alter ABA metabolism in barley: (a) Overexpression of *AtNCED6* that encodes the key enzyme for ABA biosynthesis, and (b) RNAi silencing to repress the endogenous *ABA 8'-hydroxylase* genes as a means to curtail ABA degradation. Both transgene constructs were placed under control of the barley *Lea (late embryogenesis abundant)* B19.3 promoter, which is induced in vegetative tissues during post-anthesis drought stress. Two independent homozygous transgenic lines of each construct were used for evaluation of the physiological performance under terminal drought stress. The lines will be referred to as LN for the *HvLea::AtNCED6* construct and LOHi for *HvLea::Hv8'-hydroxylase* RNAi construct, respectively. In general, transgenic plants performed better than the parental line (referred to as wild-type WT) under stress. Both LN and LOHi lines, possessed a higher relative leaf water content at 4 DAS (Fig. 3A). While stress had a negative impact on assimilation in WT, the transgenic LN lines had higher assimilation rate and LOHi lines suffered to a lesser extent (Fig. 3B). Instantaneous WUE (assimilation/transpiration) was not reduced in three of the four transgenic lines when they were subjected to drought stress. There was even an increase in LN transgenic lines (Fig 3C). This contrasted the reduction seen in WT and one of the two LOHi lines. These results show effective coordination of assimilation with the balanced transpiration in LN plants under drought stress.

Expression of the transgene *AtNCED6* in flag leaves was detected as early as 0.5 DAS, and highest expression was observed at 8 DAS in both overexpression lines (LN39 and LN51), with nearly 2-fold increase under stress in comparison with control conditions (Fig. 4A). In the RNAi lines (LOHi236 and LOHi272), reduction in *HvABA8’OH1* transcript levels in flag leaves was observed mainly under long-term stress (12 DAS), with a clear reduction of 52%...
and 42% in LOHi236 and LOHi272 lines, respectively. Among three \textit{HvABA 8’-hydroxylase} genes, only \textit{HvABA8’OH-1} is induced under stress in WT (Fig. 4B inset). Therefore, we focused on \textit{HvABA8’OH-1}.

We measured the ABA content in the flag leaf of WT and transgenic lines under control and drought stress (0.5-12 DAS). This analysis showed that, under the conditions of short-term drought stress at 2 DAS, much more ABA was accumulated in \textit{AtNCED6} overexpression lines than in WT or in \textit{ABA hydroxylase} RNAi lines (Fig. 4C). In WT, the highest level of ABA response was observed two days later than in LN39 (4 DAS, 17-fold) and these WT plants maintained higher ABA levels under long-term stress (12 DAS). In contrast, by 4 DAS onwards LN39 plants had reduced their ABA levels and under prolonged stress maintained the ABA content at a near-basal level. This was also reflected in LN39 transgenic line where downregulation of endogenous \textit{HvNCED1} gene is noted (Supplemental Fig. S5 and Supplemental Table S3). In the LOHi line, ABA levels did not reach as high as in WT under long-term stress (4-12 DAS).

To understand the dynamics between ABA biosynthesis and degradation under short-term and long-term stress, we analyzed ABA catabolites in WT and in the ABA hydroxylase repression line (LOHi236) at 2 DAS and 12 DAS. In the short term, the two major ABA degradation products PA and DPA increased similarly in WT and LOHi236 (2.8- and 1.8-fold for PA; 1.8- and 1.5-fold for DPA, respectively; Fig. 4D). While, PA (9.2-fold) and DPA (6.4-fold) reached very high levels under long-term stress in WT plants (Fig. 4D), these degradation products accumulated to a lesser extent at 5.5 and 4.8 fold, respectively, in the LOHi236 RNAi plants.

**Expression dynamics of the ABA receptor complex is modulated differentially in transgenic lines with altered ABA flux under terminal drought**

Having determined that \textit{AtNCED6}-overexpressing lines and \textit{ABA 8’-hydroxylase} RNAi lines differ with respect to altered ABA flux and homeostasis under short-term versus long-term stress during the grain filling period, we investigated regulation of \textit{HvPYR/PYL} family members (Fig. 5). In general, \textit{HvPYR/PYL} members (exception of \textit{HvPYR/PYL1} and \textit{HvPYR/PYL5}) of that family were down regulated in WT and in both transgenic lines under stress (Fig. 5). Under short-term stress (2 DAS), \textit{HvPYR/PYL 5} was up regulated in the flag leaf of line LN39; this coincided with ABA accumulation reaching its peak level. The
transcript levels did not change in the RNAi line. In WT, upregulation of HvPYR/PYL5 was found at 4 DAS when the ABA level had peaked (Fig. 5). Under prolonged drought stress (12 DAS) there were no stress-associated differences in the expression of HvPYR/PYL1 and HvPYR/PYL5 in any of these lines, but transgenic lines show highest repression in LN39 and LOHi236 transgenic lines under long-term stress (Fig. 5 and Supplemental TableS3). These results suggest that WT and transgenic line LN39/LOHi236 likely differ in their source tissues with respect to ABA-binding and/or ABA-sensitivity under short-term drought stress.

In general, transcriptional upregulation under stress was observed for the type 2C protein phosphatases (Supplemental Fig. S6). Notably, the upregulation of PP2Cs in LN39 and LOHi236 was mainly confined to short-term drought stress and in particular the induction of PP2C1, 4 and 5 transcript accumulation was moderate in LOHi236 during 12 DAS. The SnRK2-related protein kinase genes (HvPKABA1/SnRK2.1 and HvSnRK2.7) were upregulated under short-term drought stress in LN39. The relative expression levels of HvPKABA1/SnRK2.1 and HvSnRK2.7 in LOHi236 line were lower during mid-term stress compared to WT and LN39. Notably, this line produced less ABA under stress in comparison to WT.

Three-dimensional modelling of HvPYR/PYL protein structures

As outlined in Methods, we constructed three-dimensional models for three HvPYR/PYL proteins in I-TASSER (Roy et al. 2010): We selected HvPYR/PYL5 (that shows highest homology to Arabidopsis AtPYR1/RCAR11) as well as HvPYR/PYL1 and HvPYR/PYL2 that show some differences in the amino acid sequence of the gate and latch regions, respectively (Fig. 6A). The general structure of the predicted models (best scores) has an α-β-α2-β6-α topology (Fig. 6A) and exhibit very similar helix-grip folds compared to AtPYR1 and AtPYL2, a characteristic of the START protein superfamily (Iyer et al., 2001). Interestingly, HvPYR/PYL5 has an additional α-helix in the N-terminal region (residues 5 – 12) but lacks the small helical segment 3 that is usually followed by six β-strands (Fig. 6B). The largest α-helix consisting of 30 amino acids is located at the C-terminal part of the protein. HvPYR/PYL1, unlike HvPYR/PYL5, does not have β-strands 2 and 3; in HvPYR/PYL2 these two β-strands are predicted as very short segment of only three amino acid residues (Fig. 6A, C and D). Additionally, like HvPYR/PYL5, HvPYR/PYL1 does not contain α-helix 3. The sequence and structure of the gate loop (between β-strands 3 and 4) and the latch region (between β-strands 5 and 6) of HvPYR/PYL5 are identical to those present in
Arabidopsis PYR/PYL/RCARs (Fig. 6B, middle). In contrast, the gate and latch regions differ slightly in HvPYR/PYL1 (Fig. 6C, middle and Supplemental Fig. S1A), and the latch region differs in HvPYR/PYL2 (Fig. 6D, middle and Supplemental Fig. S1A). Although the primary structure does differ slightly, the 3D modeling indicates that the gate and latch regions can have loop-like structures. Fig. 2B-D indicates putative ligand binding sites in the gate and latch regions. It will be interesting to determine if the predicted structural differences influence functional properties.

**Physical interaction of the ABA receptor components**

A yeast two-hybrid (Y2H) assay was conducted to examine interactions between selected HvPYR/PYLs and HvPP2C4. HvPYR/PYL1, HvPYR/PYL2 and HvPYR/PYL5 were fused to the GAL4 DNA binding domain (GBD) and HvPP2C4 was fused to the GAL4 activation domain (GAD) in order to circumvent self-activation of the Y2H gene reporters. After co-transforming the cloned genes in pairs into the yeast strain MaV203, interaction between receptor and phosphatase was tested on medium with (100 µM ABA) or without ABA supplementation. In this study HvPYR/PYL1 interacted with HvPP2C4 in the presence of ABA while a weaker interaction was noted in the absence of ABA. HvPYR/PYL5-HvPP2C4 interaction was ABA-dependent (Fig. 7). In contrast, no interaction was found between HvPYR/PYL2 and HvPP2C4 regardless of ABA addition. Note the amino acid changes in HvPYR/PYL2 in the core latch region that is necessary for ABA binding (Supplemental Fig. S1A). This region seems to be important for interaction with PP2C. In summary, all three tested HvPYR/PYL proteins behaved differently in the Y2H screening.

To validate the interactions *in planta*, bimolecular fluorescence complementation (BiFC) analysis was employed (Fig. 8). Fluorescence was detected predominantly in the cytoplasm and nucleus for the combinations of dimer formation of HvPYR/PYL5, HvPP2C4-HvPYR/PYL5 and HvPP2C4-HvPKABA1/SnRK2.1 complexes. This finding is in agreement with the interaction of Arabidopsis ABI1-AtPYL9, which takes place in the nucleus and cytosol (Ma et al., 2009). However, in rice it was shown recently that the complex of OsPYL/RCAR5-OsPP2C30 formed exclusively in the nucleus (Kim et al., 2011). Adding ABA to the protoplasts caused no changes in the interaction. Imposing desiccation stress with polyethyleneglycol or mannitol treatment of protoplasts also did not change the interactions (Supplemental Fig. S7). The interaction of HvPYR/PYL5 with HvPP2C4 noted using BiFC (Fig. 8), fits with Y2H data. However, in contrast to the Y2H results, no interaction between
HvPYR/PYL1 and HvPP2C4 was detected (data not shown). Additionally, we could demonstrate interaction between HvPP2C4 and HvPKABA1/SnRK2.1 by BiFC (Fig. 8). This combination could not be tested in Y2H due to self-activation problems with both proteins (data not shown). In summary, a complete ABA signalling complex consisting of barley HvPYR/PYL5, PP2C4 and PKABA1/SnRK2.1 is formed in planta, suggesting a highly conserved mechanism in monocots and dicots.

DISCUSSION

Selection for higher WUE using quantitative trait loci (QTL) analysis have been undertaken as approaches to improve drought tolerance (Chen et al., 2011; Zhengbin et al., 2011). However, the genes for the trait have not yet been isolated in cereals. From a physiological perspective, it is well known that ABA accumulation in response to drought reduces transpirational water loss and help plants survive, but under long-term stress it is at the expense of photosynthetic productivity (Yoo et al., 2010). Hence, understanding the fine regulation of stomatal functions by ABA under short-term versus long-term stress is important for devising strategies to improve WUE and drought tolerance in crops (Sreenivasulu et al., 2012). There is clear evidence from studies on isolated leaf epidermis or guard cell protoplasts for the participatory role of ABA signaling complex in affording ABA sensitivity and reduction of water loss. However, the impact of any differences in the ABA content and that of the activity of the ABA signaling components on plant growth and development under conditions of prolonged stress are unclear (Ben-Ari, 2012; Merilo et al., 2013). In our study, we have addressed this by investigating a pair of elite breeding lines of barley: a line that senesces sooner under drought (senescing line; LP110) and a line that remains green for a longer duration (stay-green; LP103) despite the stress. In addition, we have used in this study transgenic barley lines that have been engineered for drought-inducible ABA production or diminution of ABA catabolism to alter ABA homeostasis.

Importance of ABA homeostasis in improving WUE under long-term drought stress

The stay-green and senescing lines differed not only in their assimilation performance under stress but also in ABA metabolism. The senescing line synthesized far greater levels of ABA than the stay-green line under short-term stress and it continued to maintain a high level of ABA and ABA catabolites under long-term stress. Consistent with the observations that ABA
catabolism is triggered to degrade excess ABA (Cutler and Krochko, 1999), we found that ABA and also ABA catabolites accumulated in a senescing breeding line of barley under drought stress (Seiler et al., 2011). This indicates a greater flux in ABA metabolism, affecting WUE and assimilation negatively in the senescing line. In contrast, the relatively modest increase of ABA in the stay-green line was held in check over long-term stress and this homeostasis appeared to help the line sustain WUE and assimilation. Overaccumulation of ABA or constitutive activation of ABA signalling reduces WUE and thus growth (Pizzio et al., 2013; Sreenivasulu et al., 2012; Zhao et al., 2013). For example, overexpression of NCED under the control of rbcS3C promoter, which is highly active in photosynthetic tissues, causes leaf yellowing, reduced chlorophyll content and reduced growth rate (Gittins et al., 2000; Tung et al., 2008). The elevated ABA flux in our senescing line might have resulted in low xanthophyll content. These results emphasize that engineering WUE enhancement in barley entails more subtle changes to ABA metabolism that requires, as a prerequisite, delineation of the ABA signalosome components.

Since we found ABA homeostasis to influence instantaneous WUE distinctly in elite breeding lines, we manipulated endogenous ABA levels in transgenic barley under the control of a drought-inducible promoter. The rise in ABA accumulation under short-term stress in LN39 carrying chimeric AtNCED correlated with the expression of the transgene and also with remodulated expression of HvNCED1 and HvNCED2 genes (Fig. 4, Supplemental Fig. S5). While these plants responded by increasing their ABA content in the short term, they reprogrammed ABA metabolism to maintain a near-basal level over the long-term stress through apparent feedback control via downregulation of HvNCED genes. These events leading to the consequence of held in check the ABA levels under long-term stress. This type of a control over ABA homeostasis was lacking in WT where HvNCED2 expression seemed to contribute to the increase in ABA from 4 to 8 DAS and HvNCED1 expression during 8 and 12 DAS (Supplemental Fig. S5). Thus in WT higher ABA levels remained throughout the stress period (Fig. 4C).

The net accumulation of ABA in LOHi236 (RNAi construct of ABA 8'-OH) was not as high as in AtNCED transgenic line at the early stages (2 DAS). In WT, long-term stress caused an increase in ABA flux but in LOHi236 where ABA catabolism has been repressed to some extent the stress condition had a modest positive effect on net ABA production (Fig. 4D). Taken together with the observations that drought-induced AtNCED6 expression was
associated with downregulation of \textit{HvABA8’OH} genes and that this resulted in improved assimilation and WUE under terminal drought reinforces the notion that plants under long-term stress benefit from modulated ABA homeostasis. These results support the inference that net rate of ABA accumulation in the WT is highly and upwardly responsive to drought, while the dynamics of it is controlled tightly in the transgenic lines enhancing WUE.

Expression dynamics of ABA perception genes under short-term versus long-term drought stress in lines that show differences in ABA flux

Given that many potential members of the ABA signalosome were identified in barley, it was of interest to elucidate their functional aspects. The highest ABA content (342 ng g$^{-1}$ DW) seen in WT barley flag leaf was at 4 DAS, a time point at which higher expression of \textit{PYR/PYL5} in flag leaf was witnessed. However, as a consequence of transgenic expression of \textit{AtNCED} the ABA levels reached 428 ng g$^{-1}$ DW already at 2DAS and under these conditions \textit{PYR/PYL1} and \textit{PYR/PYL5} was also induced (Fig. 5). This suggests that differences in ABA levels can modulate the ABA signalosome by altering the expression of specific receptors in the family. In the RNAi line, where there was a moderate increase in ABA content that was less in comparison to WT, there were no changes in the transcript level. In Arabidopsis, it has been reported that increase in ABA via either exogenous supply or enhanced production under water deficit conditions downregulates \textit{AtPYL8, AtPYL4, AtPYR1} and \textit{AtPYL1} expression; the other members remain constant or show slight upregulation (Santiago et al., 2009b; Szostkiewicz et al., 2010). We found in WT barley no change in the expression of six \textit{HvPYR/PYL} genes under short-term stress but downregulation under prolonged stress (Fig. 5). In the senescing type of barley breeding line, elevated flag leaf ABA content downregulated the transcription of ABA receptors when the plants reached midpoint in drought stress, but \textit{HvPYR/PYL} transcription was again upregulated when the ABA level had been reduced in long-term stress (Fig. S4). No such adjustments in receptor gene expression was found in the stay-green type that maintained ABA homeostasis under long-term stress. Collectively, all these results point to different levels of ABA in short-term versus long-term stress having an impact on perception by various PYR/PYL members that appear to have specialized functions with respect to signalling dynamic cellular ABA content (Miyakawa et al., 2012; Okamoto et al., 2013).

Most barley \textit{PP2C} and \textit{SnRK2} members were prominently upregulated under stress in the senescing and stay-green types, WT and LN39 transgenic line (Supplemental Fig. S4, S6).
regardless of the differences in the ABA content. A similar situation has been reported in Arabidopsis, where \textit{PP2Cs} are elevated while the majority of \textit{PYR/PYL} members are down regulated upon exogenous ABA treatment (Santiago et al., 2009b; Szostkiewicz et al., 2010). These authors also reported that ABA-related stress conditions or treatments alter the ratio of PP2C to PYR/PYL at both transcript and protein levels. Recently, it was postulated that an increase in this ratio might be necessary for activation of the downstream ABA signalling cascade under stress conditions (Chan, 2012). It is interesting to note that \textit{PP2C:PYR/PYL} transcript ratio in WT was preferentially higher under stress, and this correlated with reduced WUE. The RNAi line that had a lower ABA flux than WT under stress showed an unaltered \textit{PP2C:PYR/PYL} ratio; a similar situation was also found in the stay-green line that had a reduced ABA flux.

**Receptor combinations and ABA responses under post-anthesis drought stress**

\textit{PYR/PYL}, PP2C and SnRK2 families comprise many members, and various combinations of them in receptor complexes are conceivable. Structural differences in the members do impact WUE and drought tolerance. In Arabidopsis, overexpression of \textit{PYL4} \textit{A194T} mutant affords stress tolerance (Pizzio et al., 2013); overexpression of \textit{AtPYL1} which is known to differ from other PYLs in its ABA binding pocket leads to improved drought tolerance (Zhao et al., 2013). Thus, some of the fine differences and expression levels in the signalosome components can have physiological consequences.

As shown in this study on barley and in other studies on Arabidopsis (Szostkiewicz et al., 2010; Pizzio et al., 2013; Zhao et al., 2013), the compositions of the receptor complexes affect sensitivity to and selectivity for ABA and impact WUE. The transcriptional activation of a subset of receptors (\textit{HvPYR/PYL1} and \textit{HvPYR/PYL5}) by elevated ABA under short-term stress in LN39 suggests that this complex is pertinent to mediate ABA signalling under these conditions. \textit{HvPYR/PYL5} belongs to subfamily 3 that includes the well characterized \textit{AtPYR1} and \textit{AtPYL1}. These form dimers in Arabidopsis (Miyazono et al., 2009; Santiago et al., 2009b; Yin et al., 2009; Dupeux et al., 2011). Our Y2H and YFP complementation experiments suggest that HvPYR/PYL5 forms a homodimer (Fig. 8). The 3D model for HvPYR/PYL5 protein presented in Fig 6 includes the ABA-binding residues located within the domains that form two loops around the ABA molecule (Melcher et al., 2009; Santiago et al., 2009a). It is known that the Ser residue of the proline gate (“SGLPA”) directly interacts with a catalytic Glu residue of AtABI1 (Miyazono et al., 2009). Thr is present instead of Ser
in HvPYR/PYL1, and it did not dimerize in our analyses. Dimerization might prevent basal activation of the signalling pathway in absence of ABA as the interfaces of the receptors for homodimerization and PP2C-binding are largely overlapping (Dupeux et al., 2011). After binding of ABA, the dimeric proteins dissociate to form the signalling complex. While monomeric receptors have a competitive advantage for binding to ABA and PP2Cs, monomeric and dimeric receptors can form high-affinity complexes with PP2C (Ma et al., 2009; Santiago et al., 2009b).

The monomeric proteins of Arabidopsis, AtPYL5, PYL6 and PYL8 show partial interaction with PP2C in absence of ABA. The dimeric receptors such as PYR1 and PYL1 do not interact under these conditions in Arabidopsis. Our findings in barley suggest a similar scenario as in Arabidopsis; barley PYR/PYL5 known to participate in homodimer formation, interacted with HvPP2C4 only in presence of ABA. In contrast, HvPYR/PYL1 showed weak interaction even in absence of ABA, hinting that the in planta oligomeric state of these two putative receptors is different. AtPYR1 residue H60 plays a key role in determining the oligomeric state of ABA receptors and a H60P substitution leads to destabilization of the PYR1 dimer (Dupeux et al., 2011). HvPYR/PYL1 and HvPYR/PYL2 both contain a “P” residue at the corresponding position (H77 in HvPYR/PYL1 and H53 in HvPYR/PYL2), and this would explain the ABA-independent interaction of PYR/PYL1 with PP2C4 in the Y2H assays. The C-terminus of HvPKABA1/SnRK2 includes an Asp-rich domain (Domain II) that is known to be required for both ABA-dependent activation of the kinase (Belin et al., 2006) and its interaction with ABI1 (Yoshida et al., 2006), and we found interaction between HvPP2C4 and HvPKABA1/SnRK2.1.

The overall implication is that plants employ PYR/PYL-ABA-PPC2 to fine-tune specific ABA signalling pathways in response to the prevailing levels of ABA (Santiago et al., 2012). The three major findings from our study supports general occurrence of this scenario: (1) The PYR/PYL, PP2C and SnRK2 gene family members are generally conserved across the monocot/dicot divide while exhibiting variation at the binding sites in selected members. (2) A drought-sensitive line of barley (senescing type) and the stay-green type differ remarkably in their regulation of PYR/PYLs and also in their ABA levels over the course of post-anthesis drought stress. The drought sensitive plants that had high concentrations of ABA senesced while the stay-green type plants modulated their PYR/PYL expression in the face of lower steady-state levels of ABA and survived longer under terminal drought. (3) Transgenic lines
in which the ABA flux was modulated by expression of NCED (or an RNAi construct of ABA8'OH) under the control of a drought-inducible promoter corroborated the connectivity between ABA homeostasis, WUE and PYR/PYL5:PP2C4 complex. Taken together, the results offer interesting insights into the combinations of active receptor complexes during post-anthesis drought in barley and raise the prospects for improving WUE through conventional breeding of appropriate lines or though targeted transgenic approaches for fine manipulations of ABA flux and perception.

MATERIAL AND METHODS

Barley gene annotation of ABA signalling pathway
Barley genes encoding proteins involved in ABA signalling were identified by a Blastn, Blastx and tBlastx homology search of the 50,000 unigenes represented in the HarvEST database (harvest.ucr.edu) with the known Arabidopsis and rice ABA signalling sequences retrieved from the PubMed and TIGR databases, respectively (http://www.ncbi.nlm.nih.gov; http://rice.plantbiology.msu.edu/). The five best hits of each Blast search were selected, and used to identify corresponding full cDNA sequences, using 24K full-length barley cDNA database (Matsumoto et al., 2011). The in silico translation product of each cDNA was queried for the presence of conserved domains identified in Arabidopsis. Multiple sequence alignments were performed using ClustalW (DNAsstar) and ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2; (Larkin et al., 2007)).

Screening germplasm and breeding material for drought tolerance
A panel of 16 barley lines was screened for their yielding capacity under terminal drought (Supplemental Fig. S2). The plots subjected to terminal drought stress each comprised 50 plants and were randomly arranged in three replications. Well watered control plots were also raised in parallel. A rain-out shelter was installed over the droughted plots at anthesis, and one week later, irrigation was withheld until maturity. The following season, stay-green LP103 (variety QUENCH) and senescing LP110 (variety PASADENA) were re-grown in a ten replicate trial conducted in the same way as above. Grain yield per plot and thousand grain weight (TGW) from both the irrigated and drought stressed plants was obtained (at least ten replicate plots per entry, Fig. 2C).

Production of transgenic material
Vector construction: The Hordeum vulgare Lea B19.3 promoter (740 bp) was amplified from barley genomic DNA using Lea forward 5'-CCGTGTGCACATATACGAT-3' and reverse 5'-TGCACGCTGCTGGGACC-3' primers and high-fidelity DNA Polymerase (Roche, Mannheim, Germany). \textit{AtNCED6} was amplified with high-fidelity DNA Polymerase (Roche, Mannheim, Germany) from Arabidopsis genomic DNA (\textit{NCED} genes lack introns) using gene-specific primers of \textit{NCED6} (forward 5'-CCACCATGCAACACTCTTTCGT-3' and reverse 5'-GATCAGAAATTTGTTCCCTAAC-3'). The following PCR conditions were used: one cycle at 94 °C (2 min), 30 cycles at 94°C (15 s), 56°C (30 s) and 72°C (2 min for \textit{NCED6}, 1 min for \textit{Lea} promoter) followed by extension at 72°C (7 min). Both amplified products were cloned into pCR™4-TOPO® vector (Invitrogen, Karlsruhe, Germany) and confirmed by sequencing. Using the TOPO clones as templates, the promoter region was amplified with primers containing \textit{PstI} and \textit{EcoRI} restriction sites at the 5'- and 3'-end, respectively, and further cloned into pNOS-ABM vector (DNA Cloning Service, Hamburg, Germany) containing the \textit{A. tumefaciens nos} terminator downstream of the multiple cloning site used. \textit{AtNCED6} coding region was amplified with gene-specific primers containing \textit{HindIII} sites from the TOPO vector and subsequently cloned into \textit{Lea-pNOS1} (pNOS-ABM with \textit{Lea} promoter). The complete expression cassette was excised using \textit{SfiI} restriction enzyme and cloned into the binary barley transformation vector p6U (DNA Cloning Service, Hamburg, Germany). Correct orientation was confirmed by sequencing.

For the repression of \textit{HvABA8’OH-1} under control of \textit{Lea B19.3} promoter an RNAi approach was undertaken. First, \textit{Lea} promoter was amplified with primers containing \textit{SpeI} restriction sites and ligated into pNOS-ABM to create \textit{Lea-pNOS2}. A 500 bp fragment of \textit{HvABA8’OH-1} was amplified from the corresponding barley EST clone (HS06M03_contig211107) using primers \textit{HvABA8’OH-1} forward 5'-TGCTCGAGTGGATGGTCAAGTTC-3' and reverse 5'-TTCGGATCCAGGAAGACATAGAT-3' with \textit{XhoI}/\textit{SpeI} restriction sites for the sense and \textit{HvABA8’OH-1} forward 5'-TGGTCGACTGGATGGTCAAGTTC-3' and reverse 5'-TTCAGTTCAGGAAGACATAGAT-3' with \textit{SalI}/\textit{BamHI} restriction sites for the antisense fragment, respectively. The chosen fragment is placed in a rather conserved region of \textit{HvABA8’OH-1} so it is likely that all three \textit{ABA8’OH} genes will be suppressed. Upon PCR amplification [one cycle at 94°C (2 min), 30 cycles at 94°C (15 s), 55°C (30 s) and 72°C (30 s) followed by extension at 72°C (7 min)], both amplified products were subcloned into the pAxi vector derived from pNOS-ABM through insertion of a 200 bp intron flanked by restriction sites. Correct clones were verified by sequencing. The RNAi cassette was subsequently excised from pAxi using \textit{PstI}/\textit{SalI} and ligated into \textit{Lea-pNOS2} (see above).
complete RNAi expression cassette was further excised using SfiI restriction enzyme and cloned into p6U.

Transformation of barley and production of homozygous lines: Stable transformed barley plants (cv. Golden Promise) were obtained by Agrobacterium-mediated transformation following a protocol reported previously (Hensel et al., 2009). In brief, immature embryos were dissected from caryopses harvested 12-16 days after flowering. Upon inoculation and co-culture with Agrobacterium strain AGL-1 harboring an appropriate binary vector as specified above, the explants were grown on media supplemented with hygromycin to provide selective conditions. Rooted regenerants tested positive for the presence of the hpt gene were then established in soil and cultivated in a phytochamber providing 12h day-length at 14/12°C day/night, respectively. At booting stage, the plants were transferred to a glasshouse cabin (16 hours light period, 18/16°C day/night) and grown until maturity.

For the pLea:NCED6 construct (NCED6 overexpression), single-copy homozygous plants were identified by conventional segregation analysis across generations based on hpt-specific PCR and DNA gel blot. Two independent homozygous lines (T3 generation) harboring a single insertion of the transgene were used for the experiments. For the pLea:ABA8’OH RNAi construct, doubled haploid (DH) plants were produced using embryogenic pollen cultures generated from primary transgenic plants following the protocol previously described by Coronado et al. (2005).

Growth conditions of transgenic and breeding material

For the experimental analyses, wild type (WT) Hordeum vulgare (cv. Golden Promise) and homozygous transgenic plants (pLea:NCED6 and pLea:ABA8’OH RNAi) were cultivated in a growth chamber (phytochamber) with a 16 h light/8 h dark cycle at 20/15°C, respectively. Spikes were labelled at anthesis and drought stress was imposed and these plants were maintained at 10% soil moisture level by monitoring soil moisture using a moisture meter HH2 coupled with soil moisture sensor SM200 probes (Delta T devices Ltd., England). Another batch of plants from a given experiment was continuously watered and treated as unstressed control. Two replications were maintained by growing them independently and additional technical replications were maintained by the pool of samples collected from average of five plants. The flag leaves as well as first leaves were harvested from WT and transgenic lines at 2, 4, 8 and 12 days after stress imposition (DAS) from stress and control plants. Imposition of stress took place at 4 days after flowering. These stages were chosen to
cover short and long duration of stress. Material obtained from this batch of plants was used for measuring hormones, gene expression profiling and various physiological experiments. As described above, the contrasting breeding material (stay-green and senescing plants) was cultivated, drought stress was imposed at 8 DAF and flag leaf samples were harvested at 4, 8, 12 and 16 DAS.

**Physiological traits**

Infra red gas analysis were carried out on individual fully emerged flag leaves of (a) breeding material of stay-green and senescing during 12 and 16 days after flowering (DAF, corresponding to 4 and 8 DAS) and (b) flag leaf of transgenic and wild type (WT) plant material during 4 DAS using a LCpro+ device (ADC Bioscientific Ltd, Great Amwell, England). A constant supply of 400 ppm CO₂ (flow rate 200 µmol s⁻¹) was provided by a CO₂ cartridge and a photon flux density of 900 µmol m⁻² s⁻¹ by a mixed red/blue LED light source mounted above the leaf chamber head. The net assimilation rate (A), internal CO₂ concentration (Ci), stomatal conductance (gs) and transpiration rate (E) were all recorded from five individual plants growing in both well watered and drought stressed conditions, with four technical replications per measurement. The measurements were only taken once the internal CO₂ concentration had stabilized (2-3 min after insertion of the leaf within the device). The instantaneous WUE was calculated from the ratio between the assimilation rate and the transpiration rate.

**Phytohormone measurements**

The content of ABA and certain of its degradation products and glucose esters was measured along with that of cytokinin, gibberellins and auxin hormone analysis. These were assessed by HPLC electrospray ionization tandem mass spectrometry (carried out at the Plant Biotechnology Institute, Saskatoon, Canada). The assays were calibrated using deuterated internal standards, as described elsewhere (Chiwocha et al., 2003; Kong et al., 2008).

**Quantitative real time PCR (qRT-PCR) analysis**

RNA was isolated from breeding material as well from WT and transgenic lines from two independent biological replications and two technical replications (pooled from five plants) using the TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) and RNeasy columns (Qiagen, Hilden, Germany). The RNA was converted to cDNA following Seiler et al. (2011). Gene-specific primers (targeting 25 ABA signalling genes) were designed using Primer Select
software (DNASTar), and the relevant sequences are given in Supplemental Table S1. The reactions were performed in 384 wells plates with an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR® Green to monitor dsDNA synthesis. For a more detailed protocol, see Seiler et al. (2011). The amplification profile consisted of a denaturation step (50°C/2 min, 95°C/10 min), followed by 45 cycles of 95°C/15 s, 60°C/60 s. Amplicon dissociation curves were recorded after cycle 45 by heating from 60°C to 95°C with a ramp speed of 1.9°C min⁻¹. Data were collected from cycles 3-15 to generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔRₙ) versus cycle number, using SDS2.2.1 software (Applied Biosystems, Germany). An Rn threshold of 0.2 was applied to obtain Cₜ values. To allow comparisons between different PCRs or templates, Cₜ values for each gene were normalized to the Cₜ value of the reference gene (elongation factor 1α, EST clone HZ42K12). PCR efficiency was calculated from the slope of the exponential phase of the amplification, following the suggestions made by Ramakers et al. (2003). Transcription levels are presented in the form 2⁻ΔΔCₜ, where ΔΔCₜ represents the difference between the Cₜ values of the target and the reference genes. Primer sequences can be found in supplementary table S1.

In transgenic barley plants, the mRNA expression levels of AtNCED6 gene, two HvNCED and three HvABA8’OH endogenous genes were monitored following gene specific primers: AtNCED6 (forward 5’-GACAAAGGTTATGTAATGGGG-3’ and reverse 5’-CTTGTTCCCTTCAACTGATTC-3’); HvNCED1 (forward 5’-CCAGCACAATACTCGATTC-3’ and reverse 5’-GAGAGTTGATGATGATGAA-3’); HvNCED2 (forward 5’-CATGGAAAGGAAAGTTG-3’ and reverse 5’-GAAGCAAGTGTGAGCTAAC-3’); HvABA8’OH-1 (forward 5’-AGCACGGAGCCTCCAAGTC-3’ and reverse 5’-TGAGAATGCCTACGTAGTG-3’); HvABA8’OH-2 (forward 5’-GAGATGCTGGTGCTCATC-3’ and reverse 5’-ACGTCGTCGCTCGATCCAAC-3’); HvABA8’OH-3 (forward 5’-CCGGCGGCAGCGTCTTCTTCTTC-3’ and reverse 5’-GTGTGCTGCCGCTCAGGGTGC-3’). qRT-PCR was performed as described above.

Yeast two-hybrid assay
The full-length coding regions of barley PYR/PYL1, PYR/PYL2, PYR/PYL5, PP2C4 and PKABA1/SnRK2.1 genes were initially cloned into pCR®8/GW/TOPO® TA vector (Invitrogen, Karlsruhe, Germany) and further introduced into pDEST22 (GAL4 activation domain, AD) and pDEST32 (GAL4 DNA binding domain, BD) vectors using GATEWAY technology following the manufacturer's instructions (ProQuest™ Two-hybrid system with
Gateway® Technology, Invitrogen, Karlsruhe, Germany). Clones containing the PP2C4 (AD), PP2C4 (BD), PYR/PYL1 (AD), PYR/PYL1 (BD), PYR/PYL2 (AD), PYR/PYL2 (BD), PYR/PYL5 (AD), PYR/PYL5 (BD), PKABA1/SnRK2.1 (AD) and PKABA1/SnRK2.1 (BD) fusions were validated by sequence analysis and subsequently used in the yeast two-hybrid assay. Specific combinations of AD and BD plasmids were co-transformed into the *Saccharomyces cerevisiae* strain MaV203 (MATα; leu2-3,112; trp1-901; his3A200; ade2-101; gal4Δ; gal80Δ; *SPAL10ΔUASGAL1::URA3*; *GAL1::lacZ*; *HIS3ΔUASGAL1::HIS3@LYS2*; *can1R*; *cyh2R*), using a lithium acetate/polyethylene glycol protocol described by Gietz and Woods (2006), and the transformed cells spread on SC-Leu-Trp media plates. Auto-activation levels of yeast transformants harboring PP2C4 (BD), PYR/PYL1 (BD), PYR/PYL2 (BD), PYR/PYL5 (BD) and PKABA1/SnRK2.1 (BD) were determined using SD medium lacking Leu and His (−leu, −his), to which 0, 10, 25, 50, 75, or 100 mM of the His biosynthesis inhibitor 3-AT was added (Durfee et al., 1993). Interactions were determined by growth of double yeast colonies on to three selective medium plates: SC-Leu-Trp, SC-Leu-Trp-His + 50 mM 3-AT and SC-Leu-Trp-His + 50 mM 3-AT + 100 µM ABA (mixed stereoisomers, Sigma, Germany). The empty vectors pDEST22 or pDEST32 or Krev1 or RalGDS-wt or RalGDS-m1 or RalGDS-m2 were co-transformed as positive and negative controls as mentioned in the manufacturer’s instructions. The selection plates were incubated at 30 ºC and growth of the colonies was assessed after 3 days. The screening experiment was performed in triplicate.

**Bimolecular fluorescence complementation (BiFC) analysis and transient gene expression in Arabidopsis protoplasts**

*HvPP2C4* was cloned into the pSPYNE vector which contains the N-terminal 155 amino acids of YFP, and *HvPYR/PYLs* were cloned into the pSPYCE vector, containing the C-terminal 86 amino acids of YFP. To test for dimer formation, *HvPYR/PYLs* were additionally cloned into pSPYNE vector. To check interaction of PP2C4 with PKABA1/SnRK2.1 of the SnRK2 family, *HvPKABA1/SnRK2.1* and *HvPP2C4* were cloned into pSPYCE and pSPYNE vectors, respectively. Combinations of BiFC constructs were co-transformed into Arabidopsis protoplasts and fluorescence was detected.

*PYR/PYL1, PYR/PYL2, PYR/PYL5, PP2C4 and PKABA1/SnRK2.1 in pCR®8/GW/TOPO® TA vector* (previous section) were subsequently recombined via an LR recombination into the binary plant transformation vectors pSPYNE and pSPYCE (Walter et al., 2004) resulting in fusion with the N- or C-terminal portion of the yellow fluorescent protein (YFP) sequence, respectively. Arabidopsis protoplast isolation and transient expression assays were performed.
as described in (Yoo et al., 2007). Plants were grown in soil under short-day conditions (8 h light/16 h dark at 20°C and 18°C, respectively) for 4 weeks. Freshly isolated leaf mesophyll protoplasts (0.5 ml) were co-transfected with various pairs of plasmid DNA (pSPYNE/pSPYCE) and pUGW15-CFP which was used as transformation control (Nakagawa et al., 2007). The total amount of DNA was 50 µg in 1:1:1 ratio. YFP fluorescence was evaluated 1 d after transfection (incubation in dark) using LSM 710 Laser Scanning System (Zeiss, Oberkochen, Germany). To investigate a possible ABA response, 5 µM ABA (mixed stereoisomers, Sigma, Germany) was applied after transfection and then additionally incubated for 1 h at room temperature. For stress treatment, protoplasts were centrifuged at 200 x g for 1 min and suspended in media (Yoo et al., 2007) containing 10 % PEG or 800 mM mannitol, respectively, followed by 1 h incubation at room temperature.

**Molecular modelling of barley PYR/PYLs**

The three-dimensional models of the complete amino acid sequence of barley HvPYR/PYL1, 2 and 5 were created by the I-TASSER server (Roy et al., 2010). From the proposed models we selected the one with best scores, downloaded structural overlays with Arabidopsis analogs and predicted ligand binding sites from the I-TASSER server and further processed it by using the PyMOL Software (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC). The complete results including coordinates of the models along with Z-score significance could be searched at I-Tasser database (http://zhanglab.ccmb.med.umich.edu/I-TASSER/search.html) with the following IDs for HvPYR/PYL1 (S91589), HvPYR/PYL2 (S91695) and HvPYR/PYL5 (S91499).

**Statistical analysis**

Values derived from several biological replicates were used to calculate standard deviations, and statistical significance was assessed using the Student’s t test and one-way ANOVA across genotypes for a given treatment at α = 0.05 or 0.01 with Tukey post-hoc test using SPSS software package (IBM). In addition two way ANOVA was also performed to identify the signified difference between accessions (stay-green versus senescing line; WT versus transgenic lines) and condition (control versus drought stress) with P values of the f-test. Letters a, b, c and x, y, z represent statistical differences under control and stress conditions, respectively. Bars with similar or no letters indicate no statistical difference among genotypes under a given treatment.
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FIGURE LEGENDS

Figure 1. Phylogenetic trees of different families of ABA signalling. Protein alignments were done using ClustalW (MegAlign, DNASTar) with sequences from Arabidopsis, rice and barley. Subfamilies are shaded in different colors.

Figure 2. Phenotype of two contrasting barley genotypes under control and drought stress conditions. A, B: Plants of the stay-green (LP103) and senescing genotype (LP110) after 3 weeks of stress and respective control plants. C, D: Yield related parameters showing mean values ± SD with n=10; E-H: Assimilation, stomatal conductance, transpiration rate and instantaneous water use efficiency (assimilation/transpiration) of the two genotypes measured in flag leaf at 4 and 8 DAS. The graphs show mean values ± SD with n=10. I: ABA metabolites PA, DPA and ABA-GE measured in flag leaves 8 DAS. J: ABA levels in flag leaves after different stress duration in stay-green and senescing genotype. The graphs I and J show mean values ± SD with n=2. K: Expression of HvPYR/PYL5 and HvPP2C4 in flag leaves at 8 and 12 DAS. The graphs show mean values from two replicates of qRT-PCR-experiments from biological independent material (n=2) with an additional two technical replications. Relative mRNA levels to reference gene HZ42K12 are shown by white and grey bars (mean ± SD), TGW, thousand grain weight; ABA, abscisic acid; PA, phaseic acid; DPA, diphaseic acid; ABA-GE, ABA glucose ester. DW, dry weight. Statistical analysis was carried out across genotypes for a given treatment using one-way ANOVA at \( \alpha = 0.05 \) with Tukey post-hoc test (E-H, K). Letters a, b and x, y represent statistical differences under control and stress conditions, respectively. Bars with similar or no letters indicate no statistical difference among genotypes under a given treatment. Two-way ANOVA performed to identify the signified difference between accessions (stay-green versus senescing lines) and condition (control versus drought stress) with P values of the f-test; \( \alpha = 0.05 \) (*) and 0.01 (**) . For further details refer Supplemental Table S3.

Figure 3. Physiological performance of the transgenic lines LOHi and LN compared to wild type (WT) under control and drought stress conditions. A: Relative leaf water content (RWC) estimated at 4 days after stress (DAS). B, C: assimilation rate and calculated instantaneous water use efficiency [WUE, assimilation (A)/transpiration (E)] in WT and transgenic plants. Mean values ± SE are given with n = 15 for all the parameters. Open and filled bars represent control and stress conditions, respectively. Statistical significant differences among genotypes and a given treatment have been calculated using one-way ANOVA at \( \alpha = 0.01 \) with Tukey post-hoc test. Letters a, b, c and x, y, z represent statistical differences under control and stress conditions, respectively. Additional information of two-way ANOVA is provided in Supplemental Table S4.

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SUPPLEMENTAL DATA

Supplemental Figure S1A. Amino acid sequence alignment of the Arabidopsis PYR1/RCAR11 protein with putative barley PYR/PYL orthologous proteins.

Supplemental Figure S1B. Amino acid sequence alignment of barley and Arabidopsis type-2C protein phosphatases.

Supplemental Figure S1C. Amino acid sequence alignment of the Arabidopsis SnRK2.2, 2.3 and 2.6 proteins with barley SnRK2 orthologous proteins.

Supplemental Figure S2. Field screening of different barley lines.

Supplemental Figure S3. Differential expression of putative ABA biosynthesis, degradation and deconjugation genes in two barley contrasting genotypes under terminal drought stress analyzed by qRT-PCR.

Supplemental Figure S4. ABA, ABA metabolites and differential expression of putative ABA signaling genes in two barley contrasting genotypes under terminal drought stress.

Supplemental Figure S5. Relative expression levels of barley endogenous genes HvNCED1, HvNCED2 and ABA8’OH1-3.

Supplemental Figure S6. Differential expression of putative ABA signaling genes in barley wild type (WT) and transgenic plants under terminal drought stress analyzed by qRT-PCR.

Supplemental Figure S7. Interaction of ABA receptor components using different stress treatments analyzed by BiFC. Pairs of PP2C4-YFPN / PYR/PYL5-YFPc, PYR/PYL5-YFPN / PYR/PYL5-YFPc and PKABA1/SnRK2.1-YFPN / PP2C4-YFPc were transformed into Arabidopsis protoplasts. Fluorescence images were taken 1 d after transformation. The pictures are YFP, bright field, autofluorescence (auto) and merged images, scale bar = 10 µm.

Supplemental Table S1. List of genes putatively involved in ABA signalling/biosynthesis and primer sequences used for qRT-PCR.

Supplemental Table S2. Levels of ABA, ABA metabolites and other hormones in flag leaf and seeds under control and stress conditions at 8 days after stress imposition (DAS). Hormone levels are given in ng g⁻¹ dry weight including standard deviation (SD). Only one of the two biological replicates gave a detectable value, thus no SD was calculated. n. d. not detected. Asterisks in brackets indicate statistical significant differences (Student’s t-test) between control and stress with * p ≤ 0.05. Two-way ANOVA statistics is presented in Supplemental Table S3.

Supplemental table S3. Statistical significant differences in expression of ABA biosynthesis and signalling genes between contrasting breeding lines. Two-way ANOVA performed to identify the signified difference between accessions (stay-green versus senescing lines) and condition (control versus drought stress) with P values of the f-test. Statistical significance is indicated at α = 0.05 yellow colour and 0.001 with pink colour.
Supplemental table S4. Statistical significant differences in expression of ABA biosynthesis and signalling genes between genotypes, ABA metabolites and physiological traits of transgenics. Two-way ANOVA performed to identify the signified difference between accessions (WT versus transgenic lines) and condition (control versus drought stress) with P values of the f-test. Statistical significance is indicated at $\alpha = 0.05$ yellow colour and 0.001 with pink colour. One-way ANOVA at $\alpha = 0.05$ with Tukey post-hoc test have been used to calculate statistically significant differences between genotypes under a given treatment indicates by letters a, b, c. ns, not significant.
Table 1. List of genes putatively involved in ABA signalling

The table shows the following details: HarvEST unigene ID, full-length cDNA ID, Affymetrix ID, full-length/partial, open reading frame (ORF) size, predicted molecular mass for the deduced proteins, predicted subcellular localization, genomic sequence information (Assembly1 Morex ID) and derived 5' upstream region of the translational start site.

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stay-green control

senescing stress

senescing control

stay-green stress

Assimilation (A)

Transpiration rate (E)

Water use efficiency (instantaneous)

Yield per plot

TGW per plot

PA

DPA

ABA

ABA-GE

HvRCAR5

HvPP2C4

ABA

PA

DPA

ABA-GE

HvRCAR5

HvPP2C4

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