RESEARCH PAPER

ABI1 regulates carbon/nitrogen-nutrient signal transduction independent of ABA biosynthesis and canonical ABA signalling pathways in Arabidopsis

Yu Lu1, Yuki Sasaki1, Xingwen Li1, Izumi C. Mori2, Takakazu Matsuura2, Takashi Hirayama2, Takeo Sato1 and Junji Yamaguchi1,*

1 Faculty of Science and Graduate School of Life Science, Hokkaido University, Kita-ku N10-W8, Sapporo 060-0810, Japan
2 Institute of Plant Science and Resources, Okayama University, Chuo 2-20-1, Kurashiki, 710-0046 Okayama, Japan

* To whom correspondence should be addressed. E-mail: jjyama@sci.hokudai.ac.jp

Received 21 November 2014; Revised 14 January 2015; Accepted 2 February 2015

Abstract

Plants are able to sense and mediate the balance between carbon (C) and nitrogen (N) nutrient availability to optimize metabolism and growth, described as the C/N response. To clarify the C/N signalling mechanism, C/N-insensitive plants were obtained from an Arabidopsis FOX hunting population, which over-expresses full-length cDNAs for individuals. The resulting cni2-D (carbon/nitrogen insensitive 2-dominant) plant was found to overcome the post-germination growth checkpoint and to expand green cotyledons in disrupted high C/low N stress conditions. The CNI2 gene encodes ABI1, a phosphatase type 2C protein, which negatively regulates abscisic acid (ABA) signal transduction. Over-expressors of ABI1 were found to be insensitive to disrupted C/N stress, whereas the loss-of function mutant abi1-2 was hypersensitive, suggesting that ABI1 plays an essential role in the plant C/N response. By contrast, the C/N-dependent growth phenotype observed in wild-type plants was not associated with endogenous ABA content. Accordingly, the ABA-insensitive mutant abi1-1, which could not bind to the ABA–ABA receptor complex, was not insensitive and restored normal sensitivity to high C/low N stress. The canonical ABA signalling mutants abi4 and abi5 were also sensitive to disrupted C/N stress. Further gene expression analysis demonstrated that several genes in the SnRK2s and SnRK1s pathways are transcriptionally affected by high C/low N stress in wild-type plants regardless of the lack of increased endogenous ABA contents, whereas the expression of these genes were significantly suppressed in ABI1 over-expressors. Taken together, these results suggest direct cross-talk between C/N and non-canonical ABA signalling pathways, regulated by ABI1, in plants.

Key words: Abscisic acid, C/N balance, FOX hunting system, nutrient signal, SnRK, sugar signal.

Introduction

Plant growth and development are controlled by many environmental factors and stresses, including nutrition, light, drought, and osmotic stress. Carbon and nitrogen are essential for plants, being constituents of nutrients and metabolites that provide energy and serve as constitutive molecular backbones. Moreover, these constitutive molecules also possess hormone-like functions, transducing signals to regulate plant growth and development (Krouk et al., 2010; Smeekens et al., 2010; Stitt et al., 2010). In addition to absolute amounts of cellular carbon (C) and nitrogen (N), the relative C/N
balance, has been found critically to affect plant growth and development (Coruzzi and Zhou, 2001; Martin et al., 2002; Sato et al., 2009). Several genome-wide investigations have shown that carbon and nitrogen metabolites and signalling co-operatively control various pathways involved in plant growth and development, such as glycolysis/gluconeogenesis, the pentose-phosphate pathway, protein synthesis, protein degradation, protein targeting, and the regulation of protein activity (Palenchar et al., 2004; Gutiérrez et al., 2007). Despite the physiological importance of the C/N response, the molecular mechanisms mediated by C/N signals remain unclear.

To assess the molecular mechanisms mediating plant C/N responses, transgenic plants were screened for novel gain-of-function using the Arabidopsis FOX (Full-length cDNA Over-e-xpressing) hunting system, which consists of independent transgenic lines expressing full-length cDNAs under the control of the CaMV promoter (Ichikawa et al., 2006). A novel C/N-insensitive mutant plant was isolated called carbon/nitrogen insensitive 1-D (cni1-D), and it was found that the CNI1 gene encoded the ubiquitin ligase ATL31 (Sato et al., 2009). Over-expression of ATL31 rescued plants from post-germination development arrest under extremely high C/low N stress conditions (Sato et al., 2009). Subsequent analysis showed that ATL31 interacts with and ubiquitinates 14-3-3 and regulates plant growth via 14-3-3 degradation in response to C/N status (Sato et al., 2011).

The phytohormone abscisic acid (ABA) is critical for plant growth in response to environmental challenges such as drought, salt, and osmotic stress. Additional exogenous ABA delayed germination and arrested plant growth, similar to plants exposed to excess sugar stress (Dekkers et al., 2008; Umezawa et al., 2009). Genetic approaches have identified several sugar-insensitive mutants (Smeekens, 2000; Rook and Bevan, 2003), with many of these mutants found to be defective in ABA biosynthesis or ABA signalling, including gin1/ABA2 (Laby et al., 2000), gin5/ABA3 (Rolland et al., 2002), and sunbl/ABI4 (Huijser et al., 2000). These findings showed close positive interactions between sugar and ABA signalling. Although the sugar–ABA response has been thoroughly investigated (Acevedo-Hernández et al., 2005; Rolland et al., 2006; Rook et al., 2006), the relationship between C/N stress and ABA signalling has not yet been clarified.

To assess the molecular mechanisms involved in C/N signalling in higher plants further, C/N response mutants were screened and a novel FOX transgenic plant, cni2-D (carbon/nitrogen insensitive 2-dominant) was isolated which was able to survive under extremely high C/low N stress conditions. The CNI2 gene encodes a type 2C protein phosphatase, ABI1, a negative regulator of ABA signalling. ABI1 is a central component of ABA signalling transduction, with its phosphatase activity inhibiting several SnRK2 proteins (Umezawa et al., 2009; Vlad et al., 2009). ABI1 and the ABA-receptor complex bind to ABI1 and inhibit its function when ABA is present (Park et al., 2009), resulting in the activation of SnRK2s kinase activity and ABA signal transduction.

This study investigated the physiological function of ABI1 at the post-germination growth checkpoint in response to C/N, demonstrating that ABI1 negatively regulates C/N signalling. By contrast, quantification of ABA amounts and genetic analysis demonstrated that C/N signalling is not mediated by ABA biosynthesis and the canonical ABA signalling pathway that regulates sugar signalling through ABI1 and ABI5. These results provide new insight into the cross-talk between C and N signalling and its effect on the non-canonical ABA signalling pathway under the control of ABI1 protein.

Materials and methods

Plant materials and growth conditions

Wild-type Arabidopsis thaliana Columbia-0 (Col-0) and all other plant material used in this study were grown under the conditions described previously by Sato et al. (2009). The Arabidopsis FOX hunting population was provided by RIKEN (Ichikawa et al., 2006). The ABA insensitive mutants ab1t-1 (Leung et al., 1994; Meyer et al., 1994), ab1-2 (SALK_072009; Saetz et al., 2006), ab1-102 (CS3387; Laby et al., 2000), and abi5-1 (line ID: CS8105; Finkelstein, 1994) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, OH, USA). Surface-sterilized seeds were plated on modified MS medium. After stratification for 3 d at 4 ºC in the dark, the plates were incubated at 22 ºC with a 16/8 h light/dark cycle.

Isolation of the cni2-D plant

The cni2-D plant was isolated by screening Arabidopsis FOX lines with selection medium containing 300 mM glucose and 0.1 mM nitrogen as described previously (Sato et al., 2009). The identity of the ABI1 gene was determined by PCR using T-DNA primers that amplify the inserted cDNA fragment (Ichikawa et al., 2006). The resulting PCR fragments were cloned into the pCR.2.1 vector (Invitrogen, http://www.invitrogen.com) and sequenced.

C/N response assay

Surface-sterilized seeds were sown on MS medium modified with different concentrations of glucose and total nitrogen, as described by Sato et al. (2009). The number of green-coloured cotyledons was counted 7 d after sowing. For transient limited-nitrogen treatment, seedlings were transferred to medium containing 0.3 mM nitrogen after being grown for 7 d in control medium containing 3 mM nitrogen.

Plasmid constructions and plant transformation

A full-length ABI1 cDNA fragment was amplified by PCR using the primers described in Supplementary Table S1 at JXB online. The fragment was sequenced and cloned into the pENTR/D-TOPO vector (Invitrogen) to generate the plasmid pENTR/ABI1. Full-length ABI1 cDNA was subsequently introduced into the pMDC83 T-DNA binary vector (Curtis and Grossniklaus, 2003), according to the Gateway instruction manual (Invitrogen), placing the full-length ABI1 gene under the control of the 35S promoter (35S-ABI1). This 35S-ABI1 construct was used to transform Arabidopsis as described by Sato et al. (2009).

Transcript level analysis

Total RNA was isolated from plants as described by Sato et al. (2009), and 500 ng RNA were reverse transcribed to cDNA with Super Script II (Invitrogen). RT-PCR analysis was performed with normalized cDNA samples for appropriate cycles, using the primer sets described in Supplementary Table S1 at JXB online.
Results

Isolation of a cni2-D transgenic plant able to tolerate C/N stress conditions

To assess the molecular mechanisms involved in the plant C/N response, FOX hunting populations were screened using

![Fig. 1](image-url)

**Fig. 1.** Isolation of cni2-D transgenic plant and C/N responses of 35S-ABI1. (A) Screening of Arabidopsis FOX hunting population with medium containing 300 mM glucose (Glc) and 0.1 mM nitrogen (N). (B) Genomic PCR using primers for the pBIG vector (GS17) and ABI1 (top panel) and RT-PCR of ABI1 mRNA transcripts (middle panel) in WT and cni2-D plants. 18S rRNA was used as an internal control (bottom panel). (C) Genomic PCR using primers for inserted GFP and ABI1 (top panel) and RT-PCR of ABI1 mRNA transcripts (middle panel) in WT and 35S-ABI1 plants. 18S rRNA was used as an internal control (bottom panel). (D) Post-germination growth phenotypes of WT and 35S-ABI1 transgenic plants grown under normal (100 mM Glc/3 mM N) and high C/low N stress (200 mM Glc/0.3 mM N) conditions. Representative seedlings of four independent 35S-ABI1 lines are shown (see Supplementary Fig. S1 at JXB online). Images were taken 7 d after germination. (E) RT-PCR analysis of RBCS1-B, CHS, and PAP1 mRNA transcripts in WT and 35S-ABI1 plants grown under the same conditions as in (D). 18S rRNA was used as an internal control. WT, wild type (Col-0).
for further analysis. Seeds of WT and 35S-ABI1 plants were sown in normal (100 mM Glc/3 mM N) and high C/low N stress (200 mM Glc/0.3 mM N) media. Although WT plants showed growth defects in this C/N stress medium, 35S-ABI1 plants expanded green cotyledons and continued post-germination growth (Fig. 1D; see Supplementary Fig. S1 at JXB online).

The transcript levels of marker genes responsible for the C/N response were analysed. In WT plants, the expression level of the photosynthesis-related RBCS1-B gene was lower in high C/low N stress conditions than in normal conditions, whereas expression of this gene in 35S-ABI1 plants was equal under both conditions (Fig. 1E). By contrast, the induction of the anthocyanin biosynthesis genes PAP1 and CHS under high C/low N stress conditions was suppressed in 35S-ABI1 plants (Fig. 1E). These results demonstrate that over-expression of the ABI1 gene causes the cni2-D phenotype, which is resistant to high C/low N stress.

Over-expressor and loss-of-function mutant of the ABI1 gene show reciprocal phenotypes under C/N stress condition

To evaluate the function of ABI1 in the plant C/N response further, growth of the 35S-ABI1 and the loss-of-function mutant abi1-2 was examined in parallel under several C/N stress conditions. Seeds of WT plants and 35S-ABI1 and abi1-2 mutants were grown at constant nitrogen concentration (0.3 mM) with various glucose concentrations (0, 100, and 200 mM) and at constant glucose concentration (200 mM) with various nitrogen concentrations (0.3, 1, and 3 mM). Almost all WT, 35S-ABI1, and abi1-2 plants grew normally and expanded green-coloured cotyledons in 0 mM Glc/0.3 mM N medium (Fig. 2A). The post-germination growth of WT plants was inhibited and the number of individuals’ expanded green cotyledons was reduced to 22% in 100 mM Glc/0.3 mM N medium (Fig. 2A, C). The growth of WT plants was more severely inhibited and less than 5% of the WT plants showed any expansion of green cotyledons in 200 mM Glc/0.3 mM N medium. By contrast, the greening ratio of 35S-ABI1 plants was 52% in 100 mM Glc/0.3 mM N and 32% in 200 mM Glc/0.3 mM N (Fig. 2A, C), indicating that these plants were insensitive to increased glucose when nitrogen was limited. Growth inhibition was enhanced in the abi1-2 mutants, with the greening ratio decreased to 6% in 100 mM Glc/0.3 mM N and 0% in 200 mM Glc/0.3 mM N (Fig. 2A, C). The growth inhibition due to increased Glc was not due to osmotic stress, because the WT and abi1-2 plants did not show anthocyanin accumulation or growth defects in media containing 0.3 mM N with 100 or 200 mM mannitol (see Supplementary Fig. S2 at JXB online).

Fig. 2. Post-germination growth of 35S-ABI1 and abi1-2 plants under different C/N conditions. (A) Post-germination growth phenotype of WT, 35S-ABI1, and abi1-2 plants germinated on media containing 0.3 mM N with 0, 100, and 200 mM Glc. Images were taken 7 d after germination. (B) Post-germination growth phenotype of WT, 35S-ABI1, and abi1-2 plants germinated on medium containing 200 mM Glc with 0.3, 1, and 3 mM N. Images were taken 7 d after germination. (C) Greening ratios of WT, 35S-ABI1, and abi1-2 seedlings; growth conditions are described in (A). Each treatment involved 20–40 seedlings. Means ±SD of three independent experiments are shown. (D) Greening ratios of 35S-ABI1 and abi1-2 seedlings grown under the conditions shown in (B). Each treatment involved 20–40 seedlings. Means ±SD of three independent experiments are shown. WT, wild type (Col-0). Asterisks indicate significant differences determined by Dunnet analysis (P < 0.05).
The effects of exogenous nitrogen availability on these mutants were examined in the presence of a constant amount of sugar. Although most WT, 35S-ABI1, and abi1-2 plants could grow normally and expanded green cotyledons in 200 mM Glc/3 mM N medium, WT plant growth was relatively inhibited and showed a decreased greening ratio (30%) in 200 mM Glc/1 mM N. This inhibition was more apparent in the abi1-2 mutant (11%), while 35S-ABI1 plants were insensitive (78%) (Fig. 2B, D). Severe growth defects of WT and abi1-2 mutants were observed in 200 mM Glc/0.3 mM N, while 35S-ABI1 was insensitive. Taken together, these results clearly demonstrate the reciprocal phenotypes of 35S-ABI1 and abi1-2 in response to increased Glc and limited N availability in the medium, suggesting that ABI1 plays an essential role in regulating plant growth in response to C/N status.

**ABA biosynthesis is not associated with the plant growth defect in response to C/N status**

ABI1 is a phosphatase that is directly repressed by interaction with the ABA receptor PYR1/RCAR in the presence of ABA, resulting in the activation of ABI1 signalling. The endogenous amounts of ABA have been reported to be increased by excess sugar in the medium, leading to growth arrest of Arabidopsis seedlings and indicating that endogenous sugar levels positively affect ABA biosynthesis. However, the relationship between C/N availability and ABA biosynthesis has not been determined. To determine whether the C/N-induced growth defect is caused by an increased ABA level, endogenous ABA contents were measured in WT Arabidopsis seedlings grown under each C/N condition. WT seedlings were grown in medium containing different concentrations of glucose (0, 100, 200, and 300 mM) and nitrogen (0.3, 1, 3 mM) for 7 d (Fig. 3A) and the ABA amounts were quantified by LC-MS analysis. ABA content was 5 ng mg⁻¹ FW in seedlings grown in 0 mM Glc/3 mM N and was significantly increased to 12, 20, and 63 ng mg⁻¹ FW during growth in medium containing 3 mM N with 50, 100, and 200 mM glucose, respectively (Fig. 3B). Similar Glc-dependent increases in ABA content were observed at all N concentrations, although the effects were lower at lower N concentrations (Fig. 3B). Surprisingly, ABA contents were decreased in response to limited N. In the presence of 100 mM glucose, ABA contents were estimated to be 20, 15, and 10 ng mg⁻¹ FW in seedlings grown in the presence of 3, 1, and 0.3 mM N, respectively. Similar patterns were observed in other N-modified media containing 50 mM and 200 mM Glc. Interestingly, the ABA contents of seedlings grown in 100 mM Glc/3 mM N and 200 mM Glc/0.3 mM N were similar (Fig. 3B), despite their growth phenotypes being totally different (Fig. 3A).

To confirm that nitrogen limitation directly affects ABA biosynthesis, WT plants grown in control medium (100 mM Glc/3 mM N) for 7 d were transferred to N-deficient medium (100 mM Glc/0.3 mM N). After 3 d, the ABA content of plants grown in N-deficient medium was slightly but significantly lower than that of plants in control conditions (Fig. 4A), similar to the results observed at stable low N (Fig. 3B). Transfer to N-deficient medium also increased the expression of Gln1.4 mRNA, which encodes a cytosolic glutamine synthase, a typical marker induced by N starvation, confirming that the endogenous N level of plants was limited (Fig. 4B). By contrast, the level of NCED3 mRNA, which encodes a key enzyme for ABA biosynthesis, was not altered after the transfer (Fig. 4B). These results indicate that the growth defect observed in high C/low N stress medium is not due to an increased ABA content, suggesting that more complex regulatory mechanisms are involved under ABI1 control to mediate C/N conditions.

**ABA-insensitive mutants, abi1-1, abi4, and abi5, are not insensitive to C/N stress conditions**

Since the C/N stress response phenotype did not associate with the endogenous ABA content, the C/N response in the abi1-1 mutant was examined. In abi1-1, the Gly180 residue is replaced by Asp, with the mutated ABI1 protein unable to bind to the ABA-receptor complex, resulting in constitutive inactivation of SnRK2 proteins (Santiago et al., 2012). Thus, the abi1-1 mutant was not insensitive to C/N stress and exhibited growth inhibition with anthocyanin accumulation, similar to WT plants (Fig. 5A). This result also suggests that the C/N response mediated by ABI1 is independent of ABA.

---

Fig. 3. ABA content in response to C/N status. (A) Post-germination growth phenotype of WT plants grown in C/N medium containing combinations of 50, 100, and 200 mM Glc and 3, 1, and 0.3 mM N. (B) Endogenous level of ABA of WT plants grown under the C/N conditions indicated in (A) and 0 mM Glc/3 mM N. Seedlings were harvested 7 d after germination. Means ±SD of four independent experiments are shown. WT, wild type (Col-0). Asterisks indicate significant differences in response to limited N condition determined by Tukey analysis (P <0.05).
biosynthesis and is probably regulated by an alternative signalling cascade unlike typical ABA signalling cascades. To evaluate the C/N-responsive signalling pathway associated with ABI1, the C/N responses of \textit{abi4} (\textit{abi4-102}) and \textit{abi5} (\textit{abi5-1}) loss-of-function mutants were tested, since these genes encode key canonical transcriptional factors under the control of ABI1-SnRK2s (Raghavendra et al., 2010). It had previously been shown that these \textit{abi4} and \textit{abi5} mutants are not resistant to the extremely high C/low N stress condition (300 mM Glc/0.1 mM N) used in \textit{cni} mutant screening (Sato et al., 2009). The detailed C/N responses of these mutant to relatively milder high C/low N stress conditions (200 mM Glc/0.3 mM N) was therefore re-examined and the ratio of green-coloured cotyledons was quantified. Both \textit{abi4} and \textit{abi5} mutants were sensitive to C/N stress, with both having similar greening ratios to WT plants (Fig. 5B, C).

These results strongly suggested that ABI1 controls plant C/N responses, but that these responses are mediated through a non-canonical typical ABA signalling pathway such as ABI4 and/or ABI5.

Expression of ABA-related marker genes is differentially affected in response to C/N and is controlled by ABI1 regulation

To assess the C/N-mediated signal transduction pathway under ABI1 control, transcript analysis was performed in WT and \textit{ABI1} over-expressor (35S-\textit{ABI1}) plants. The expression of \textit{CHS} was about 13-fold higher in WT plants grown under high C/low N stress (200 mM Glc/0.3 mM N) than under control (100 mM Glc/3 mM N) conditions, whereas \textit{CHS} induction was clearly suppressed in 35S-\textit{ABI1} plants (Fig. 6A), confirming that ABI1 functions in C/N response. Moreover, the expression of \textit{NCED3}, which encodes an ABA-biosynthetic enzyme, was not increased by high C/low N stress in WT plants (Fig. 6B), a finding consistent with the results of ABA quantification (Fig. 3B).

The expression of \textit{NCED3} was slightly higher in 35S-\textit{ABI1} than in WT plants, suggesting that ABA content is not responsible for the insensitive phenotype of 35S-\textit{ABI1} plants under the high C/low N stress condition. Evaluation of the expression of several ABA-responsive marker genes in the SnRK2s pathway (Mizoguchi et al., 2010) showed that \textit{RD29b} expression in WT plants was about 6-fold higher under high C/low N stress than under control conditions, but that \textit{RD29b} expression was slightly lower in 35S-\textit{ABI1} plants under stress conditions (Fig. 6C). Similar expression patterns were observed for the \textit{LEA3-4} and \textit{TSPO} genes (Fig. 6D, E). By contrast, the levels of expression of \textit{RAB18}, \textit{AREB1}, and \textit{ABF3} were not increased by high C/low N stress and were similar in WT and 35S-\textit{ABI1} plants (Fig. 6F–H). These results suggested that C/N stress activates some, but not all, of the ABA signalling cascade involving SnRK2s. SnRK1s-regulated marker genes, such as...
as DIN6 and SEN5 (Baena-González et al., 2007; Rodrigues et al., 2013) were also evaluated since SnRK1s are important for energy homeostasis and recently reported to be direct targets of ABI1 (Rodrigues et al., 2013). Transcript analyses showed that the levels of expressions of DIN6 and SEN5 in WT plants were significantly decreased in response to high C/low N stress, but were not affected in 35S-ABI1 plants (Fig. 6I, J). Taken together, these results suggest that high C/low N stress affects specific ABA-related signal transduction cascades under the control not only of SnRK2s but also of SnRK1s, and is independent of ABA biosynthesis.

**Discussion**

**Identification of ABI1 as a C/N-response regulator**

The cni2-D plants isolated in this study were insensitive to disrupted C/N-nutrient stress conditions, with ABI1 being the gene responsible for the phenotype of these plants (Fig. 1). Over-expression of ABI1 caused the successful post-germination growth of these plants under high C/low N stress condition, while the loss-of-function mutants showed hyper-sensitive responses and severe growth inhibition when compared with WT plants (Fig. 2), clearly demonstrating the importance that ABI1 plays in C/N signal regulation in Arabidopsis. ABI1 encodes a protein phosphatase and functions as an essential negative regulator of ABA signal transduction. It has been reported that ABA is involved in multiple stress signal mediations including the sugar and osmotic signals during post-germination growth. Sugar and osmotic stresses enhance ABA biosynthesis followed by inhibition of ABI1 and then activation of the ABA signalling cascade (León and Sheen, 2003; Jossier et al., 2009; Fujii et al., 2011). Interestingly, however, ABA quantification analysis showed that endogenous ABA content is not correlated with growth inhibition in response to C/N status (Fig. 3). This finding was consistent with results showing that the abil-1 mutant, in which mutated ABI1 is unable to bind to the ABA-receptor complex, resulting in the constitutive inactivation of SnRK2s, was not resistant to C/N stress (Fig. 5). In addition, loss-of-function mutants of ABI4 and ABI5 were not insensitive to high C/low N stress conditions (Fig. 5), despite being resistant to high sugar stress and exogenous ABA at normal N concentration (Arenas-Huertero et al., 2000). These results indicated that the high C/low N stress treatment used in this study is distinguishable from osmotic stress and that the C/N signalling cascade is not redundant to the canonical sugar- and ABA-signalling pathway under ABI1 control.
ABA biosynthesis in response to N and C/N status

It was found that ABA biosynthesis is affected by C/N status but is not associated with plant growth phenotype in response to C/N (Fig. 3). ABA content was not increased under high C/low N stress conditions, although sugar promotes ABA biosynthesis. Both continuous and transient nitrogen limitation did not enhance the expression of NCE D3, which encodes a key enzyme in ABA biosynthesis, under both high C/low N and limited-N conditions (Figs 4, 6).

Since little was known about the relationship between ABA biosynthesis and nitrogen availability, the results of this study are complicated. Related studies have reported that ABA biosynthesis in aerial parts of cucumbers is affected by limited nitrogen, but that this effect is dependent on time after treatment and developmental stage (Oka et al., 2012). In Arabidopsis, transcription analysis showed that the expression of ABA biosynthetic genes could be activated by nitrogen starvation as well as by sugar supplementation (Yang et al., 2011). By contrast, the ABA contents of shoots and roots were similar in Arabidopsis plants grown at high and low nitrogen conditions (Kiba et al., 2011). Thus the effect of nitrogen on ABA biosynthesis may depend on developmental stage, N-treatment condition, and plant species, suggesting that a complex system regulates ABA biosynthesis in response to nitrogen availability. Although the direct participation of the ABA signalling pathway in nitrogen signal mediation remains unclear, genetic studies have demonstrated that the ABA pathway is involved in regulating plant development in response to nitrogen status. ABA-insensitive mutants such as abi4 and abi5 and ABA-deficient mutants were shown to be less sensitive to the inhibitory effects of high nitrate medium on lateral root formation (Signora et al., 2001). Further studies are needed to determine the physiological function of the ABA pathway in mediating nitrogen availability and disrupted C/N stress.

C/N signalling cascade under ABI1 control

The results in this study raise the question about how ABI1 regulates post-germination growth in response to C/N. To explore the unknown signalling pathway mediated by C/N response under ABI1 control, transcript levels of several ABA-signalling marker genes regulated by the SnRK2s pathway were examined. Interestingly, although endogenous ABA contents were not up-regulated, the levels of expression of R D29b, LEA3-4, and TSP O were increased by high C/low N stress, and suppressed in ABI1 over-expressing plants (Fig. 6), suggesting a direct cross-talk between C/N and the ABA signalling pathway under ABI1 control. On the other hand, the levels of expressions of other marker genes, R AB18, AREB1, and ABF3, were not affected by high C/low N stress, indicating that C/N signals are mediated by specific ABA signalling pathways. In addition, the involvement of the SnRK1s, a family of essential kinases associated with various transcriptional events and metabolic reprogramming, restoring homeostasis, and inducing tolerance to energy starvation stress (Baena-González and Sheen, 2008; Lunn et al., 2014) was investigated. Recent studies showed that, besides SnRK2s, ABI1 could also directly target SnRK1s (Rodrigues et al., 2013) and SnRK1s is involved in ABA signalling (Jossier et al., 2009). Our transcript analyses demonstrated that high C/low N stress decreased the expression of DIN6 and S E N5, both of which are typical SnRK1s-responsive marker genes, in WT plants, but which effect was clearly suppressed in the ABI1 over-expressor (Fig. 6). This finding suggests the importance of the SnRK1s pathway in C/N signal mediation under ABI1 control. In responding to cellular energy status, SnRK1s target key components involved in protein synthesis and autophagic degradation (Smeekens et al., 2010). Moreover, SnRK1s proteins have been reported to phosphorylate various 14-3-3 targeting proteins involved in primary carbon and nitrogen metabolism, including nitrate reductase (NR) and sucrose 6-phosphate synthase (SPS) (Comparot et al., 2003; Smeekens et al., 2010), suggesting a strong relationship to the C/N response. Our previous study revealed that 14-3-3 is targeted by ATL31 for ubiquitination and is essential for post-germination growth regulation in response to C/N (Sato et al., 2011). Alternatively, a proteomics analysis showed that the SPS enzyme was precipitated along with ABI1 protein (Nishimura et al., 2010), suggesting that ABI1 may regulate metabolic enzyme activity. Further biochemical and genetic analyses are required to understand the detailed functions of ABI1 and downstream SnRK proteins in the plant C/N response.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Table S1. PCR Primers used for genotype and transcript analyses.

Supplementary Table S2. Primers used for quantitative RT-PCR analyses.

Supplementary Fig. S1. C/N response phenotype of ABI1 over-expressors.

Supplementary Fig. S2. Osmotic stress response of abi1-2 mutants.

Acknowledgements

We thank Drs Matsui and Ichikawa of the RIKEN Plant Science Center and RIKEN Bioresource Center for the Arabidopsis FOX lines and Arabidopsis cDNA. This work was supported by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research Nos 251250104, 262921884, and 266604604 to JY and 24770035 to TS. This work was also supported by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) as part of Joint Research Programme implemented at the Institute of Plant Science and Resources, Okayama University in Japan. LY was supported by a Special Grant Programme for Young Foreign Scientists in Basic Science (Hokkaido University Faculty of Science: 2014–2015) and MEXT Honours Scholarship for Privately Financed International Students (2014).

References

Acevedo-Hernández GJ, León P, Herrera-Estrella LR. 2005. Sugar and ABA responsiveness of a minimal RBCS light-responsive unit is mediated by direct binding of AB4. The Plant Journal 43, 506–519.
inducible gene expression. Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K.

Meyer K, Leube MP, Grill E. regulated by carbon:nitrogen availability. Martin T, Oswald O, Graham IA.

calcium-modulated protein phosphatase. Leung J, Bouly J-P, Meimoun P, Arjmand A, Lessard P, Hawley J.

adaptation to fluctuating environments. Krouk G, Crawford NM, Coruzzi GM, Tsay Y-F.

The Plant Journal 1) has a central role in sugar and ABA signalling in vivo. Genes & Development 19, 8, 1399–1409.

Arabidopsis SUCROSE UNCOUPLED-6. Arenas-Huertero F, Arroyo A, Zhou L, Sheen J, León P, Sheen J.

A central integrator of transcription networks in plant stress and energy signalling. Nature 448, 939–942.

Baena-González E, Sheen J. 2008. Convergent energy and stress signalling. Trends in Plant Science 13, 417–424.

Comparot S, Lingia G, Martin T. 2003. Function and specificity of 14–3–3 proteins in the regulation of carbohydrate and nitrogen metabolism. Journal of Experimental Botany 54, 585–604.

Coruzzi GM, Zhou L. 2001. Carbon and nitrogen sensing and signaling in plants: emerging ‘matrix effects’. Current Opinion in Plant Biology 4, 247–253.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiology 133, 482–469.

Dekkers BJW, Schuurmans J a MJ, Smeekens SCM. 2008. Interaction between sugar and abscisic acid signalling during early seedling development in Arabidopsis. Plant Molecular Biology 67, 151–167.

Finkelstein R. 1994. Mutations at two novel Arabidopsis ABA response loci are similar to the abi3 mutations. The Plant Journal 5, 765–771.

Fujii H, Verslues PE, Zhu J-K. 2011. Arabidopsis decuple mutant reveals the importance of SnRK2 kinases in osmotic stress responses in vivo. Proceedings of the National Academy of Sciences of the United States of America 108, 1717–1722.

Gutiérrez RA, Lejay LV, Dean A, Chiaromonte F, Shasha DE, Coruzzi GM. 2007. Qualitative network models and genome-wide expression data define carbon/nitrogen-responsive molecular machines in Arabidopsis. Genome Biology 8, R7.

Huijser C, Kortstee A, Pego J, Weisbeek P, Wisman E, Smeekens S, Dekkers BJW, Schuurmans J a MJ, Smeekens SCM. 2008. Genome-wide patterns of carbon and nitrogen regulation of gene expression validate the combined carbon and nitrogen (CN)-signaling hypothesis in plants. Genome Biology 5, R91.

Park S-Y, Fung P, Nishimura N, et al. 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science 324, 1068–1071.

Raghavendra AS, Gonugunta VK, Christmann A, Grill E. 2010. ABA perception and signalling. Trends in Plant Science 15, 395–401.

Rodrigues A, Adamo M, Crozet P, et al. 2013. ABI1 and PP2CA phosphatases are negative regulators of snf1-related protein kinase1 signaling in Arabidopsis. The Plant Cell 25, 3871–3884.

Rolland F, Baena-Gonzalez E, Sheen J. 2006. Sugar sensing and signaling in plants: conserved and novel mechanisms. Annual Review of Plant Biology 57, 675–709.

Rolland F, Moore B, Sheen J. 2002. Sugar sensing and signaling in plants. The Plant Cell 14 (Suppl), S185–S205.

Rook F, Bevan MW. 2003. Genetic approaches to understanding sugar-response pathways. Journal of Experimental Botany 54, 495–501.

Rook F, Hadingham SA, Li Y, Bevan MW. 2006. Sugar and ABA response pathways and the control of gene expression. Plant, Cell and Environment 29, 425–434.

Saez A, Robert N, Maktabi MH, Schroeder JI, Serrano R, Rodriguez PL. 2006. Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatase types 2C ABI1 and HAB1. Plant Physiology 141, 1389–1399.

Santiago J, Dupeux F, Betz K, Antoni R, Gonzalez-Guzman M, Rodriguez L, Marquez JA, Rodriguez PL. 2012. Structural insights into PYR/PYL/RCAR ABA receptors and PP2Cs. Plant Science 182, 3–11.

Sato T, Maekawa S, Yasuda S, et al. 2009. CNI1/ATL31, a RING-type ubiquitin ligase that functions in the carbon/nitrogen response for growth phase transition in Arabidopsis seedlings. The Plant Journal 60, 852–864.

Sato T, Maekawa S, Yasuda S, Domeki Y, Sueyoshi K, Fujimura M, Fukao Y, Goto DB, Yamaguchi J, Maekawa S, Yasuda S, et al. 2009. CNI1/ATL31, a RING-type ubiquitin ligase that functions in the carbon/nitrogen response for growth phase transition in Arabidopsis seedlings. The Plant Journal 60, 852–864.

Smeekens S. 2000. Sugar-induced signal transduction in plants. Annual Review of Plant Physiology and Plant Molecular Biology 51, 49–81.

Smeekens S, Ma J, Hanson J, Rolland F. 2010. Sugar signals and molecular networks controlling plant growth. Current Opinion in Plant Biology 13, 274–279.

Stitt M, Lunn J, Usadel B. 2010. Arabidopsis and primary photosynthetic metabolism - more than the icing on the cake. The Plant Journal 61, 1067–1091.

Umezawa T, Nakashima K, Miyakawa T, Kuromori T, Tanokura M, Shinozaki K, Yamaguchi-Shinozaki K. 2010. Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. Plant and Cell Physiology 51, 1821–1839.

Umezawa T, Sugiyama N, Mizoguchi H, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K. 2009. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. Proceedings of the National Academy of Sciences, USA 106, 17588–17593.

Vlad F, Rubio S, Rodrigues A, Sirichandra C, Belin C, Robert N, Leung J, Rodriguez PL, Laurière C, Merlot S. 2009. Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in Arabidopsis. The Plant Cell 21, 3170–3184.

Yang Y, Yu X, Song L, An C. 2011. ABI4 activates DGA1 expression in Arabidopsis seedlings during nitrogen deficiency. Plant Physiology 156, 873–883.