Interaction between sugar and abscisic acid signalling during early seedling development in Arabidopsis

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Abstract Sugars regulate important processes and affect the expression of many genes in plants. Characterization of Arabidopsis (Arabidopsis thaliana) mutants with altered sugar sensitivity revealed the function of abscisic acid (ABA) signalling in sugar responses. However, the exact interaction between sugar signalling and ABA is obscure. Therefore ABA deficient plants with constitutive ABI4 expression (aba2-1/35S::ABI4) were generated. Enhanced ABI4 expression did not rescue the glucose insensitive (gin) phenotype of aba2 seedlings indicating that other ABA regulated factors are essential as well. Interestingly, both glucose and ABA treatment of Arabidopsis seeds trigger a post-germination seedling developmental arrest. The glucose-arrested seedlings had a drought tolerant phenotype and showed glucose-induced expression of ABSCISIC ACID INSENSITIVE3 (ABI3), ABI5 and LATE EMBRYOGENESIS ABUNDANT (LEA) genes reminiscent of ABA signalling during early seedling development. ABI3 is a key regulator of the ABA-induced arrest and it is shown here that ABI3 functions in glucose signalling as well. Multiple abi3 alleles have a glucose insensitive (gin) phenotype comparable to that of other known gin mutants. Importantly, glucose-regulated gene expression is disturbed in the abi3 background. Moreover, abi3 was insensitive to sugars during germination and showed sugar insensitive (sis) and sucrose uncoupled (sun) phenotypes. Mutant analysis further identified the ABA response pathway genes ENHANCED RESPONSE TO ABA1 (ERA1) and ABI2 as intermediates in glucose signalling. Hence, three previously unidentified sugar signalling genes have been identified, showing that ABA and glucose signalling overlap to a larger extend than originally thought.

Keywords Abscisic acid · ABA insensitive · Glucose insensitive · Seedling development · Sugar signalling

Abbreviations
ABA Abscisic acid
ABI ABA insensitive
ApL ADP-glucose pyrophosphorylase large subunit
ERA Enhanced response to ABA
GIN Glucose insensitive
HXK Hexokinase
ISI Impaired sucrose induction
LEA Late embryogenesis abundant
PC Plastocyanin
SIS Sugar insensitive
SUN Sucrose uncoupled

Introduction

Photosynthesis provides plants with sugars that play a central role in the plant life cycle as energy sources, storage molecules, structural components or intermediates for the synthesis of other organic molecules. Next to these metabolic functions, sugars act as signalling molecules with
hormone-like properties. Both hexoses and disaccharides are able to induce signalling via different pathways (Jang and Sheen 1997; Loreti et al. 2001; Rolland et al. 2006). In plants glucose has been shown to affect many processes, including germination, early seedling growth, flowering and senescence (Gibson 2000, 2005; Smeeckens 2000; Rolland et al. 2006). Moreover, glucose feeding of Arabidopsis seedlings affected the expression of many genes as shown by micro array studies (Price et al. 2004; Villadsen and Smith 2004; Li et al. 2006). Sugar-induced signal transduction has been shown to control gene expression via diverse mechanisms that include transcription, translation, and modification of mRNA and protein stability (Rolland et al. 2006).

Genetic analysis showed that sugar signalling in plants is closely associated with plant hormone biosynthesis and signalling, in particular with that of abscisic acid (ABA, for review see Finkelstein and Gibson 2001; Gazzarini and McCourt 2001; León and Sheen 2003; Rook et al. 2006; Dekkers and Smeeckens 2007). Four screens for sugar response mutants i.e. sucrose uncoupled (sun), impaired sucrose induction (isi), glucose insensitive (gin) and sugar insensitive (sis) identified ABA deficient mutants (i.e. aba2/isi4/gin1/sis4 and aba3/gin5) and ABA insensitive (abi4/sun6/isi3/gin6/sis5) as sugar insensitive (Arenas-Huertero et al. 2000; Huijser et al. 2000; Laby et al. 2000; Rook et al. 2001). ABI4 encodes an AP2 domain containing transcription factor that binds a CE1-like element present in many ABA and sugar regulated promoters (Finkelstein et al. 1998; Niu et al. 2002; Acevedo-Hernandez et al. 2005). These observations linked sugar regulation to ABA signalling. However, there are many more examples of co-regulation of sugar and ABA in plants. The expression of many genes is co-regulated by sugar and ABA. A study of Li et al. (2006) showed that 14% of the ABA upregulated genes are induced in response to glucose as well, in total nearly 100 genes. These included genes in stress responses, carbohydrate and nitrogen metabolism and signal transduction. Another group of nearly 40 genes was repressed by both ABA and glucose. In addition the authors identified a group of genes that showed a synergistic upregulation when treated with both ABA and glucose, including the ApL3 (ADP pyrophosphorylase large subunit) gene, which is involved in starch biosynthesis. An earlier study showed that ABA was not able to induce the ApL3 gene but that ABA in combination with sugar boosted transcription levels in comparison to sugar treatment alone (Rook et al. 2001). Interestingly, Akhihiro et al. (2005) found a similar ABA/sugar interaction for the regulation of ApL3 expression and starch biosynthesis in rice (Oryza sativa). There are many other examples of processes and genes that are regulated by sugar and ABA. E.g. the ABA, stress and ripening-induced (ASR) protein in grape (Vitis vinifera) that binds to the sugar response boxes in the promoter of the monosaccharide transporter VvHT1. ASR mRNA expression is responsive to sugar and is modulated by ABA (Cakir et al. 2003). Both sugar and ABA signals also regulate the maize (Zea mays) invertase gene, Ivr2 (Trouverie et al. 2004). Thus ABA/sugar interactions were reported for a multitude of processes and in different plant species indicating that these interactions are physiologically relevant. Therefore, a thorough understanding of these sugar and ABA interactions is of importance.

An important strategy for the isolation of sugar response mutants makes use of the effect of exogenous supplied sugars on early seedling development in Arabidopsis Although the use of high sugar concentrations (>300 mM) possibly are above the physiological range, it provided an efficient screening method for mutants (reviewed by Rognoni et al. 2007). Such elevated sugar concentrations arrest early seedling development, which is characterized by the absence of cotyledon greening and leaf formation. The difference in phenotype between arrested and non-arrested seedlings was exploited to isolate sugar insensitive mutants that showed proper seedling development on high glucose (gin) and sucrose (sis) media. In addition to the aforementioned role of ABA biosynthesis and signalling such sugar response mutants revealed a role for HEXOKINASE1 (HXK1/GIN2) and ethylene signalling in the GIN response pathway (Zhou et al. 1998; Gibson et al. 2001; Cheng et al. 2002; Moore et al. 2003). ABA deficiency caused by either mutations in ABA1, ABA2 or ABA3 (Koornneef et al. 1998), resulted in a clear gin phenotype but analysis of ABA insensitive mutants resulted in more ambiguous phenotypes. In addition to abi4, only abi5 and abi8 showed a sugar insensitive phenotype, while for abi1-1, abi2-1 and abi3-1 no or only a weak phenotype was reported (Arenas-Huertero et al. 2000; Huijser et al. 2000; Laby et al. 2000; Brocard et al. 2002; Brocard-Gifford et al. 2004). Nevertheless, a role for ABI3 in sugar signalling during early seedling development was hinted too by later studies. E.g. ABI3 over expression lines were sugar hypersensitive (Finkelstein et al. 2002; Zeng and Kermode 2004) and abi3 mutants were insensitive to glucose in combination with ABA (Nambara et al. 2002). This insensitivity appeared to be allele specific and the assay used was different from other sugar sensitivity assays. However, in a more recent report WT glucose sensitivity during early seedling development for the abi3 mutant was observed (Yuan and Wysocka-Diller 2006). Thus it is unclear how and to what extend sugar and ABA signalling overlap.

ABA by itself blocked post-germination early seedling development, in addition to the role of ABA in the glucose-induced seedling arrest (Lopez-Molina et al. 2001). Similar
to glucose-arrested seedlings, ABA-arrested seedlings lacked greening and leaf formation. The ABA-induced seedling arrest is dependent on ABI3 and ABI5 activity and both abi3 and abi5 mutant do not arrest early seedling growth in response to ABA (Lopez-Molina et al. 2002). This ABA sensitivity is confined to a limited time window after the start of germination (Lopez-Molina et al. 2001; Lopez-Molina et al. 2002) Adding ABA after this sensitive phase to seedlings no longer affects vegetative development. Similarly, the glucose-induced seedling arrest could only be triggered in a similar limited time frame after sowing (Gibson et al. 2001).

Here a study of the role of ABA biosynthesis and ABA signalling in sugar response pathways is presented. In particular this study was focussed on the glucose-induced early seedling developmental arrest (the GIN pathway). Analysis of transgenic ABA deficient lines with ectopic ABI4 expression (aba2-1/35S::ABI4) suggested that additional ABA-controlled factors are necessary to arrest early seedling development in response to glucose. ABI3 expression and protein stability are controlled by ABA and ABI3 is a key component in the ABA mediated pathway that blocks early development (Lopez-Molina et al. 2002; Zhang et al. 2005). Therefore, we investigated the role of ABI3 in the GIN pathway. ABI3 encodes a B3 domain transcription factor that contains four conserved domains, one acidic activation domain (A1) and three basic domains (B1, B2 and B3, Giraudat et al. 1992; Finkelstein et al. 2002). ABI3 directs seed specific gene expression and is required for induction of desiccation tolerance and seed dormancy and represses genes related to seed germination (Ooms et al. 1993; Hoecker et al. 1995; Nambara et al. 2000; Suzuki et al. 2001; Finkelstein et al. 2002). Other functions for ABI3 include regulation of flowering, resource allocation, lateral root growth in response to auxin and serve as a signal transduction component downstream of PHYB signalling in seeds (Robinson and Hill 1999; Suzuki et al. 2001; Brady et al. 2003; Mazzella et al. 2005). Here we show that multiple abi3 mutants display a gin phenotype. The glucose insensitive phenotype observed for abi3 mutants was comparable to that of other known gin mutants like abal, ab4 and ab5. Moreover, abi3 mutants showed a reduced expression level of glucose-regulated genes. In addition, physiological and molecular experiments led to the conclusion that the glucose-arrested seedlings mimic ABA-arrested seedlings. This was supported by the observations that two other ABA response mutants, i.e. era1 and abi2-1, showed altered glucose responsiveness as well. The identification of ABI3 and two other ABA response loci as components in GIN signalling suggests that glucose and ABA signalling share more components than originally anticipated.

Material and methods

Plant material

Arabidopsis plants were grown in a climate chamber at 22°C with 70% humidity and a 16 h/8 h light/dark cycle (Sylvania GRO-LUX fluorescent lamps; Technische Unie, Utrecht) for seed production. WT and mutant seeds that were compared in all experiments presented were produced together and stored under identical conditions. Seeds were dry-stored in paper bags at room temperature for at least a month before use in our experiments. The transgenic aba2-1/35S::ABI4 lines were constructed as follows. ABI4 cDNA sequence was PCR amplified and cloned in pDONR vector and sequenced. Using Gateway cloning ABI4 was cloned behind the Cauliflower Mosaic Virus 35S promoter in the pGD625 vector. The pGD625 vector containing 35S::ABI4 was introduced in Agrobacterium which were used to transform aba2-1 plants by the floral dip method (Clough and Bent 1998).

Germination assays

All germination assays were performed on 0.5 MS: half-strength Murashige and Skoog medium (pH 5.8), including vitamins, solidified with 0.8% plant agar (Duchefa, Haarlem, The Netherlands). Before plating, seeds were surface-sterilized in 20% (v/v) commercial bleach (Glorix) for 15–20 min and rinsed four to five times with sterile water. After a three days stratification period at 4°C in the dark, plates (with or without sugars present as indicated) were incubated in the growth chamber (22°C and 16 h/8 h light/dark cycle). To investigate the effect of stratification on sugar-free (0.5 MS) media seeds were transferred to sugar media after this stratification period. Control experiments showed that transferring of seeds did not affect seed germination. Germination defined as radicle emergence from the seed coat, was scored daily for 3–8 days. Experiments were performed in duplo, each plate containing 50–100 seeds, and every experiment was repeated one to three times.

Sugar response assays

For gin assay experiments seeds were sterilized, sown on 0.5 MS media and stratified for three days at 4°C in the dark. Hereafter seeds were collected and sown on 0.5 MS media supplemented with 1% sucrose (control) or on control media with the indicated amount of sugar unless otherwise indicated. Different seed batches show slightly different responses and therefore both 7% and 8% glucose
media were used. Plates were incubated in the growth chamber (22°C and 16 h/8 h light/dark cycle) for up to two weeks. Seedlings were scored *gin* when green cotyledons emerged. To determine the *isi* phenotype of WT Col, *aba2-1*, L6 and L10 the seeds were stratified first for three days on sugar-free 0.5 MS media. After stratification the seeds were sown on 100 mM sucrose media. After four days plant material was harvested and *ApL3* expression levels determined. For the *sun* phenotype analysis seeds were sterilized, sown on 0.5 MS and stratified for three days at 4°C in darkness. After stratification seeds were transferred to plates containing 0.5 MS or 0.5 MS with the indicated amount of sugar. Seeds were light-treated for at least one hour before they were incubated in the dark (wrapped in three layers of aluminium foil) at 22°C. After two days plant material was harvested for *PC* expression analysis by qRT-PCR.

Gene expression analysis

WT Ler-0, *abi3-1* and *abi3-5* seeds were stratified for three days and germinated on 0.5 MS media. Seed batches were germinated to approximately 70% (*t* = 0) before the start of the different treatments. At this stage all WT seedlings were developmentally arrested in response to elevated glucose concentrations and ABA treatment. For RNA isolation and qRT-PCR analysis plant material was ground using mortar and pestle in liquid nitrogen and additionally bead-beaten in a mikro-dismembrator S (B. Braun Biotech International, Germany). RNA was isolated according to Schuurmans et al. (2003). Additionally, RNA was purified using RNeasy columns (Qiagen USA, Valencia, CA). RNA samples were DNase treated, checked for the absence of DNA by PCR and used for cDNA synthesis and qRT-PCR analysis as described before by van Dijken et al. (2004). For expression analysis by qRT-PCR either a 5′ FAM/3′ TAMRA labelled probe (with TaqMan® 2× Universal PCR Master Mix, Applied Biosystems, Foster City, CA) or SYBR®Green technology (Power SYBR®Green PCR Master Mix, Applied Biosystems, Foster City, CA) was used. Relative quantitation of gene expression is based on the comparative *C*T method (User Bulletin No. 2: ABI PRISM 7700 Sequence Detection System, 1997) using the *AtACT2* (ACT2) as reference gene. The *EM1* primers were obtained for the CATMA database (http://www.catma.org, *EM1* forward primer ID: 3a44750.5, *EM1* reverse primer ID: 3a44750.3). The sequences of primers and probes that were used for gene expression analysis are listed in Table 1. The qRT-PCRs were run on a ABI-prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).

Results

Transgenic ABA deficient lines with constitutive expression of *ABI4* are glucose insensitive

Screens for sugar insensitive mutants revealed the importance of ABA and ABA signal transduction, in particular *ABI4* (Arenas-Huertero et al. 2000; Huijser et al. 2000; Laby et al. 2000; Rook et al. 2001). To study the interaction between ABA biosynthesis and *ABI4* in sugar signalling we generated transgenic lines with constitutive *ABI4* expression driven by a CaMV 35S promoter in an ABA deficient (*aba2-1*) background. *ABI4* expression in these transgenic *aba2-1* plants is independent from plant sugar status. Two lines homozygous for the 35S::*ABI4* construct were selected, i.e. lines L6 and L10. Both lines germinated normally but showed a stunted growth phenotype (Fig. 1a) similar to what has been reported previously for severe *ABI4* overexpression lines (Söderman et al. 2000). Expression analysis on control media showed that *ABI4* expression is high in L6 and L10 and exceeded glucose-induced *ABI4* expression in developmentally arrested WT Col seedlings by approximately 5–8 fold (Fig. 1b).

The *gin* phenotype of Col, *aba2-1*, L6 and L10 was tested by growing the lines for two weeks on control, 8% sorbitol and 8% glucose media. All lines showed greening on control (con) and sorbitol (sorb, osmotic control) media as expected (Fig. 1c). On glucose the Col seedlings showed an arrested phenotype whereas *aba2-1* showed a *gin* phenotype as reported before (Fig. 1c, León and Sheen 2003). Also *ABI4* expression levels in glucose-arrested Col seedlings were enhanced whereas in *aba2-1* *ABI4* expression was very low. The L6 and L10 *aba2-1/35S::ABI4* lines have very high *ABI4* levels but, importantly, display a *gin* phenotype (Fig. 1c). The *gin* phenotype of *aba2-1* and the transgenic lines could be reverted to WT by the addition of 100 nM ABA as was reported before for *aba* mutants (Fig. 1c, Arenas-Huertero et al. 2000). Thus, in an ABA deficient background *ABI4* overexpression does not restore wild type sensitivity to glucose.

Sucrose feeding induces *ApL3* gene expression in Arabidopsis seedlings. This sugar regulation of the *ApL3* promoter has been used as basis for a screen for sugar response mutants. Rook et al. (2001) reported on *isi* sugar response mutants that were unable to fully activate *ApL3* expression in response to sucrose. The identification of *aba2/isi4* and *abi4/isi3* as mutants with an impaired sucrose induction phenotype revealed a role for ABA biosynthesis and signalling in control of *ApL3* expression (Rook et al. 2001). To study the sugar-induced *ApL3* expression in WT Col, *aba2-1*, L6 and L10 stratified seeds were sown on 100 mM sucrose media. After four days...
plant material was harvested and ApL3 expression levels determined. Sucrose feeding resulted in an approximate six fold induction of ApL3 in Col seedlings (Fig. 1d). The induction of ApL3 is 25% lower in the aba2-1 seedlings, which is in agreement with the isi phenotype of aba2/isi4 (Rook et al. 2001). In the L6 and L10 aba2-1/35S::ABI4 lines the ApL3 expression level on sugar-free control media already exceeds that of sugar-treated WT seedlings. Sugar feeding of L6 and L10 seedlings further boosted ApL3 expression 7–9 fold higher than sugar-treated WT seedlings (Fig. 1d).

Glucose and ABA trigger a similar developmental early seedling arrest

High sugar concentrations arrest early seedling development in Arabidopsis. After germination vegetative seedling growth is blocked, characterized by the absence of cotyledon greening and leaf formation. Equimolar concentrations of an osmotic control did not block development in this early seedling stage (Fig. 2a; Zhou et al. 1998; Laby et al. 2000; Rognoni et al. 2007). Previously, Lopez-Molina et al. (2001) showed that treatment with ABA arrested this post-germination switch from embryonic to vegetative growth as well. ABA-arrested seedlings showed neither greening nor leaf formation similar to glucose-arrested seedlings (Fig. 2a). In comparison to ABA-arrested seedlings, glucose-arrested seedlings were larger due to the longer hypocotyl and larger cotyledons (Fig. 2b). Moreover, significant root growth was observed in glucose-arrested seedlings, which is absent in ABA-arrested seedlings (Fig. 2a, b). Further, in glucose-arrested seedlings often a pink colouration was observed, indicative for anthocyanin accumulation that was absent in ABA-arrested seedlings (Fig. 2a, b).

ABA-arrested seedlings possess an enhanced resistance to drought stress compared to non-arrested seedlings (Lopez-Molina et al. 2001). A comparison was made between the drought resistance phenotype of glucose- and ABA-arrested seedlings. Control (non-arrested) seedlings did not survive a drought treatment of 2.5 h (Fig. 2c). ABA-treated seedlings showed a near 100% survival rate after 6 h of drought, the longest treatment tested in our experiments. Glucose-arrested seedlings showed an intermediate drought resistance. After 2.5 h of drought stress, glucose-treated seedlings showed a nearly 90% survival rate and after 6 h of drought the survival rate was 50% compared to 0% survival observed for control seedlings. Thus, a clear drought resistant phenotype was observed for glucose-arrested seedlings.

The ABA-induced early seedling developmental arrest depends on functional ABI3 and ABI5 genes. abi3 and abi5 mutants did not block early seedling development in response to ABA. Moreover, WT seedlings exposed to ABA showed strongly enhanced ABI3 and ABI5 protein levels (Lopez-Molina et al. 2001, 2002). Both genes encode transcriptional regulators important for seed development and LEA gene expression (Koornneef and Karssen 1994; Parcy et al. 1994, Finkelstein and Lynch 2000; Carles et al. 2002). Lopez-Molina et al. (2002) showed that during the early seedling developmental arrest several LEA genes were re-induced as well. This re-induction of seed transcriptional regulators and LEA gene expression suggested that a late embryogenesis program is re-initiated by ABA treatment, inducing a drought tolerant phenotype in such arrested seedlings (Lopez-Molina et al. 2002). It was investigated whether the same transcriptional regulators and LEA genes were expressed by glucose signalling during early seedling development. A role for ABI5 has been proposed in GIN signalling and ABI5 expression is sugar responsive (Arenas-Huertero et al. 2000; Laby et al. 2000; Brocard et al. 2002; Arroyo et al. 2003). ABI5 expression thus is a good positive control in this experiment. Gene expression upon glucose feeding was studied by germinating seeds on control medium until the batch reached 70% germination (time

### Table 1 Primers and probes used for gene expression analysis

| Gene   | Forward primer        | Reverse primer        | Probe               |
|--------|-----------------------|-----------------------|---------------------|
| ACT2   | gctgagagattcagatgccca| atgggagctgctggaatccac| agtcttgtcagccctcgttgc|
| ABI3   | cacagccagagtctctcttt | tggctatgtggacacagca  | cttgaatctccacgcatgcc|
| ABI4   | cgttgggtcaggtctatcaca| cggcatgacccatgacaaa  | acctcatacagcttgggga  |
| ABI5   | gagggtgcgttgggttt   | ggtcttaaagctgcaacca  | tccatattgtgctcaccacgc|
| EM1    | agatggagacacaaaggag  | tgtgtgtaaacttgactacg  ||
| EM6    | gtaggagggcacaagagag  | tgtgctcacatctgtaa    ||
| RAB18  | gacaactctcacaagagaag| gtacccacacagcatcatac ||
| ApL3   | cagagagatcggctattt   | ggaactttaggtgctcatt  | cccagaaacactcgtgattacccg|
| PC     | tggtgaggggcttttccttt | catggccatcgtcattcaca | aaagctgagcttgcttgccac|

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point zero, \( t = 0 \). At this time point a sample was collected for RNA isolation while the rest of the seeds were transferred to control, sorbitol, glucose or ABA containing media for an additional 24 h. After 24 h samples were collected for gene expression analysis. Using quantitative RT-PCR (qRT-PCR), the expression of \( ABI3 \), \( ABI5 \), \( AtEM1 \), \( AtEM6 \) and \( RAB18 \) was studied in response to the different treatments. Gene expression was studied in three independent experiments. In each experiment the gene expression level at \( t = 0 \) was set to one (Table 2). Some variation in gene expression levels between the three experiments was observed, likely due to the fact that for each experiment independently generated seed batches were used. Generally, continued growth on control media after \( t = 0 \) resulted in a decreased expression of all five genes (Table 2). In contrast, treatments with sorbitol, glucose and ABA enhanced the expression of all genes studied, except for the sorbitol treatment in the second experiment. In this particular experiment gene expression decreased on sorbitol, however, this decrease was less pronounced compared to the control treatment. ABA treatment resulted in the re-induction of \( ABI3 \), \( ABI5 \) and the LEA genes, which is in line with results reported by Lopez-Molina et al. (2002). Also glucose exposure significantly induced the expression levels of all five genes although the expression levels are in general somewhat lower than in response to ABA. Thus \( ABI3 \) is upregulated by glucose as well, which indicated a possible role for \( ABI3 \) in glucose responsiveness. The observation that the expression levels of the genes studied are in general lower after sorbitol treatment compared to glucose treatment indicates that the glucose-induced expression is not an osmotic response.

Expression studies using seedlings grown for five days on control, sorbitol (7%) or glucose (7%) media confirmed the induction of these genes (Fig. 3). In five days old glucose-arrested seedlings enhanced expression levels were detected for \( ABI3 \) and \( ABI5 \) as well as the LEA genes \( AtEM1 \), \( AtEM6 \) and \( RAB18 \). The induction of these genes by sorbitol was roughly five fold lower (Fig. 3). After 72 h of growth on ABA-free media, seedlings lose the capacity to re-induce \( ABI3 \), \( ABI5 \) and LEA expression and to arrest early seedling development in response to ABA (Lopez-Molina et al. 2001, 2002). To study whether the induction by glucose is restricted to a similar time frame seeds were germinated and grown for three days on half-strength MS and subsequently switched for two additional days to elevated sorbitol and glucose media. The re-induction by
glucose of \( \text{ABI3} \), \( \text{ABI5} \), \( \text{EM1} \), \( \text{EM6} \) and \( \text{RAB18} \) was largely lost (Fig. 3, glc 72 h). The weaker induction by sorbitol is essentially lost after three days (Fig. 3, sorb 72 h). Thus, the glucose-response that induced \( \text{ABI3} \), \( \text{ABI5} \) and \( \text{LEA} \) expression is confined to a similar time frame as observed for ABA. This is in agreement with the finding that glucose regulation of \( \text{ABI4} \) and \( \text{ABI5} \) expression was dependent on developmental stage (Arroyo et al. 2003) and that sugar arrests early seedling development only in a small 2–3 day time frame after sowing (Gibson et al. 2001).

In conclusion, the glucose-induced block of early seedling development mimics the ABA-induced arrest of vegetative development in young seedlings. Both glucose- and ABA-arrested seedlings have a drought resistant phenotype and show enhanced expression levels of seed expressed genes. The observation that \( \text{ABI4} \) overexpression is unable to restore WT glucose sensitivity in the \( \text{aba2-1} \) mutant shows that WT ABA biosynthesis is essential for \( \text{GIN} \) response. Apparently, next to \( \text{ABI4} \) additional factors which are under control of ABA are important for \( \text{GIN} \) signalling. Two candidates are \( \text{ABI3} \) and \( \text{ABI5} \). Both play a role in the ABA-induced early seedling developmental arrest and gene expression and protein stability of both transcription factors is affected by ABA (Lopez-Molina et al. 2001; Lopez-Molina et al. 2002; Lopez-Molina et al. 2003; Zhang et al. 2005; this study). A role for \( \text{ABI5} \) in glucose signalling has been established; \( \text{abi5-1} \) has a \( \text{gin} \) phenotype (although somewhat weaker compared to \( \text{abi4} \)) and \( \text{ABI5} \) expression is regulated by sugar (Arenas-Huertero et al. 2000; Laby et al. 2000; Brocard et al. 2002; Arroyo et al. 2003; this study see Table 2). However, a role for \( \text{ABI3} \) is controversial (for review Finkelstein and Gibson 2001; Leon and Sheen 2003; Gibson 2004; Rognoni

### Table 2

| Drying Time (h) | Con | ABA | Con | ABA | Con | ABA |
|----------------|-----|-----|-----|-----|-----|-----|
| 0              | 100 | 100 | 100 | 100 | 100 | 100 |
| 1              | 27  | 100 | 97  | 100 | 100 | 89  |
| 2.5            | 0   | 100 | 87  | 0   | 100 | 85  |
| 6              | 0   | 100 | 62  | 0   | 91  | 45  |

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Fig. 2 Glucose and ABA signalling arrest early seedling development and induce a drought tolerant phenotype. Ler-0 seedling phenotypes are shown after growth for twelve days on control (con, half-strength MS + 1% sucrose), or on con medium supplemented with either 7% sorbitol (sorb), 7% glucose (glc) or 10 \( \mu \text{M} \) ABA (ABA) (a). A close-up image of a glc-arrested seedling (left) and an ABA-arrested seedling (right) is shown (b). Developmentally arrested seedlings have a drought tolerant phenotype. Seeds were grown on control (con) or on con media containing either 10 \( \mu \text{M} \) ABA (ABA) or 7% glucose (glc) for nine days. At day nine the seedlings were subjected to a drought treatment as indicated (c). Hereafter seedlings were allowed to recover on control media for five days after which survival was scored. The survival percentage following drought treatment is presented (c).

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Fig. 3 The glucose-regulated expression of \( \text{ABI3} \), \( \text{ABI5} \) and \( \text{LEA} \) genes is restricted to a limited time frame after the start of germination. The expression of \( \text{EM1} \), \( \text{EM6} \), \( \text{RAB18} \), \( \text{ABI3} \) and \( \text{ABI5} \) genes was quantified by qRT-PCR after germination and growth for five days on different media. Ler-0 seeds were plated for five days on control media (con, half-strength MS + 1% sucrose), or on con medium supplemented with either 7% sorbitol (sorb) or 7% glucose (glc). The values are an average of three experiments and in each experiment the expression on glc was set to one. It was investigated whether this gene expression regulation is limited to a small time window after the start of germination as has been shown for ABA regulation (Lopez-Molina et al. 2002). Therefore seeds were germinated on con media for three days and thereafter switched to either sorb (sorb 72 h) or glc media (glc 72 h).
abi3 mutants are glucose insensitive

The glucose insensitive phenotype of WT Ler-0 and the abi3 mutant was investigated by plating seeds of both genotypes on media with elevated glucose concentrations (6–8% glucose). Initially, two abi3 mutant alleles were tested, i.e. the weak abi3-1 allele (producing normal, drought tolerant seeds) and the strong abi3-5 allele (producing green, drought intolerant seeds, Ooms et al. 1993). As expected WT Ler-0 early seedling development was arrested in response to high glucose concentrations. In contrast, approximately 60–100% (differed between experiments) of both abi3-1 and abi3-5 seedlings showed greening on high glucose media, suggesting that abi3 is a gin mutant, (Fig. 4a). All genotypes showed greening on control and osmotic control media (Fig. 4a, control and 7% sorbitol, respectively). The gin phenotype was observed for abi3 mutants in several experiments using independently grown and harvested seed batches (data not shown). Next, the strength of the gin phenotype of abi3-1 was assessed in comparison to that of the ABA deficient mutant aba1-1, which is in the Ler-0 background as well. The aba1-1 mutant, like other ABA deficient mutants tested, was shown to possess a gin phenotype (Arenas-Huertero et al. 2000; Huijser et al. 2000; Laby et al. 2000). A similar percentage of seedling greening was observed for aba1-1 and abi3-1 when grown on glucose media (Fig. 4b) suggesting that both mutants possess a comparable gin phenotype.

Six additional mutant alleles (abi3-8, abi3-9, abi3-10, abi3-11, abi3-12, and abi3-13) were obtained (a kind gift of Dr. E. Nambara) and tested for their gin phenotype. These abi3 mutants were tested along with two known gin mutants, abi4-3 and abi5-7 in the gin assay. All these mutants were retrieved from a screen for mutants insensitive to the unnatural (+)-ABA (Nambara et al. 2002). The ABA insensitivity of the mutants was tested by plating the mutants on half-strength MS media containing 1% sucrose + 10 μM ABA. The ABA insensitivity observed was in agreement with that reported by Nambara et al. (2002) (see Table 3). The abi3-8, abi3-9, abi3-10, abi4-3 and abi5-7 mutants had a strong ABA insensitivity, the abi3-12 and abi3-13 showed a weaker phenotype. We did observe 40% greening for abi3-13 on ABA media while originally no abi phenotype was observed for this allele (Nambara et al. 2002). These mutants were isolated on (+)-ABA and the (+)-ABA insensitivity does not necessarily parallel (+)-ABA insensitivity (Nambara et al. 2002). Next, these mutants were plated on control, sorbitol- and glucose-containing media, respectively. On 7% glucose media strong gin phenotypes were observed for abi4/abi6 and abi5 mutants as shown by the high percentages of greening, in agreement with earlier reports (Arenas-Huertero et al. 2000; Huijser et al. 2000; Laby et al. 2000). Again a strong gin phenotype was observed for abi3, in agreement with our observation using abi3-1 and abi3-5 mutants. Five out of the six additional abi3 mutants tested show a gin phenotype. For four abi3 mutants the gin phenotype was comparable to that of abi4-3 and abi5-7 (Table 3). The strong ABA insensitive abi3 mutants are strong gin mutants as well whereas the mutants with a weaker phenotype (abi3-11 and abi3-12) show a weaker phenotype on glucose media. However, exceptionally abi3-13 shows a weak abi phenotype but a strong gin phenotype. The abi3-11 mutant lacks an ABI insensitive phenotype.
Glucose-induced expression of ABI4, ABI5 and RAB18

Glucose-induced early seedling developmental arrest is associated with expression of ABI3, ABI4, ABI5 and a subset of LEA genes (Arenas-Huerta et al. 2000; Cheng et al. 2002; Arroyo et al. 2003; this study). The involvement of ABI3 in glucose-regulated gene expression was tested for three genes ABH4, ABI5 and RAB18 as a representative of the LEA genes. For expression analysis the weak abi3-1 and the strong abi3-5 mutants were used.

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Glucose-induced expression of ABI4, ABI5 and RAB18 is reduced in abi3

Table 2: Relative expression levels of ABI3, ABI5, EM1, EM6 and RAB18 after glucose treatment

| Gene | Treatment | Exp. 1 | Exp. 2 | Exp. 3 |
|------|-----------|--------|--------|--------|
| ABI3 | t = 0     | 1      | 1      | 1      |
|      | con       | 0.14   | 0.09   | 0.06   |
|      | sorb      | 2.2    | 0.19   | 1.7    |
|      | glc       | 9.0    | 4.5    | 7.9    |
|      | ABA       | 17     | 8.3    | 6      |
| ABI5 | t = 0     | 1      | 1      | 1      |
|      | con       | 0.48   | 0.20   | 0.29   |
|      | sorb      | 8.7    | 0.41   | 12     |
|      | glc       | 19     | 13     | 19     |
|      | ABA       | 40     | 41     | 23     |
| EM1  | t = 0     | 1      | 1      | 1      |
|      | con       | 0.26   | 0.34   | 0.20   |
|      | sorb      | 74     | 0.71   | 164    |
|      | glc       | 79     | 72     | 300    |
|      | ABA       | 749    | 742    | 200    |
| EM6  | t = 0     | 1      | 1      | 1      |
|      | con       | 0.22   | 0.14   | 0.15   |
|      | sorb      | 36     | 0.38   | 78     |
|      | glc       | 51     | 40     | 173    |
|      | ABA       | 450    | 132    | 237    |
| RAB18| t = 0     | 1      | 1      | 1      |
|      | con       | 0.16   | 0.12   | 0.00   |
|      | sorb      | 77     | 0.46   | 1.7    |
|      | glc       | 324    | 41     | 6.2    |
|      | ABA       | 6186   | 333    | 15     |

The relative expression levels of five genes were determined using quantitative RT-PCR. The gene expression levels were measured at t = 0 and after a 24 h treatment on control media (con, half-strength MS + 1% sucrose) and on con media supplemented with either 8% sorbitol (sorb), 8% glucose (glc) or 10 μM ABA (ABA) media. The expression level at t = 0 was set equal to 1. The experiment was performed three times and the results for each experiment are indicated.

and did not show a gin phenotype either. In conclusion, gin phenotype analysis of eight abi3 alleles confirms the glucose insensitive phenotype for abi3. Moreover, the gin phenotype of abi3 is comparable to that of other known gin mutants like aba1, abi4 and abi5.

Glucose-induced expression of ABI4, ABI5 and RAB18 is reduced in abi3

Glucose-induced early seedling developmental arrest is associated with expression of ABI3, ABI4, ABI5 and a subset of LEA genes (Arenas-Huerta et al. 2000; Cheng et al. 2002; Arroyo et al. 2003; this study). The involvement of ABI3 in glucose-regulated gene expression was tested for three genes ABH4, ABI5 and RAB18 as a representative of the LEA genes. For expression analysis the weak abi3-1 and the strong abi3-5 mutants were used.

Seedlings of all three genotypes were grown for 24 h on control media or on control media containing 8% glucose.

Both RAB18 and ABI5 expression were sensitive to glucose addition. In WT seedlings, glucose treatment induced these two genes on average 14 times in comparison to t = 0 (Fig. 5a, c). However, on glucose the expression levels of both genes were much reduced in abi3-1 and abi3-5 compared to WT (Fig. 5a, c). Glucose treatment did not result in a clear induction of ABI4, especially when compared to ABI5 and RAB18. Instead ABI4 expression was roughly maintained to the same level observed for t = 0 but was enhanced when compared to the control treatment which displayed a strong reduction of ABI4 expression (Fig. 5b). ABI4 transcripts were 2–8 fold lower in the abi3 mutant backgrounds upon glucose treatment. Thus, the abi3 mutation did not allow full glucose-induced expression of RAB18, ABI4 and ABI5. Interestingly, the reduced expression is most pronounced in the strong abi3-5 mutant background.

Already at t = 0 reduced expression levels for all three genes were observed in the abi3 mutant backgrounds. This was expected since the ABI transcription factors were found to affect each others expression in seeds (Söderman et al. 2000). Moreover, ABI3 is an important regulator of LEA gene expression (Parcy et al. 1994; Kermode 2005). To rule out that this reduced expression is responsible for the phenotype observed after glucose treatment the gene expression ratios of WT over mutant were determined for t = 0 and after glucose feeding. For each gene the expression level of WT is divided by the expression level of each mutant. These values show the fold difference of expression between WT and both abi3 mutants (Fig. 5d). E.g. the level of RAB18 expression is 9 fold higher in WT compared to abi3-1 at t = 0. After glucose treatment the fold difference for RAB18 expression was 38. Except for the ABI4 expression ratio of Ler-0 over abi3-1, all WT/ mutant expression ratios showed increased values after glucose exposure. This confirms that both mutants have a reduced ability to control glucose-regulated gene expression.

Other sugar insensitive phenotypes of abi3

The sugar response phenotype of the abi3-1 mutant was investigated in the sis, sun and glucose-induced delay of germination assays. High sucrose concentrations block early seedling development similar to high glucose concentrations. High sucrose resistant mutants are known as sis mutants (Laby et al. 2000). Several mutants affected in the same genes were retrieved from gin and sis screens, e.g. gin1/sis4/aba2, gin6/sis5/abi4 and gin4/sis1/ctrl. The sis phenotype of WT Ler-0, abi3-1 and abi3-5 was analysed by plating these genotypes on 13% sucrose media. Both
abi3 mutants show a sucrose insensitive phenotype (Fig. 6a, b) and can thus be considered as sis as well as gin mutants.

Dijkwel et al. (1997) previously reported on sugar insensitive mutants that have been isolated using the sun screen. Dark-grown seedlings transiently express photosynthesis genes, including plastocyanin (PC, Dijkwel et al. 1996). This transient increase of photosynthesis gene expression is repressed on sucrose media. In sun mutants sucrose did not repress this transient PC gene expression in dark grown seedlings (Dijkwel et al. 1997). sun6 is such a mutant which was shown to be allelic to abi4 (Huijser et al. 2000). A possible sun phenotype of abi3-1 was investigated by quantifying PC mRNA levels in two days old, dark-grown seedlings using qRT-PCR. Elevated PC expression was observed in sucrose-treated seedlings of sun6/abi4-3 compared to its WT PC-LUC parent confirming its sun phenotype (Fig. 6c). Interestingly, the abi3-1 mutant showed enhanced PC expression as well and, therefore, abi3 has a sun phenotype (Fig. 6c). abi4 mutants displayed a nine fold increased PC expression level in sugar-treated seedlings compared to WT. In the abi3 mutants the PC expression level is only two fold increased, indicating that abi3 is a weak sun mutant.

Sugars delay seed germination in Arabidopsis. ABA plays a role in this glucose-induced delay of germination (Ullah et al. 2002; Price et al. 2003; Dekkers et al. 2004; Chen et al. 2006). Despite the involvement of ABA, several abi mutants (abi1-1, abi2-1, abi4 and abi5) are normally sensitive to glucose during germination (Price et al. 2003; Dekkers et al. 2004). Sugar insensitive mutants like gin6/abi4 and gin2/hxk1 that are insensitive to the glucose-induced early seedling developmental arrest are sensitive to glucose during germination (defined as radicle emergence). This indicates that the glucose response during germination (which delays radicle protrusion) and early seedling growth (which arrests seedling development) are two distinct processes (Price et al. 2003; Dekkers et al. 2004). The, abi3-1 and abi3-5 alleles were plated on 2.5% glucose. The presence of 2.5% glucose clearly delayed germination of WT seeds. The osmotic control of 2.5% sorbitol only slightly affected germination compared to control media. Both abi3 mutants showed a clear resistance against glucose as displayed by their reduced germination delay (data not shown),
which is in agreement with observations of Yuan and Wysocka-Diller (2006) who reported on the glucose insensitivity of abi3-1 during germination.

In our experiments seeds were sterilized, plated on sugar media, and after a three day stratification period moved to the growth chamber and germination was scored the following days. Interestingly, when WT seeds were stratified on sugar-free media (half-strength MS) and after stratification transferred to sugar-containing plates, the delay of germination by 2.5% glucose was completely lost (data not shown). Thus, stratification on sugar-free media suppressed the inhibitory effect of glucose on germination. This effect of stratification was further investigated using higher (7%) glucose and sorbitol concentrations. Ler-0 and abi3-1 seeds were plated either on control, sorbitol or glucose media, stratified for three days and moved to the growth chamber. Alternatively, seeds were stratified on sugar-free control media and shifted to media containing sorbitol or glucose, and then placed in the growth chamber. Continuous treatment with 7% glucose severely delayed germination of WT seeds but affected abi3-1 significantly less as expected (Fig. 6d, e). Also in this experiment, stratification on sugar-free media strongly suppressed the germination response to glucose in WT Ler-0. Even germination of the abi3-1 mutant, which already showed a reduced sensitivity to glucose during germination could be improved by stratification on sugar-free media. The germination speed of the abi3-1 mutant after stratification on sugar-free media was similar to the osmotic control indicating that this combination completely suppressed the germination delay by glucose. The glucose response during germination was affected by stratification but not the osmotic response (sorbitol treatment). This was observed for WT Ler-0 as well as the abi3-1 mutant thereby differentiating glucose from osmotic signalling.

abi2-1 and era1-2 mutant are glucose response mutants

Our results on the physiological and molecular level indicate that glucose-arrested seedlings mimic ABA-arrested
The finding that ABI3 is important for glucose signalling in addition to ABI4 and ABI5 led us to test the glucose insensitive phenotype of two other ABA signalling mutants, i.e. abi2-1 and era1. Brady et al. (2003) proposed a genetic pathway for ERA1 and the ABI1-ABI5 genes in seed ABA responsiveness based on double mutant analysis between the era1 mutant and the abi mutants. This analysis suggested that ABI1 and ABI2 act at or upstream of ERA1, while ERA1 acts at or upstream of ABI3, ABI4 and ABI5 (Brady et al. 2003). ABI2 encodes a phosphatase 2C protein that acts as a negative regulator of ABA signalling and the abi2-1 mutation results in an ABA insensitive phenotype (Koornneef et al. 1984; Leung et al. 1997). WT Ler-0 and abi2-1 seeds were plated along with abi3-1 on control, 7% sorbitol and 7% glucose media. In addition to abi3, the abi2-1 mutant displayed a gin phenotype as well, although its phenotype is not as strong as that of abi3-1 (Fig. 7a). On control and sorbitol media all seedlings showed greening.

The ERA1 gene encodes a β subunit of a protein farnesyl transferase (Cutler et al. 1996) and acts as a negative regulator of ABA signalling. The era1-2 mutant has an ABA hypersensitive phenotype. If ERA1 exerts a similar function in glucose signalling, a glucose oversensitive (glo) phenotype is expected for the era1-2 mutant. Seeds of WT Col and era1-2 were plated on half-strength MS + 6% glucose media. This glucose concentration allowed seedling greening of the WT Col seedlings. Greening started for WT seedlings from day four onwards. However, this glucose concentration arrested early seedling development of era1-2 seedlings. Even after eleven days no greening was observed for era1-2 seedlings (Fig. 7b), which indicates that era1-2 indeed has a glo phenotype. The era1-2 mutant has a more dormant phenotype compared to WT (Cutler et al. 1996). Therefore, seed germination was synchronized as much as possible by using after-ripened seeds and employing a three day stratification period on sugar-free media. On control media (half-strength MS) germination was somewhat delayed but that did not affect cotyledon greening. On 6% glucose era1-2 germination reached similar germination levels as WT seeds despite that germination was 1–2 days delayed (data not shown). This slower germination of era1-2 does not explain the much larger difference observed for cotyledon greening on glucose media. Thus, the small difference in germination timing between era1-2 and WT is not responsible for the seedling greening phenotype.

**Discussion**

**GLUCOSE INSENSITIVE** signalling depends on a functional ABI3 gene

Our results show that ABI3 is an essential regulator of GIN signalling. This conclusion is based on three observations. Firstly, ABI3 transcript levels accumulate in response to glucose treatment. In our study seedlings were treated for a relatively short period of 24 h and showed a 5–9 fold enhanced ABI3 levels, while ABI3 expression levels dropped after 24 h growth on control media (Table 2). The osmotic control conditions induced ABI3 expression at most two fold showing that the strong induction of ABI3 by glucose is not solely due to osmotic stress. Secondly, glucose-regulated gene expression was altered in the abi3 mutant background. Glucose enhanced the expression of LEA genes and two important regulators of GIN signalling ABI4 and ABI5. In WT, glucose treatment led to a 14 fold induction of RAB18 and ABI5, and a 1.5 fold induction of ABI4. The abi3 mutation caused 2–60 fold under
Thirdly, we observed a gin phenotype for seven out of eight abi3 alleles in both Ler-0 and Col accessions. The majority of the abi3 alleles tested showed a gin phenotype that was comparable to well-known gin mutants like aba1, abi4 and abi5 (Fig. 4 and Table 3). This indicates that ABI3 is a key regulator of GIN signalling which is in agreement with previous reports showing that ABI3 overexpression (either AtABI3 or CnABI3) in Arabidopsis results in a glucose oversensitive phenotype (Finkelstein et al. 2002; Zeng and Kermode 2004). Kermode (2005) distinguished at least three functions for ABI3 in seeds. (i) dormancy induction and maintenance (ii) induction of storage proteins and desiccation/stress protectants and (iii) repression of post-germinative gene expression. The observations that abi3 mutants showed a disturbed glucose-regulated induction of RAB18 and a gin phenotype (lack of post-germinative gene repression) are in agreement with the functions defined for ABI3.

Although a role for ABI3 in sugar signalling during early seedling development was hinted by ABI3 overexpression lines (Finkelstein et al. 2002; Zeng and Kermode 2004) and the observation that abi3 mutants were insensitive to glucose in combination with ABA (Nambara et al. 2002) its role remained controversial. This because studies that investigated the gin phenotype of abi3-1 in the Ler-0 background observed at most a weak phenotype. Importantly however, Ler-0 is a sugar oversensitive accession compared to the Col accession (Laby et al. 2000) and likely, the sugar insensitive phenotype of abi3-1 has been underestimated in comparison with mutants in genetic backgrounds that show more rapid greening. Further, the absence of a clear gin phenotype for abi3-1 in earlier studies could be due to the seed material used. We observed that freshly harvested seeds were particularly sensitive to sugars during germination (data not shown). Low sugar concentrations delay germination while higher levels resulted in severely reduced germination rates. Therefore, we routinely after-ripened seeds for at least a month before these were used in our sugar signalling experiments. Seed quality changes per batch produced, which affects germination and most likely responses to internal and external stimuli as well. Hence, WT and mutant seeds used in the sugar response assays were produced simultaneously under the same growth conditions and seeds were stored under identical conditions. Moreover, we routinely stratified the seeds on sugar-free half-strength MS media before the start of the gin assays, since this procedure dramatically improves germination rates following transfer to glucose-containing media (Fig. 6d; Dekkers and Smeekens 2007). Under these conditions we were able to obtain clear gin phenotypes within 12–14 days for abi3 in the Ler-0 background.

In addition, this study identified both abi3-1 and abi3-5 as six mutants (which are insensitive for the sucrose-induced early seedling developmental arrest, Laby et al. 2000) as well. In addition, abi3 mutants are insensitive to the glucose-induced delay of germination (Yuan and Wysocka-Diller 2006; Fig. 6d, e) and abi3 is a weak sun mutant. These observations imply a broader role for ABI3 in mediating sugar responsiveness. Interestingly, another B3 domain transcription factor, HS12, was found to regulate the sugar-inducible sporamin promoter (Tsukagoshi et al. 2005). HS12 is an active transcriptional repressor. A mutation in this gene causes high sporamin promoter activity in both low and high sugar conditions.

Glucose and ABA induce a similar early seedling arrest in Arabidopsis

Glucose treatment of germinated seeds resulted in an enhanced expression of several transcriptional regulators of late seed maturation (ABI3, ABI4 and ABI5). GIN signalling depends on these glucose-induced factors as shown by mutant analysis (León and Sheen 2003; this study), LEA genes (EM1, EM6 and RAB18) were also induced by glucose treatment. The ABI transcription factors are known to regulate the expression of LEA genes (Parcy et al. 1994; Finkelstein et al. 1998; Finkelstein and Lynch 2000; Carles et al. 2002; Kermode 2005). LEA gene expression is correlated with drought and desiccation tolerance (Hoekstra et al. 2001) and its enhanced expression is in agreement with the drought tolerant phenotype observed for glucose-arrested seedlings. Interestingly, the ABA response during

### Table 3 Glucose insensitive phenotype of six abi3 alleles

| Genotype | Treatment | 7% sorb | 7% glc | 10 μM ABA | 3 μM ABA |
|----------|-----------|---------|--------|-----------|----------|
| WT Col   | con       | 100     | 99     | 2         | 0        |
| abi4-3   | 99        | 98      | 89     | 100       | 100      |
| abi5-7   | 100       | 98      | 97     | 96        | 100      |
| abi3-8   | 100       | 100     | 100    | 100       | 100      |
| abi3-9   | 100       | 100     | 100    | 100       | 100      |
| abi-310  | 100       | 100     | 100    | 100       | 100      |
| abi3-11  | 100       | 100     | 5      | 2         | 0        |
| abi3-12  | 100       | 96      | 33     | 83        | 50       |
| abi3-13  | 100       | 100     | 98     | 40        | 0        |

Seeds of WT Col, abi4-3, abi5-7 and six abi3 mutants were stratified on half-strength MS and after stratification switched to control medium (con, 0.5 MS + 1% sucrose) or con medium containing 7% sorbitol, 7% glucose or 10 μM ABA. Values are the percentage of cotyledon greening scored after 11 days. The experiment was repeated once with similar results.

* The ABA insensitivity scored for the different mutant alleles based on cotyledon greening by Nambara et al. (2002)
the post-germination developmental arrest depends on the same ABI3, ABI4 and ABI5 transcription factors. ABA similarly induces ABI3, ABI5 and a subset a LEA genes. Lopez-Molina et al. (2001; 2002) suggested that a late embryogenesis program was re-induced by ABA, which resulted in developmentally arrested, drought tolerant seedlings and that this may constitute an important checkpoint during germination and seedling development.

The developmentally arrested state could only be induced within a limited time frame and after 60–72 h ABA sensitivity was lost. Beyond this limited time window ABA did not induce ABI3 and ABI5 protein levels, nor, re-induce LEA gene transcription and did not block early seedling development. The sugar-induced seedling arrest is restricted to a similar time window (Gibson et al. 2001). Following this time window glucose failed to re-induce ABI3, ABI5 and LEA genes. Thus far it is not clear whether this time frame specifies a sugar sensitive window. Since ABA insensitivity results in a gin phenotype the loss of ABA sensitivity beyond this time frame may account for the glucose insensitivity observed. In conclusion, both glucose and ABA signalling depend on the same set of genes, induce similar transcription factors and LEA genes, result both in drought tolerant phenotypes and are sensitive for a similar time window. This indicates that both trigger a similar seedling developmental arrest.

Interestingly, the induction of desiccation tolerance in germinated radicles is limited to a small time window after germination as well. In germinated seeds with short radicles (<3 mm) desiccation tolerance can be induced while in seeds with longer radicles (>4 mm) this capacity is lost (Buitink et al. 2003). Possibly, this phenomenon depends on the same genetic components and is sensitive in a similar time window observed for glucose- and ABA-induced post-germination developmental block. A possible role was suggested for sugar signalling in desiccation tolerance induction in barrel medic (Medicago truncatula) and cucumber (Cucumis sativa) radicles (Leprince et al. 2004). Non-reducing di- and trisaccharides, e.g. sucrose, protect the structure of membranes and proteins by the formation of a glassy state (Koster 1991; Hoekstra et al. 2001). E.g. elevated sucrose concentrations were measured in desiccation tolerant radicles (Koster and Leopold 1988; Bruggink and van der Toorn 1995). Our study suggests that such elevated sugar concentrations may serve a double role, by protecting cellular structures and by acting as a signal for the induction of protectants such as LEA protein.

Interactions between sugar and ABA signalling

The early seedling developmental arrest phenotype has been extensively exploited to isolate mutants with sugar signalling defects like gin mutants (Rognoni et al. 2007). The gin2/hxk1 mutant revealed a role for HXK1 in glucose signalling. Such HXK1 signalling does not depend on its metabolic function (Moore et al. 2003) and HXK1 was shown to interact with unusual partners (a vacuolar H+-ATPase and a 19S proteasome subunit) to regulate glucose signalling (Cho et al. 2006). Other gin mutants suggested a link between glucose signalling and plant hormone biosynthesis and signalling. The lack of ABA biosynthesis and signalling (gin1/aba2, gin5/aba3 and gin6/abi4) resulted in glucose insensitivity. To study the interaction between ABA biosynthesis, ABI4 and sugar signalling we used ABA deficient lines with constitutive ABI4 expression (aba2-1/35S::ABI4). These strong ABI4 overexpression lines affected ApL3 expression (ISI pathway) and showed a stunted growth phenotype similar to what has been reported previously for severe ABI4 overexpression lines (Söderman et al. 2000), indicating that ABI4 is active in an ABA deficient background. Nevertheless, the gin phenotype of aba2-1 could not be restored by high ABI4 expression levels (Fig. 1c). Thus, ABI4 is necessary but not sufficient for GIN signalling in an ABA deficient background, indicating that other ABA regulated factors are essential for proper GIN signalling as well. Both ISI and GIN signalling rely on ABA biosynthesis and ABI4 but surprisingly, ABI4 overexpression only affected the isi phenotype of aba2-1. Thus, gin and isi sugar signalling depend on the same components but this study shows that the relationship between sugar, ABA and ABI4 is different for both sugar signalling pathways.

ABA biosynthesis and ABA signalling are required for the sugar induced seedling arrest. Genetic studies showed that two transcription factors which are involved in ABA signalling, i.e. ABI4 and ABI5, function in the sugar-induced seedling arrest (Arenas-Huertero et al. 2000; Huijser et al. 2000; Laby et al. 2000; Brocard et al. 2002). Here we show that ABI3 is also essential for GIN signalling. Glucose induces ABA biosynthesis genes (Cheng et al. 2002) and increased ABA levels were measured in developmentally arrested seedlings. ABA is able to induce ABI3 transcription (Table 2) and affects ABI3 protein stability (Zhang et al. 2005). Thus glucose may impinge on ABI3 via activation of ABA biosynthesis. ABI3 also functions in seed development and developmental timing (Rohde et al. 1999, 2000). Severe abi3 mutant embryos remain green and are unable to acquire desiccation tolerance and dormancy during seed development (Ooms et al. 1993). Such mature abi3 embryos resemble a developing seedling rather than a dormant embryo (Nambara et al. 1995). The seedling developmental arrest as induced by ABA and glucose is sensitive to developmental signals as well. Arrest of seedling development by ABA and sugars only occurs within a two to three days time window after imbibition (Gibson et al. 2001; Lopez-Molina et al. 2001).
Therefore, the phenotype of the severe abi3 mutants is probably not solely due to ABA insensitivity but to seed developmental defects and the heterochronic nature of this mutation as well.

Two additional ABA response mutants with altered glucose responsiveness, abi2 and era1, strengthen the link between glucose and ABA signalling. The abi2-1 mutant showed a gin phenotype that is weaker compared to abi3-1. Possibly additional seed developmental phenotypes of abi3 may explain the stronger phenotype of the abi3-1 mutant compared to abi2-1. However, the ABA insensitive phenotype of abi2-1 is somewhat weaker compared to abi3-1 (Koornneef et al. 1984; Finkelstein and Somerville 1990), which could explain the weaker gin phenotype as well. The era1-2 mutant is hypersensitive to ABA and shows a glucose oversensitive phenotype. Interestingly, era3 another ABA hypersensitive mutant shows a similar glo phenotype. Era3 is allelic to ethylene insensitive2 (ein2, Ghassemian et al. 2000) and also other ethylene related mutants show sugar signalling defects (Zhou et al. 1998; Gibson et al. 2001; León and Sheen 2003).

The role of ABA and ABA signalling in sugar signalling is well established. Thus far, sugar signalling was thought to rely on only a part of the ABA signalling cascade. The analysis of aba2/35S::ABI4 transgenic lines showed that ABA related factors other than ABI4 are involved in glucose signalling. Furthermore, the glucose-induced early seedling arrest mimics the ABA-induced seedling arrest. Our study indicates that all response loci tested that act in the genetic pathway regulating ABA responsiveness as proposed by Brady et al. (2003) affect glucose signalling. These observations suggest that the genetic pathways regulating glucose and ABA signalling are much more closely linked than assumed thus far. Obviously, both pathways are different as well. Genetic analysis suggested that HXK1 acts upstream of ABA signalling in glucose signalling. Moreover, the gaolaozhswangren2 mutant has a gin phenotype but is not affected in ABA signalling (Chen et al. 2004). Such mutants may provide insight in the mechanism by which glucose affects ABA signalling. Interestingly, glucose delays germination in a process that depends on ABI3 but that does not require ABI1, ABI2 ABI4 and ABI5 (Gibson 2005; Yuan and Wysocka-Diller 2006; Fig. 6d, e), showing that the tight interaction between glucose and ABA is not present in all sugar response pathways.

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