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ORIGINAL ARTICLE

Heterologous expression of Arabidopsis *ABF4* gene in potato enhances tuberization through ABA-GA crosstalk regulation

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Abstract Potato (*Solanum tuberosum* L.) tuberization is regulated by many signals, such as abscisic acid (ABA), sucrose and gibberellic acid (GA). ABA and sucrose are positive modulators, while GA is an inhibitor of the process. ABF (ABRE-binding factor) proteins are transcription factors involved in ABA and stress signaling. Previously, we reported that *S. tuberosum* StABF1 could mediate the ABA effects on tuberization. The aim of the present study was to evaluate the potential use of ABF genes to enhance tuberization and to determine the molecular mechanism involved. For this purpose, transgenic potato plants expressing the Arabidopsis *ABF4* or *ABF2* genes were generated, and their tuberization capacity and response to tuberization-related signals were analyzed in vitro. The results indicate that both ABF4 and ABF2 proteins positively regulate potato tuber induction; however, only *ABF4* expression significantly increases the number and weight of the tubers obtained, without stunting growth. ABF4 and ABF2 transgenic plants exhibit ABA hypersensitivity during tuberization, accompanied by a GA-deficient phenotype. *ABF4* expression triggers a significant rise in ABA levels in stolons under tuber-inducing conditions as compared with wild-type plants and a transcriptional deregulation of GA metabolism genes. Our results demonstrate that Arabidopsis ABF4 functions in potato ABA-GA signaling crosstalk during tuberization by regulating the expression of ABA- and GA-metabolism genes. *ABF4* gene might be a potential tool to increase tuber production, since its heterologous expression in potato enhances tuber induction without affecting plant growth.

Keywords ABF/AREB · *ABF2* · *ABF4* · Abscisic acid · Gibberellic acid · Potato · *Solanum tuberosum* · Tuberization

Abbreviations

ABA	Abscisic acid
GA	Gibberellic acid
ABF	ABRE-binding factor
AREB	ABA-response element binding factor

Introduction

ABF (ABRE-binding factor) proteins, also referred to as AREB (ABA-response element binding factor) are key regulators of abscisic acid (ABA) signaling during abiotic stress (Choi et al. 2000; Uno et al. 2000). They are basic leucine zipper (bZIP) transcription factors that bind to the ABA-responsive element (ABRE), the major *cis*-acting regulatory sequence that controls ABA-dependent gene expression (Hirayama and Shinozaki 2010). ABF proteins are activated by phosphorylation in an ABA-dependent manner to induce the transcription of target genes. The protein kinases reported to be involved in this activation are SnRK2s (SNF1-related protein kinase subfamily 2 Uno et al. 2000) and CDPKs (calcium-dependent protein kinases; Choi et al. 2005; Muñiz García et al. 2012).

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The involvement of ABF transcription factors in ABA and abiotic stress signaling has been widely described in many plant species, like *Arabidopsis thaliana* (Choi et al. 2000; Uno et al. 2000), barley (*Hordeum vulgare*; Casaretto and Ho 2005), wheat (*Triticcum aestivum*; Kobayashi et al. 2008), rice (*Oryza sativa*; Hossain et al. 2010a, b), tomato (*Solanum lycopersicum*; Hsieh et al. 2010; Orellana et al. 2010; Yáñez et al. 2009) and potato (*Solanum tuberosum*; Muñiz García et al. 2012). Despite their undoubted roles in abiotic stress response, ABF proteins have been involved in other physiological processes, such as sugar signaling (Kang et al. 2002; Kim et al. 2004), tomato fruit development (Bastías et al. 2011) and response to pathogens (Orellana et al. 2010).

Potato is one of the world's most important crops. Therefore, research on potato tuber induction that enables the understanding and genetic engineering of this process is important to increase crop yield. Potato tuber is formed from an underground stem, called stolon. In conditions that are non-inductive for tuberization, stolons often grow upward and emerge out of the soil to form a new shoot, but in tuber-inducing conditions, stolons grow underground until the tip swells to form the tuber (Jackson 1999). Tuberization is thus a process that requires the cessation of stolon apex growth, the swelling of the stolon by sub-apical radial growth and finally, the enlargement of the tuber.

There are many environmental factors that influence tuber formation, such as nitrogen levels, temperature and light (Jackson 1999). Since effects of the environmental signals on potato tuberization are essentially hormone-mediated, phytohormones have been suggested to play a prominent role in the control of tuberization (for review, see Hannapel et al. 2004; Rodríguez-Falcón et al. 2006; Sarkar 2008). Gibberellic acid (GA) is a dominant negative regulator that promotes stolon elongation and inhibits tuber formation (Kumar and Wareing 1972; Smith and Rappaport 1969). It was also reported that a decline of GA content in potato plants is associated with tuberization (Krauss and Marschner 1982; Okazawa 1960; Railton and Wareing 1973). GA has long been implicated in the photoperiodic inhibition of tuberization in species that strictly require short-day conditions for tuber formation, such as *Solanum demissum* and *Solanum tuberosum* Group Andigenum (for review see Martínez-García et al. 2001; Sarkar 2010). Conversely, ABA is regarded as a promoting hormone in tuberization. Tuber formation in ABA-containing 8 % sucrose medium (tuber-inducing conditions) started earlier than in ABA-free medium (Menzel 1980; Xu et al. 1998). Moreover, application of ABA to 1 % sucrose medium (non-inducing conditions) significantly promoted the initiation and frequency of tuber formation (Xu et al. 1998). However, the roles of ABA with respect to stolon elongation, tuber initiation and tuber development are still unclear. ABA might exert its positive effect by inhibiting stolon elongation, a prerequisite for tuberization (Vreugdenhil and Struik 1989). It has been proposed that ABA also promotes tuberization by counteracting the inhibitory effect of GA (Xu et al. 1998), possibly by decreasing GA content in developing stolons and tubers. Similarly, sucrose positively regulates tuberization by influencing GA levels (Xu et al. 1998).

Antagonism often implies the presence of crosstalk points, where two signaling pathways intersect. It has been shown that the ABA-responsive kinase PKABA1 (a member of the SnRK2 subfamily) is up-regulated by ABA and subsequently inhibits the GA pathway in barley aleurone cells (Gómez-Cadenas et al. 2001; Ho et al. 2003). In these cells, PKABA1 interacts with HvABF1 and HvABF2, which function as negative regulators of GA signaling (Schoonheim et al. 2009). These results support the hypothesis that ABF transcription factors may function as crosstalk intermediates in ABA and GA signaling pathways.

In a previous work, we reported the cloning and characterization of an ABF transcription factor from *S. tuberosum*, named StABF1. The results obtained suggest that StABF1 might be a key regulator of ABA-dependent stress signaling pathways in cultivated potato, but also may mediate the ABA effects on tuberization. Interestingly, StABF1 is phosphorylated in a calcium-dependent manner, and this phosphorylation is stimulated by ABA or tuber-inducing conditions (high sucrose/nitrogen ratio in the medium), but is inhibited by GA (Muñiz García et al. 2012), suggesting that this ABF transcription factor may act as a positive regulator of tuberization mediating the crosstalk between ABA and GA signaling pathways. These findings provide a new strategy to improve tuber production using ABF genes. The aim of the present study was to evaluate the potential use of ABF transcription factors in engineering tuberization and to determine the molecular mechanism involved in this process. For this purpose, transgenic potato plants expressing the Arabidopsis *ABF4* or *ABF2* genes were generated, and their tuberization capacity and response to tuberization-related conditions were analyzed.

Materials and methods

Generation of *35S-ABF4* and *35S-ABF2* transgenic potato plants

The coding regions of *ABF4* and *ABF2* (clones U10909 and U85579, respectively, obtained from The Arabidopsis Information Resource, http://www.arabidopsis.org) were amplified by PCR using Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, FI). The fragments were sub-cloned into the pHAP12 vector to generate the plant-expression cassettes

35S(L)CaMV-Ω enhancer-*ABF4/2*-tNOS. The intactness of the junction sequence was confirmed by DNA sequencing. The plant-expression cassette was then cloned into the binary vector pPZP-NPT (Romano et al. 2001). Transformation of potato minituber discs was performed using *Agrobacterium tumefaciens* strain EHA101. Transformation of *A. tumefaciens* was carried out by the "freeze and thaw" method described by Holsters et al. (1978). Minitubers (*Solanum tuberosum* cv. Spunta) were washed, sterilized, cut and transferred to Petri dishes containing Murashige and Skoog medium (MS; Prod Nº M519, *PhytoTechnology* Laboratories, Shawnee Mission, KS) with 20 g/l sucrose solidified with 0.7 % (w/v) agar, according to Bravo-Almonacid et al. (2012). For transformation, batches of 50 minituber discs were co-cultivated for 5 min with *A. tumefaciens*, blotted dry on sterile filter paper and transferred to semisolid MS medium supplemented with 2 % sucrose and 10 mg/l acetosyringone for 30–36 h at 24 °C in darkness. Minituber discs were washed with sterile water containing 400 mg/l cefotaxime, blotted dry on sterile filter paper and transferred to semisolid MS medium supplemented with 2 % sucrose and 400 mg/l cefotaxime, 50 mg/l kanamycin, 5.0 μM of indoleacetic acid and 5.0 μM zeatin riboside. Regeneration controls were co-cultivated with non-transformed *Agrobacterium* and finally transferred to the same medium without kanamycin. Explants were incubated at 22 °C for 48 h under 16-h photoperiod and transferred to fresh medium every 2 weeks until plants over 2 cm in height were obtained. These plants were sub-cultured in the same growth medium for root development. Rooted plants were micro-propagated in vitro. Regenerated plants carrying no plasmid but obtained at the same time from the same explants and by the same regeneration method were used as controls. Fifteen lines were confirmed as transgenic and their phenotypes were examined for preliminary characterization. From these lines, three and two independent lines of ABF4 and ABF2, respectively, were selected and characterized in this study (see below). The transgenic lines selected presented different levels of transgene expression and different degrees of tuberization phenotype in the preliminary characterization.

Plant growth conditions

Transgenic and control plants were propagated in vitro from single-node cuttings on MS medium containing 20 g/l sucrose solidified with 0.7 % (w/v) agar. They were grown in a growth chamber under a 16-h photoperiod (4,000 lx light intensity) at 22 °C.

PCR amplification of the transgenes

The primary screening of putative transgenic plants was carried out using PCR. Plant genomic DNA was isolated from leaves of in vitro plantlets as described by Murray and Thompson (1980). PCR amplification was performed using several primer pairs designed for specific transgene detection. Amplification products were separated by electrophoresis in a 1.5 % agarose gel and visualized by ethidium bromide staining. Primer sequences were as follows: P1: 5′-ATCTCCACTGACGTAAGG-3′, P2: 5′-CGGGATCCCGATGGGAACTCACAT-3′, P3: 5′-GGGTGGGATACCAGCAC-3′, P4: 5′-AGGATGAAGCAACAAGAACG-3′, P5: 5′-TGATAATCATCGCAAGACCG-3′, P6: 5′-CGGGATCCCGATGGATGGTAGTATG-3′, P7: 5′-CGGAATTCCGTCACCAAGGTCCCGA-3′.

Southern blot analysis

Genomic DNA was isolated from young leaves of in vitro-grown plants (Murray and Thompson 1980). Aliquots of DNA (10 μg) from control and transgenic plants were digested overnight at 37 °C with *Nde*I or *Xba*I (New England Biolabs, Beverly, MA), for ABF4 or ABF2 analysis, respectively. Samples were concentrated to 45 μl, separated on 0.8 % (w/v) agarose gels and blotted onto a nylon membrane (Hybond N+, GE Healthcare, Piscataway, NJ). A fragment of 790 bp corresponding to the NPTII gene amplified from the pPZP-NPT vector (Brown et al. 1993) was used as a probe for Southern hybridization. The probe was labeled with ^{32}P using the Prime-a-Gene DNA Labeling System kit (Promega, Madison, WI). The blot was hybridized at 60 °C for 16 h. Washes were carried as follows: 2× SSC/0.1 % SDS for 20 min at 45 °C, 1× SSC/0.1 % SDS for 10 min at 45 °C, 0.5× SSC/0.1 % SDS for 15 min at 50 °C and 0.1× SSC/0.1 % SDS for 30 min at 55 °C (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate). Bands were visualized using a Storm PhosphorImager (GE Healthcare).

Stolon growth conditions and in vitro tuberization

Nodal segments from in vitro control and transgenic plants were grown on MS medium containing 20 or 80 g/l sucrose (non-inducing and inducing conditions, respectively) solidified with 0.7 % (w/v) agar. Stolons were grown in a growth chamber under darkness at 22 °C. The basal MS medium was supplemented with GA_3 (5.0 μM), CCC (chlorocholine chloride, 500 mg/l) or ABA (5.0 μM).

Sensitivity to ABA was calculated as the inverted ratio of the reduction in length observed for stolons grown in 5.0 μM ABA relative to the reduction observed in stolons from regeneration control plants (100 %). GA sensitivity was calculated as the ratio of the increase in stolon length in 5.0 μM GA_3 relative to the increase observed in stolons from regeneration control plants (100 %).

Northern blot analysis and semi-quantitative reverse transcription-PCR (RT-PCR)

Samples (0.1–1 g) were collected, ground in liquid nitrogen and total RNA was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA). Total RNA (15 μg) was separated on formaldehyde 1.2 % (w/v) agarose gels and blotted onto nylon membranes (Hybond N+, GE Healthcare). Northern blots were hybridized with *Patatin* probe (País et al. 2009, 2010) labeled with ^{32}P by random priming with Prime-a-Gene DNA Labeling System kit (Promega). After sequential stringent washes (2× SSC/0.1 % SDS for 10 min, 1× SSC/0.1 % SDS for 10 min, 0.5× SSC/0.1 % SDS for 10 min, at 60 °C), bands were visualized using a Storm PhosphorImager (GE Healthcare).

Semi-quantitative RT-PCR was performed to detect transgene transcripts. RNA was isolated as described above. M-MLV Reverse Transcriptase (Invitrogen) was used for cDNA synthesis according to the manufacturer's protocol using a mix of oligo(dT)20 and random primers (Invitrogen). The newly synthesized cDNA was used as template for PCR amplification. *ABF4* expression was evaluated using the primers P2-P3 and P4-P5 described above (annealing temperature: 49 °C, 27 cycles). *ABF2* expression level was determined by PCR specific amplification of a 262-bp fragment (primer AtABF2 probe forward: 5′-GTTACTTTGGTAATGATGCC-3′, primer AtABF2 probe reverse: 5′-CGTTAAAGACTGATCTCCAAG-3′; annealing temperature: 47 °C, 22 cycles). 18S rRNA was used for normalization of mRNA expression levels (primer 18S forward: 5′-GGCATTCGTATTTCATAGTCA GAG-3′, primer 18S reverse: 5′-GGTTCTTGATTAAT GAAAACATCCT-3′; annealing temperature: 58 °C, 17–20 cycles). Alternatively, EF1-α (elongation factor 1-α) was used for normalization (primer EF1-α forward: 5′-GTATG-GTTGTGACCTTTGG-3′, primer EF1-α reverse: 5′-CAACATTCTTGACAACAC-3′; annealing temperature: 48 °C, 20–25 cycles). The described conditions were chosen to ensure that the PCR reaction was terminated within the linear range of amplification.

Northern blot bands were quantified relative to the ethidium bromide-stained ribosomal RNA (rRNA). RT-PCR bands were quantified relative to the internal control 18S rRNA or EF1-α. Quantifications were carried out using ImageJ software (http://rsb.info.nih.gov/ij/). Each experiment was performed at least three times independently.

RT-qPCR analysis

RNA was isolated and cDNA synthesis was performed as described above. The newly synthesized cDNA was used as template for PCR amplification after dilution with sterile RNAse-free water. Relative expression levels of ABA and GA metabolism genes were determined by real-time RT-qPCR on a DNA Engine Opticon sequence detector (BioRad, Hercules, CA). Potato EF1-α primers (Nicot et al. 2005) were used as a control. The primer sequences for *StGA20ox1* (AJ291453), *StGA20ox3* (AJ291455), *StGA3ox2* (AY039110) and *StGA2ox1* (EU003995) amplification were obtained from Kloosterman et al. (2007), while the primer sequences for *StZEP* (DQ206629), *StNCED1* (AY662342) and *StCYP707A1* (DQ206630) amplification were obtained from Destefano-Beltrán et al. (2006). Reactions were performed in a final volume of 20 μl containing 4 μl of 5XHOT FIREPol EvaGreen qPCR Mix Plus (Solis-BioDyne, Tartu, Estonia) and 1 or 2 μl of primer mix (containing 2.5 μM of each forward and reverse primer) for GA metabolism genes and EF1-α or ABA metabolism genes, respectively. The amount of cDNA used in each reaction was derived from 1 ng of total RNA for EF1-α amplification, and 15 ng or 5–10 ng for GA or ABA metabolism gene amplification, respectively. Reactions for ABA metabolism genes were carried out under the following conditions: 95 °C/15 min (1 cycle); 94 °C/2 min (1 cycle); 58 °C/1 min (1 cycle); 72 °C/1 min (1 cycle); 94 °C/30 s, 58 °C/30 s; 72 °C/45 s (35–40 cycles). For EF1-α and GA metabolism gene amplification, reactions were carried out under the following conditions: 50 °C/2 min (1 cycle); 95 °C/15 min (1 cycle); 95 °C/15 s; 60 °C/1 min; 72 °C/30 s (35–40 cycles).

PCR amplification of a single product of the correct size for each gene was confirmed by agarose gel electrophoresis. The amplified fragment of each gene was purified and used to generate efficiency curves. Relative fold expression for each gene was calculated by the method of Pfaffl (2001).

Measurement of ABA in *S. tuberosum* leaf and stolon extracts

To determine the ABA levels, leaves from 15-day-old in vitro grown plants or stolons obtained after 15 days of culture under tuber-inducing conditions (500 mg, approximately) were harvested in liquid nitrogen and freeze-dried. To obtain homogeneous samples, leaves and stolons from several plants were pooled for each measurement. ABA extraction was performed according to the method reported by Kelen et al. (2004). Content of ABA was determined by high-performance liquid chromatography (HPLC)as described in Iriti et al. (2009), using an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA), the column Eclipse XDB-C18, methanol:water (70:30), pH 4 as mobile phase and a flow rate of 0.5 ml/min.

Statistical analysis

Statistical analysis was carried out using the Student's *t* test. The data were considered significantly different

from the control when P values were <0.05. The asterisks in the figures indicate statistical significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$; with respect to the regeneration control (unless otherwise indicated in the figure legend).

Results

Generation of potato transgenic lines expressing *ABF4* or *ABF2*

Transgenic potato plants expressing the Arabidopsis *ABF4* or *ABF2* genes under the control of the cauliflower mosaic virus 35S promoter were generated. Representative plants growing on media containing kanamycin and showing PCR amplification of the respective transgenes (Fig. S1a) were selected for detailed examination. Southern blot analysis showed that ABF4 lines 4.2, 4.3 and 4.1 derived from independent integration events and contained one or two copies of the transgene (Fig. S1b), while ABF2 lines 2.1 and 2.2 were independent single-copy transformants (Fig. S1b). The expression of the respective transgenes was confirmed by RT-PCR (Fig. 1a). Transgene expression levels were lower in the 4.2 and 4.3 ABF4 lines than in the 4.1 line, while no significant differences were obtained between the ABF2 lines 2.1 and 2.2.

No significant phenotypic differences were observed between regeneration controls (wild type) and ABF4 plants grown in vitro (although line 4.1 resulted slightly shorter and presented smaller leaves), while ABF2 plants were shorter than control plants (Fig. 1b). ABF4 plants grown in soil exhibited normal vegetative phenotype as compared with control plants (Fig. 1c). These results indicate that *ABF4* expression has little or no effect on potato plant development, while *ABF2* expression negatively affects potato plant growth.

In vitro tuberization of ABF4 and ABF2 transgenic plants

Tuberization can be analyzed in vitro, providing a good representation of the processes occurring in vivo, with the advantages of producing tubers in a controlled environment (Ewing and Struik 1992; Hendriks et al. 1991; Koda and Okazawa 1983; Xu et al. 1998). Single-node cuttings taken from potato plants usually do not induce tubers when cultured in darkness on standard propagation media (MS medium plus 2 % sucrose); however, increasing sucrose concentration (8 %) increases the frequency of tuberization. To evaluate the effects of *ABF4* or *ABF2* expression on tuber induction, single-node cuttings from control (wild type) and transgenic plants were cultured in darkness in non-inducing (MS 2 % sucrose) or tuber-inducing (MS 8 % sucrose) medium.

Stolon length was measured, since cessation of stolon growth is a prerequisite for tuberization. After 15 days of growth under non-inducing conditions, no significant differences in stolon length were observed between 4.2 or 4.3 ABF4 lines and control plants, while line 4.1 showed shorter stolons (Fig. 2, left and S2a, upper-left panel). No signs of tuber induction were observed in stolons of ABF4 or control plants under non-inducing conditions; however, line 4.1 occasionally showed shorter stolons with sub-apical thickening (Fig. S3). The stronger phenotype of line 4.1 under non-inducing conditions is consistent with its higher transgene expression. Under tuber-inducing conditions, stolons of ABF4 plants were significantly shorter than those of control plants (Fig. 2, right and S2a, upper-right panel).

ABF2 stolons became shorter than those of control plants under non-inducing conditions as well as under tuber-inducing conditions (Fig. 2 and S2a, lower panels), in agreement with the previous observation of a stronger effect of ABF2 on plant growth inhibition.

After 15 days of culture under tuber-inducing conditions, potato plants expressing *ABF4* or*ABF2* showed higher tuber induction rates (higher number of stolons with visible swelling) as compared with control plants (Fig. 3a) and an increased transcriptional induction of the tuberization gene marker *Patatin* (Fig. 3b). No *Patatin* signal was, however, detected in stolons of control or transgenic lines grown under non-inducing conditions.

Time-course in vitro tuberization assays using single-node cuttings (Fig. 4a, b and S4) revealed that ABF4 stolons had higher tuber induction rates at all measured time points, indicating that the increased rate of tuber initiation is accompanied by a higher percentage of tubers obtained at the end of the assay. On the other hand, ABF2 stolons had higher tuber induction rates only at the beginning of the experiment (15 days), showing no significant differences from control stolons with increasing time periods (Fig. 4a, b and S4). Tubers from ABF4 plants became larger than those from control plants (Fig. 4c, d). These results indicate that the differences between ABF4 and control plants are not merely due to a different time on tuber initiation but also to the number and size of tubers obtained in vitro, while ABF2 plants only show a more rapid tuber initiation than control plants, with no differences in the number and size of tubers.

Surprisingly, at longer times of culture under non-inducing conditions (between 30 and 60 days), stolons from control plants showed no signs of tuber induction, while lines 4.1 and 4.2 presented clear sub-apical thickenings after 30 days of culture (Fig. 5a), tuber induction rates above 10 % between 30 and 45 days (Fig. 5b) and completely formed tubers at the end of the assay (60 days). This result agrees with the stronger tuber-induction observed for line 4.1 and 4.2 under tuber-inducing conditions after

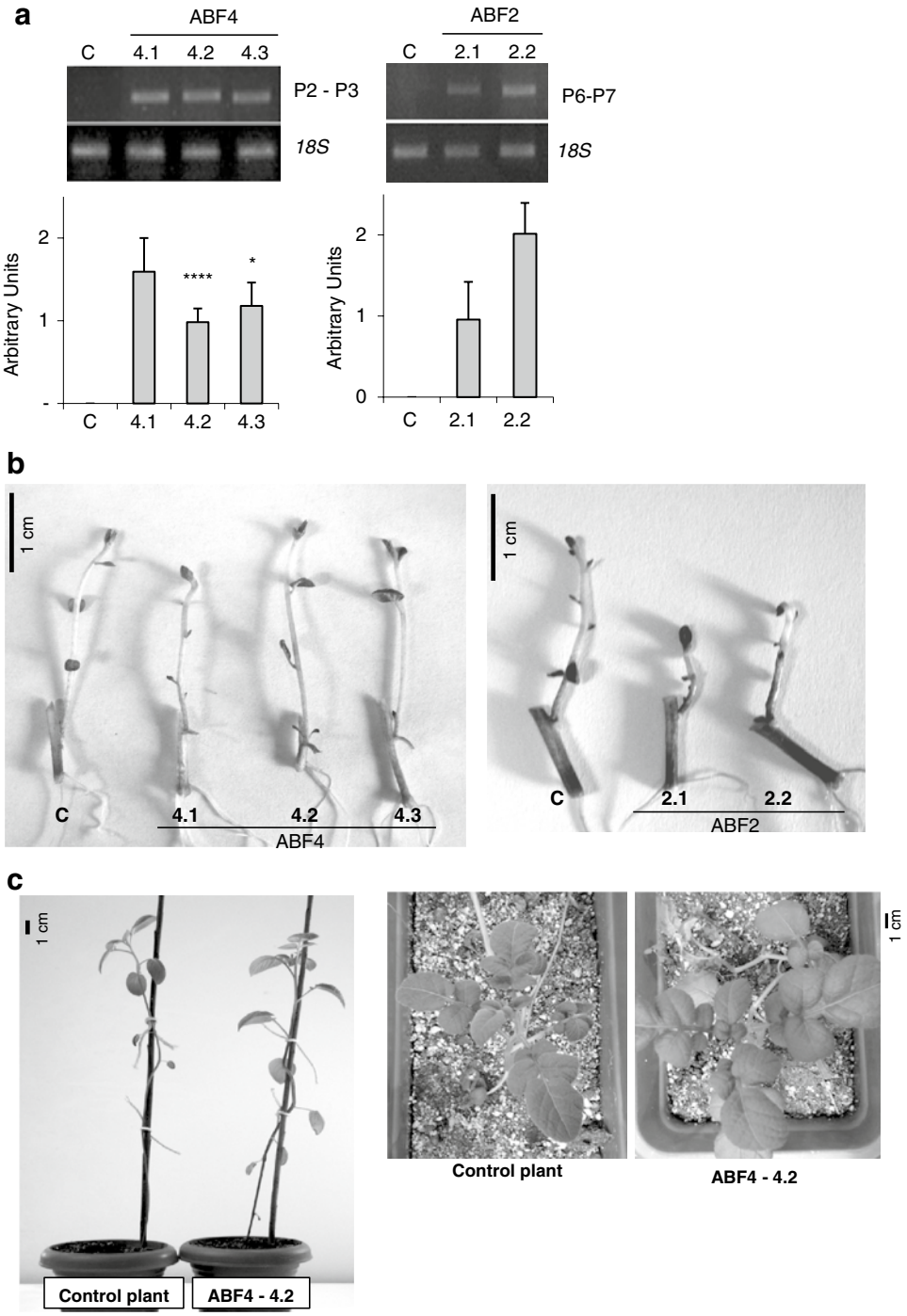

Fig. 1 Molecular analysis of ABF4 and ABF2 transgenic potato plants. **a** Transgene expression analysis by semi-quantitative RT-PCR performed using the indicated primers, displayed in Fig S1. 18S rRNA was amplified as internal control. Quantitative data of RT-PCR bands (mean ± SD) are displayed in the *bar graph*. A representative RT-PCR analysis is shown. *Asterisks* indicate statistical significance with respect to line 4.1. **b** Phenotype of ABF4 and ABF2 transgenic lines. Plants derived from nodal segments were grown for 15 days under standard conditions. **c** ABF4 4.2 and control plants generated from tubers obtained in vitro (*left*) or transferred ex vitro (*right*), grown in soil for 2 months (lines 4.1 and 4.3 are not shown but were phenotypically similar from line 4.2, although 4.1 plants resulted slightly shorter). *C* regeneration control

15 and 30 days, in comparison with line 4.3 (Figs. 3, 4a, b and S4). When control and ABF4 transgenic whole plants (corresponding to other independent transgenic lines whose characterization was not documented in this study) were cultivated in vitro on MS medium containing 2 % sucrose for 3.5 months, 86.4 % of ABF4 plants developed tubers, while none of the control plants tuberized (Fig. S5). Moreover, 18 % of ABF4 plants produced more than one tuber per plant.

The results obtained show an increased tuber induction in stolons from ABF4 and ABF2 plants after 15 days of growth under tuber-inducing conditions, at the morphological and molecular level, suggesting that ABF transcription factors act as positive regulators of high sucrose-induced tuberization, possibly modulating ABA signaling which in turn is a positive regulator of the process. Stolons from ABF4 plants are also able to tuberize under non-inducing culture conditions.

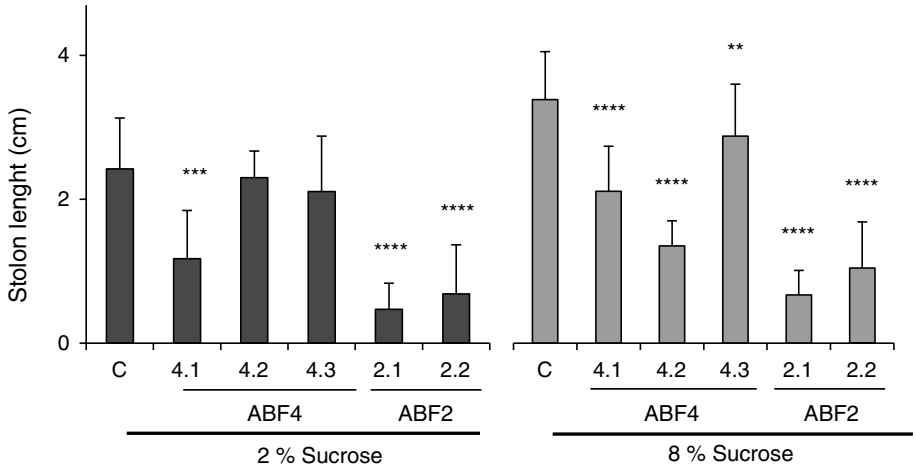

Fig. 2 Growth of stolons from ABF4 and ABF2 plants under non-inducing and tuber-inducing conditions. Stolon length of single-node cuttings from control, ABF4 and ABF2 transgenic lines under non-inducing (2 % sucrose) and tuber-inducing (8 % sucrose) conditions after 15 days of culture. Quantitative data of six independent experiments (mean ± SD) are displayed in the *bar graph*. *C* regeneration control

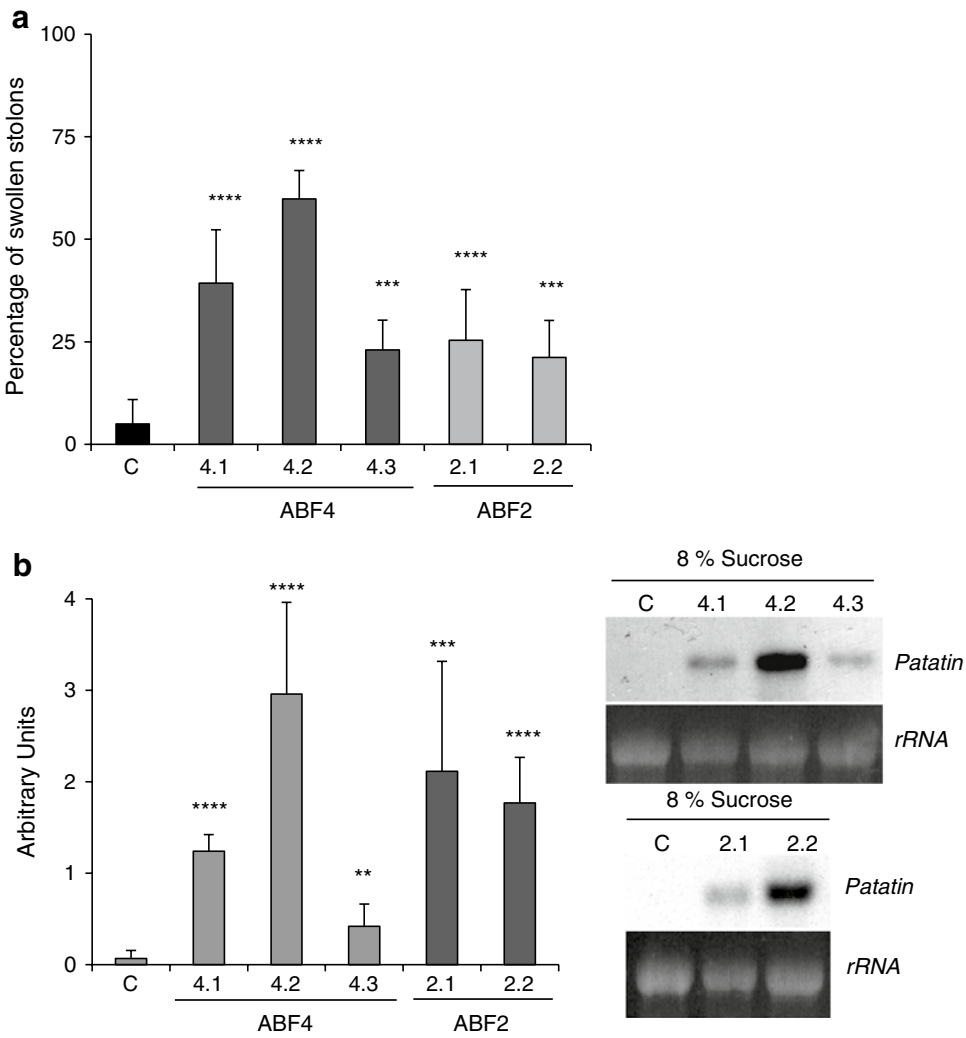

Fig. 3 In vitro tuberization of stolons from ABF4 and ABF2 plants. **a** Percentage of swollen stolons of single-node cuttings from control, ABF4 and ABF2 transgenic lines cultured under tuber-inducing conditions (8 % sucrose) for 15 days. Quantitative data of six independent experiments (mean ± SD). **b** Northern blot analysis for *Patatin* expression of RNA extracted from stolons grown in MS 8 % sucrose for 15 days. Quantitative data of Northern blot (mean ± SD) are displayed in the *bar graph* (arbitrary units). A representative blot is shown. *C* regeneration control; *rRNA* ribosomal RNA

ABA signaling in ABF4 and ABF2 transgenic plants

Since ABA has a positive role in tuberization, and ABFs are master transcription factors involved in ABA signaling, the stolon length response to ABA application was analyzed. Treatment with 5.0 μM ABA resulted in the reduction of stolon length in both control and transgenic plants, under inducing and non-inducing conditions (Fig. 6a, b,

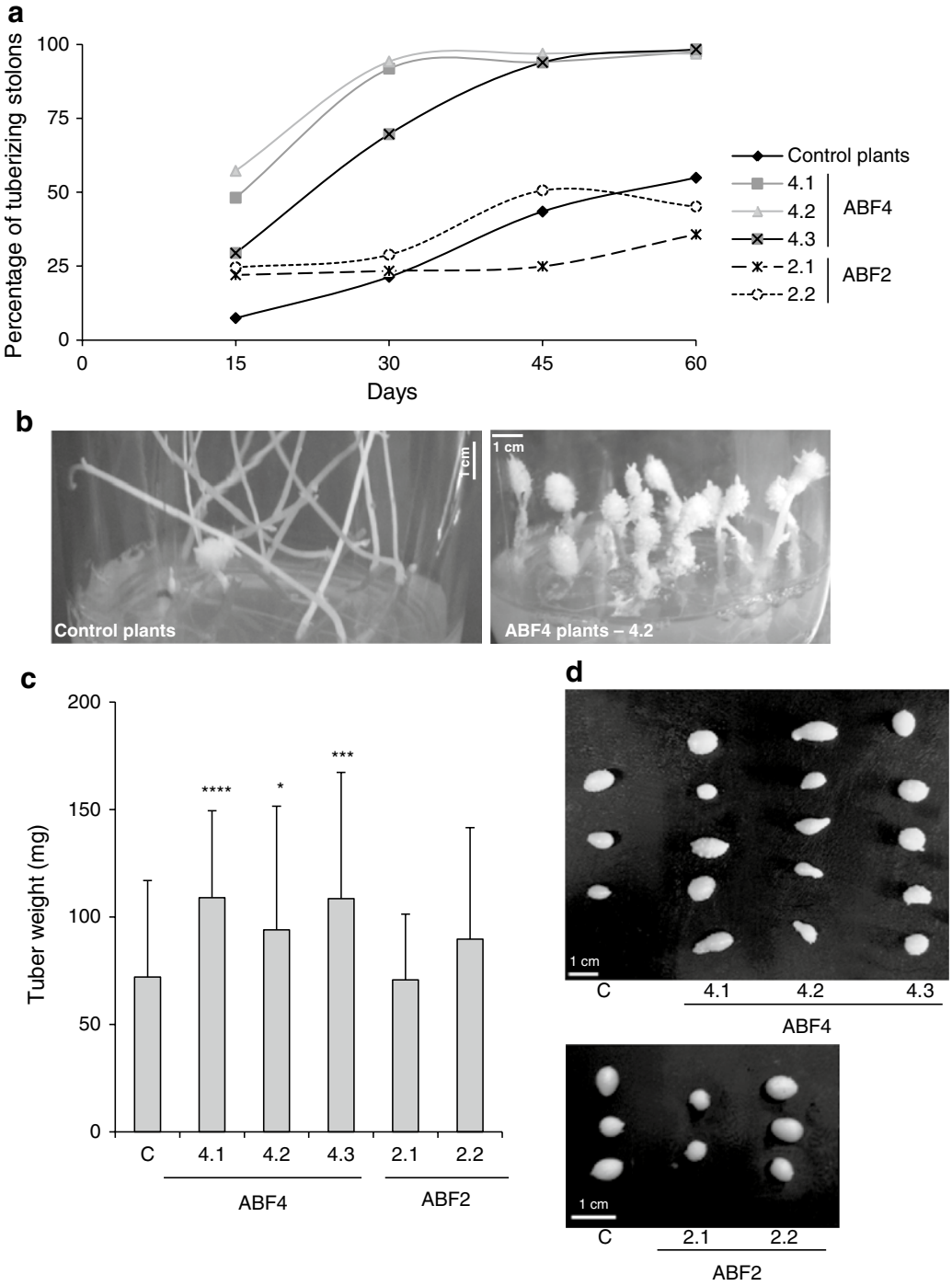

Fig. 4 Time-course tuberization rate and tuber weight of stolons from ABF4 and ABF2 plants. **a** Percentage of tuberizing stolons from control and transgenic plants was determined at the indicated times of culture in MS 8 % sucrose. The results are shown in a line graph for better visual comparison of the tuberization rate. Values are means of at least four independent experiments (statistical analysis is shown in Fig. S4). **b** Representative images of stolons from control and ABF4 line 4.2 plants after 30 days of culture in MS 8 % sucrose. **c** Weight of tubers obtained at the end of the experiment. Data are mean ± SD of all tubers obtained after 60 days of stolon culture in MS 8 % sucrose ($n = 88$ for *C*; $n = 63$ for 4.1; $n = 49$ for 4.2; $n = 30$ for 4.3; $n = 26$ for 2.1; $n = 28$ for 2.2). **d** Representative images of tubers from control and transgenic plants described in **c**. *C* regeneration control

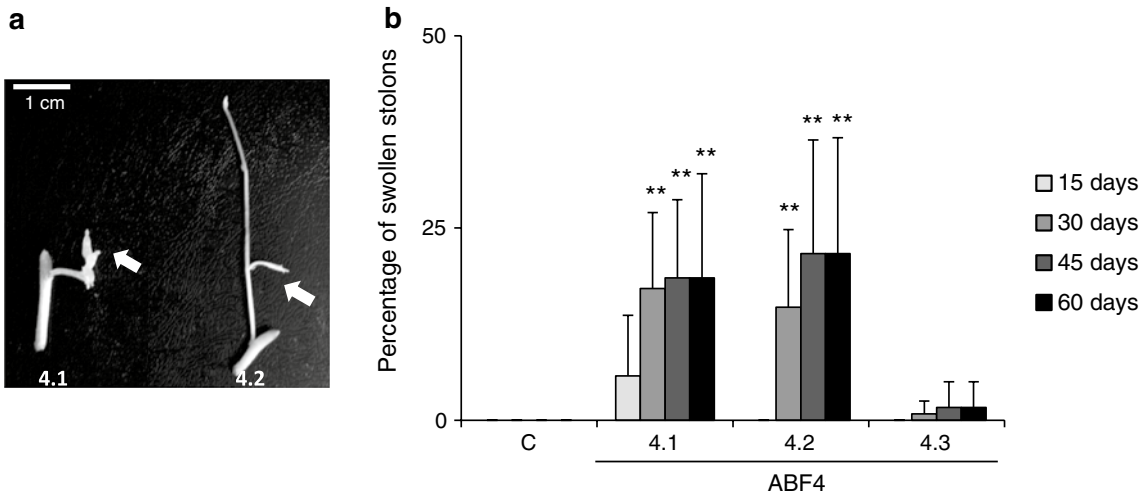

Fig. 5 Time-course tuberization rate of stolons from ABF4 plants under non-tuber inducing conditions. **a** Representative images of swelling stolons from 4.1 and 4.2 ABF4 plants after 30 days of culture in MS 2 % sucrose. **b** Percentage of swelling stolons from control and ABF4 plants cultured in MS 2 % sucrose for the indicated times. Values are mean ± SD of at least four independent experiments. *C* regeneration control

Fig. S2b); however, ABF4 and ABF2 stolons showed an increased sensitivity to ABA in both conditions (Fig. 6c). ABF2 and ABF4 plants produced shorter stolons than control plants in the absence of exogenous ABA (mainly under inducing conditions), suggesting a constitutive response to the hormone (Fig. 6a, b, Fig. S2b).

The increased sensitivity to ABA and constitutive response to the hormone is the result of an enhanced ABA signaling through ABF4 or ABF2 transcription factors in the transgenic plants. However, it could also be explained by increased endogenous ABA levels, which are controlled by transcriptional regulation of ABA metabolism genes (Fig. 7a). Expression of ABA biosynthetic genes, such as *ZEP* and *NCED*, can be activated in response to several signals and lead to an increase in the endogenous ABA content (Nambara and Marion-Poll 2005). Conversely, the expression of genes encoding degradative enzymes, such as *CYP70A*, negatively regulates ABA accumulation. In order to determine if ABF4 plants accumulate higher levels of ABA, the expression of *StZEP*, *StNCED1* and *StCYP707A1* was quantified by RT-qPCR. ABF4 stolons grown under tuber-inducing conditions (MS plus 8 % sucrose) had higher transcript levels of ABA biosynthetic genes (*StZEP* and *StNCED1*) and equal or even lower levels of *StCYP707A1* as compared with control plants (Fig. 7b, left). However, leaves from ABF4 plants grown under non-inducing conditions (MS plus 2 % sucrose), showed higher *StCYP707A1* levels than control plants accompanied by increased expression of *StZEP* and *StNCED1* genes (Fig. 7b, right), suggesting a compensatory mechanism to decrease ABA concentration in leaves. To confirm this, endogenous ABA levels were measured in control and ABF4 plants. Leaves from ABF4 plants grown under non-inducing conditions had lower ABA levels than control plants (Fig. 7c). On the other hand, ABF4 stolons obtained after 15 days of culture on tuber-inducing medium had increased ABA levels as compared with stolons from control plants (Fig. 7d).

GA response inABF4 and ABF2 transgenic plants

Since ABA effects on tuberization seem to be mediated mainly through the negative regulation of GA levels (Xu et al. 1998), the effects of exogenous GA or CCC (chlorocholine chloride, a GA biosynthesis inhibitor) were analyzed. GA application to tuber-inducing medium (MS medium plus 8 % sucrose) completely or partially reverted the short-stolon phenotype of ABF4 and ABF2 plants, respectively (Fig. 8a and S2c, left panels). As expected, tuberization was inhibited by GA in control and transgenic plants, since no signs of stolon swelling were observed after GA application. Transgenic plants exhibited an increased sensitivity to GA treatment (Fig. 8c). The differences in stolon length between control and transgenic plants were significantly minimized or disappeared (in ABF2 and ABF4 plants, respectively) as a consequence of the GA biosynthesis inhibition by CCC (Fig. 8a and S2c, right panels). Similar results were obtained when the experiment was performed under non-tuber-inducing (MS medium plus 2 % sucrose) conditions (Fig. 8b, c). These findings indicate that ABF transgenic plants exhibit a GA-deficient phenotype, suggesting that ABF actions on tuberization involve the regulation of GA endogenous levels.

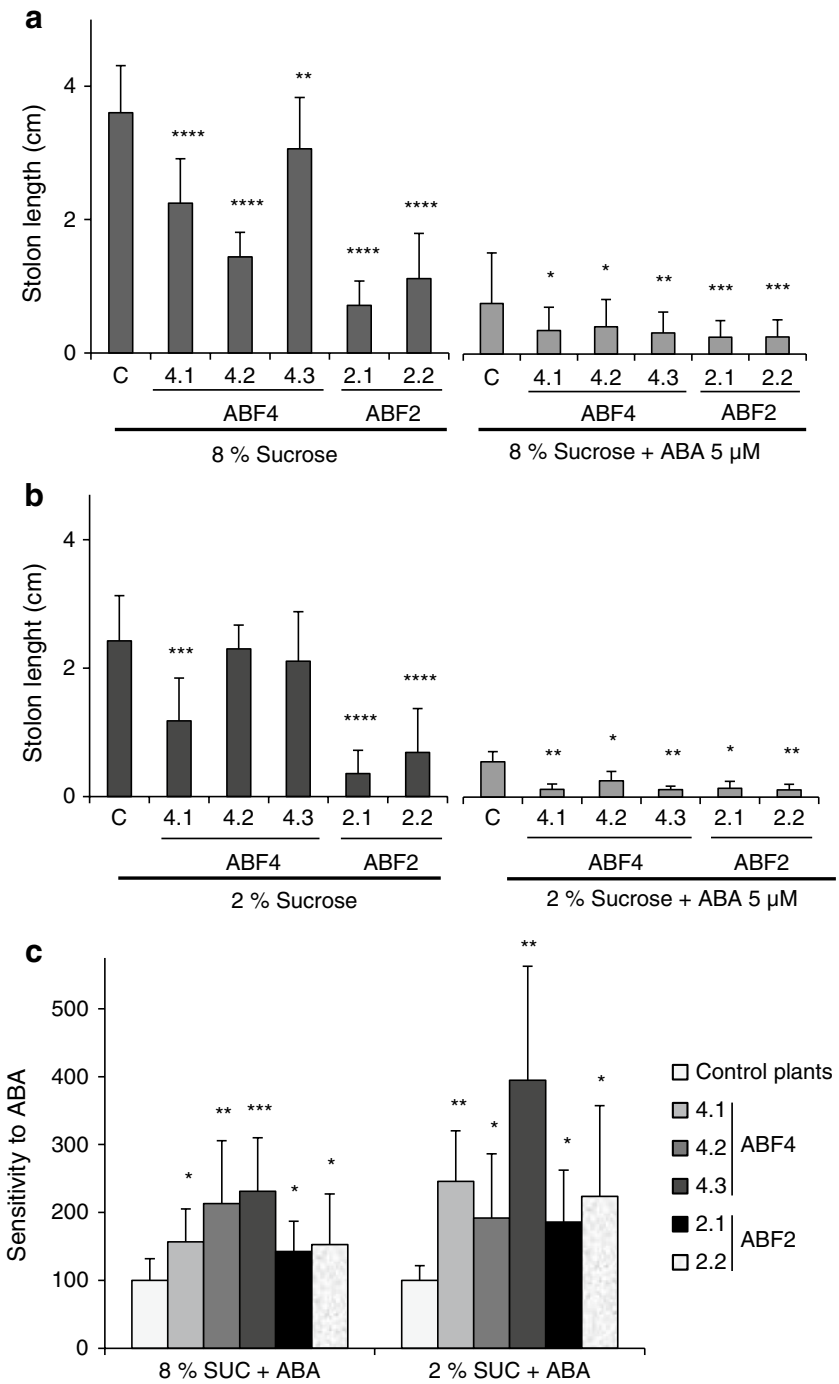

Fig. 6 Effect of exogenous ABA on the length of stolons from control and transgenic plants cultured in high sucrose tuber-inducing (8 % sucrose, **a**) or non-tuber inducing conditions (2 % sucrose, **b**). Quantitative data of five independent experiments (mean ± SD) are displayed in the *bar graphs*. **c** Sensitivity to ABA, determined as described in "Materials and methods". Values are the mean ± SD of at least four independent experiments. ABA application induced statistically significant differences in stolon length in control and ABF transgenic lines. *C* regeneration control

Levels of active GAs are regulated by the biosynthetic enzymes of the later stages of the pathway, GA20-oxidases (GA20ox) and GA 3-oxidases (GA3ox), as well as the catabolic enzymes GA 2-oxidases (GA2ox), responsible for inactivating bioactive forms of GAs (Fig. 9a; Hedden and Phillips 2000). To test the hypothesis that ABF proteins mediate the regulation of GA levels, transcriptional rates of genes involved in GA metabolism were determined by RT-qPCR in ABF4 plants. ABF4 plants showed a transcriptional deregulation of GA metabolism genes in stolons cultivated under tuber-inducing conditions (Fig. 9b, left), as well as in leaves obtained from plants grown under non-inducing conditions (Fig. 9b, right). This transcriptional deregulation includes higher levels of *StGA2ox1* that could be responsible for the GA-deficient phenotype observed in ABF transgenic plants. However, this up-regulation of the catabolic enzyme gene expression was accompanied by higher expression levels of the biosynthetic genes *StGA20ox3* and *StGA3ox2* in stolons (Fig. 9b, left) and leaves (Fig. 9b, right).

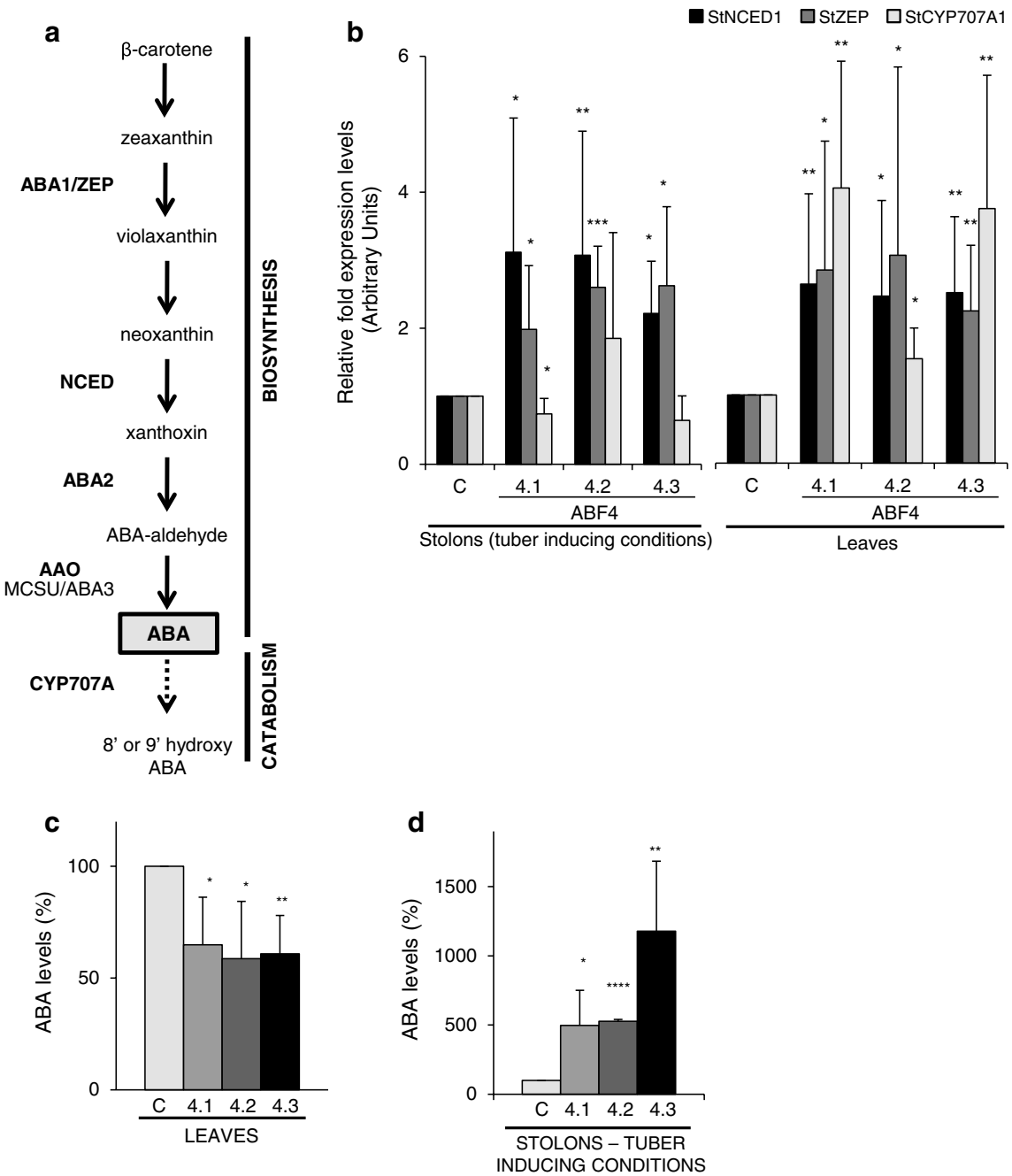

Fig. 7 ABA metabolism gene expression and ABA levels in ABF4 plants. **a** Scheme showing ABA metabolic pathway. Active ABA is indicated by a *gray filled square*. Important enzymes involved in ABA metabolism are indicated on the *left*. **b** Quantitative expression analysis (RT-qPCR) of ABA biosynthetic and catabolic genes in stolons under tuber-inducing conditions (*left*) and leaves from plants grown in MS 2 % sucrose (*right*). Data are presented as fold change of gene expression relative to control plants values, arbitrarily set as 1. Quantitative data of six independent experiments (mean ± SD) are shown in the *bar graph*. ABA levels in leaves from plants grown in MS 2 % sucrose (**c**) and stolons obtained under tuber-inducing conditions (**d**), from control and ABF4 transgenic plants. Data are presented as percentage of ABA concentration relative to control plants' values, arbitrarily set to 100 %. *Error bars* show the SD of the mean of at least three independent experiments. As reference, average ABA content in leaves and stolons (tuber-inducing conditions) from control plants was 68.2 and 3.1 ng/g fresh weight, respectively. *C* regeneration control

Since ABF2 plants exhibited a stunted phenotype, GA metabolic gene expression was determined in leaves of these lines to infer if the differences in growth between ABF2 and ABF4 plants are due to differences in GA levels. As shown in Fig. 9c, the expression profile of GA metabolic genes in ABF2 leaves was similar to ABF4 leaves.

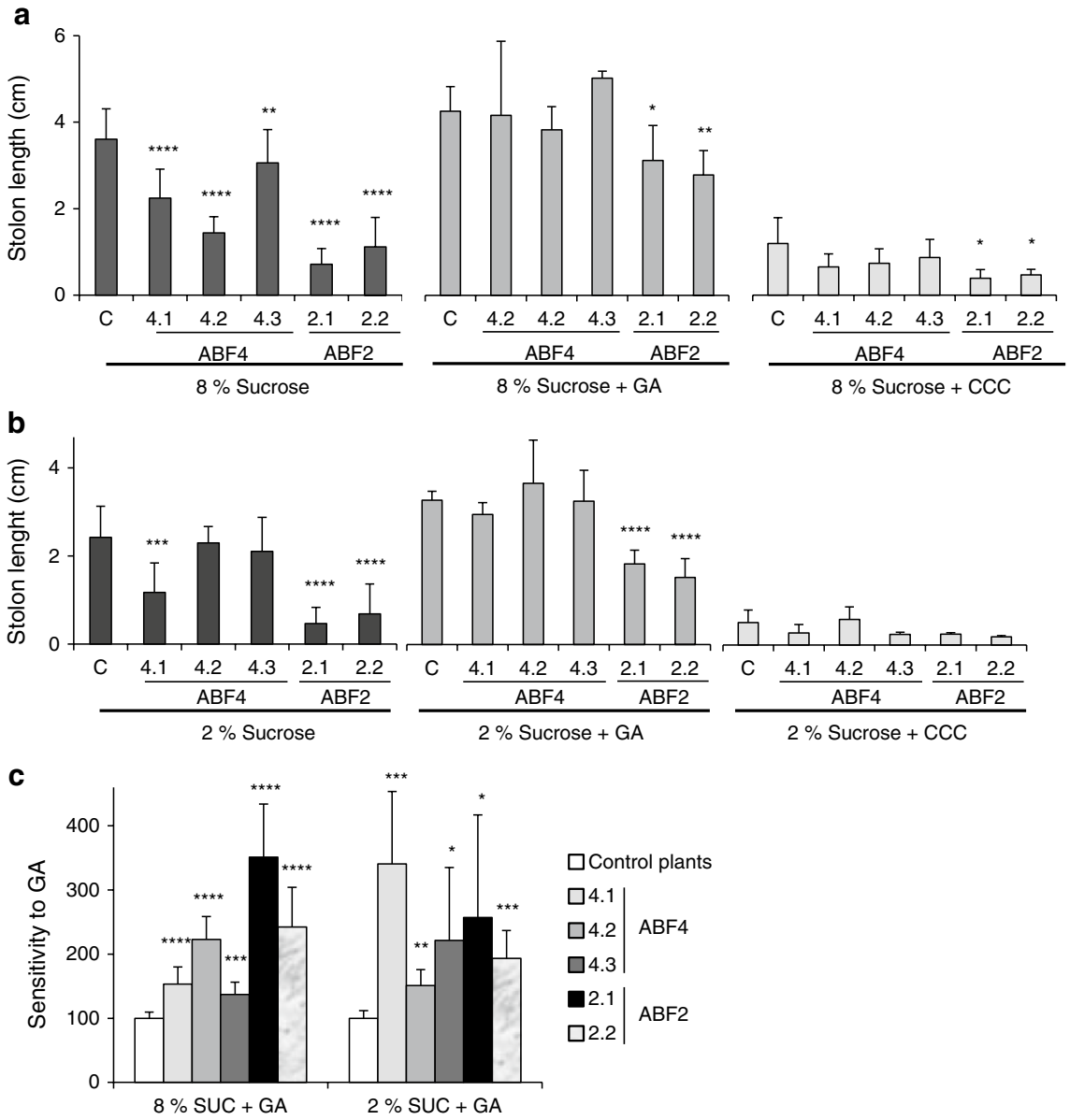

Fig. 8 Effect of exogenous GA or CCC on the length of stolons from control and transgenic plants. **a** Length of stolons from control, ABF4 and ABF2 plants cultured for 15 days under tuber-inducing conditions (8 % sucrose) in the absence or presence of GA_3 (5.0 μM) or CCC (0.5 g/l). Quantitative data of four independent experiments (mean ± SD) are displayed in the *bar graph*. **b** Stolon length after GA_3 or CCC application to non-tuber inducing MS medium (2 % sucrose). Quantitative data of at least four independent experiments (mean ± SD) are displayed in the *bar graph*. **c** GA sensitivity, determined as described in "Materials and methods". Values are the mean ± SD of at least four independent experiments. GA application induced statistically significant differences in stolon length in control and both ABF transgenic plants. CCC treatment induced statistically significant differences in stolon length in control and ABF4 plants, but stolon shortening induced in ABF2 plants resulted not statistically significant. *C* regeneration control

Discussion

Previously, we described a member of the ABF transcription factor family (StABF1) in cultivated potato and showed that it could mediate the ABA effects on tuber development (Muñiz García et al. 2012). Considering the idea that ABF proteins could positively regulate tuber formation, we generated potato plants expressing *ABF4* or *ABF2* genes from Arabidopsis, evaluated their biotechnological potential to enhance tuberization and determined the molecular mechanism involved. It would have been interesting to overexpress the *StABF1* gene in potato plants to confirm the role of StABF1 in tuberization; however, we have so far been unable to generate these transgenic plants due to technical

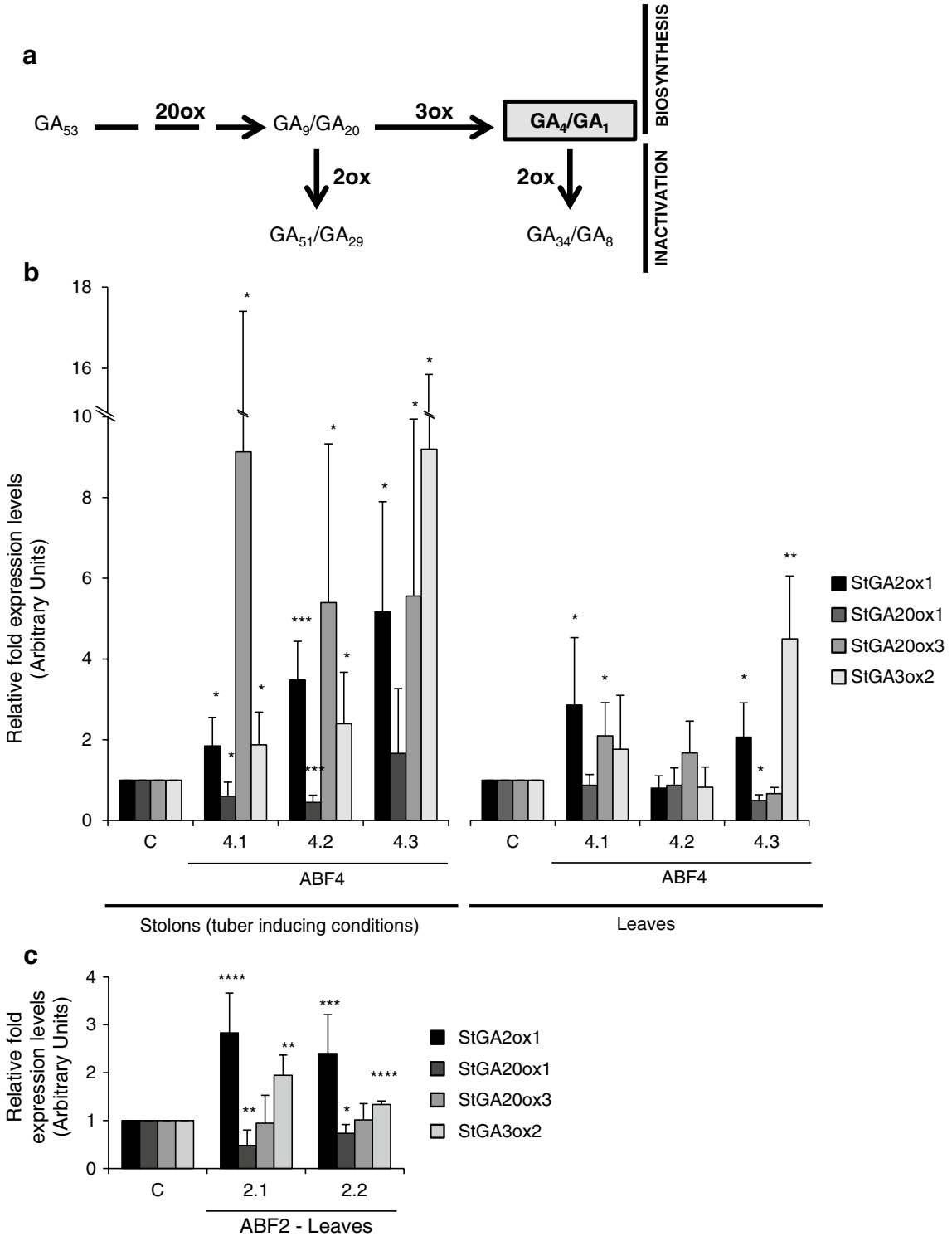

Fig. 9 Quantitative expression analysis of GA metabolism genes in ABF4 transgenic plants. **a** Scheme showing GA metabolic pathway. Active gibberellins (GA_4/GA_1) are indicated by a *gray filled square*. **b** Expression analysis (RT-qPCR) of GA biosynthetic and catabolic genes in stolons under tuber-inducing conditions (*left*) and leaves from ABF4 plants grown in MS 2 % sucrose (*right*). **c** Expression analysis of GA biosynthetic and catabolic genes in leaves from ABF2 plants grown in MS 2 % sucrose. **b**, **c** Data are presented as fold change of gene expression relative to control plants values, arbitrarily set as 1. Quantitative data of six (**b**) and five (**c**) independent experiments (mean ± SD) are shown in the *bar graph*. *C* regeneration control

difficulties. Besides their characteristic function in ABA signaling and abiotic stress response, ABF4 and ABF2 are involved in sugar signaling in Arabidopsis, with ABF2 playing a more important role in seedling growth regulation and glucose response (Kim et al. 2004).

ABF4 and ABF2 differentially affect potato plant growth. ABF2 potato plants presented a retarded growth phenotype, as observed in ABF2-overexpressing Arabidopsis plants (Kim et al. 2004), while no significant differences in the vegetative growth were observed between ABF4 and control plants (although line 4.1 was slightly smaller). It was reported previously that ABF genes were used in rice, tomato and other important crop species to enhance abiotic stress tolerance, without causing undesirable growth phenotype (Oh et al. 2005; Orellana et al. 2010).

Tuber induction in potato is a multilevel process, which integrates environmental and internal signals. Sucrose plays a complex role in tuber induction, since it not only serves as an energy source, but also regulates the expression of both metabolic and regulatory genes involved in tuber formation (Raices et al. 2003). In Arabidopsis, it has been shown that ABA has an essential function during glucose and sucrose signaling (Arenas-Huertero et al. 2000; Huijser et al. 2000; Laby et al. 2000). ABF proteins have been involved in non-osmotic sugar signal transduction, as *ABF4* or *ABF2* overexpression in Arabidopsis results in glucose hypersensitivity phenotype (Kang et al. 2002; Kim et al. 2004). In tomato, *SlAREB1* overexpression triggers hexose accumulation and induction of genes encoding a vacuolar invertase and sucrose synthase in the fruit (Bastías et al. 2011). Taken together, these pieces of evidence point out that ABF transcription factors may play an important role in sugar signaling and metabolism, which are crucial to tuberization.

Our results show that increased sucrose levels elicit higher rates of tuber-inducing stolons after 15 days in ABF transgenic plants. This effect was observed at the morphological and molecular levels, indicating that ABF2 and ABF4 are involved in the developmental switch between shoot and tuber formation triggered by high sucrose. These results support our previous report suggesting that StABF1 could act as a positive regulator of high sucrose-induced tuberization (Muñiz García et al. 2012). A differential role for ABF4 and ABF2 was observed based on the time-course analysis of the tuberization process. *ABF4* expression affects the overall process, increasing the number and weight of tubers, while *ABF2* expression seems to affect only the time of tuber initiation. ABF4 plants are able to tuberize even under non-inducing conditions, while ABF2 plants are unable to do so. These results indicate that ABF4 has a prominent role in the positive regulation of tuberization and might be a good candidate for genetic engineering to enhance tuber production.

Differences in vegetative growth and tuberization induction between ABF4 and ABF2 lines were expected, since ABF4 and ABF2 in Arabidopsis were reported not to have equivalent regulatory functions in seedling growth regulation and glucose response (Kim et al. 2004).

Stolons from ABF4 and ABF2 transgenic plants show an increased sensitivity to ABA as a result of an enhanced ABA signaling through the ABF transcription factors, indicating that ABF proteins act as positive regulators of the hormonal control of tuberization. The enhanced stolon growth inhibition by ABA in transgenic plants occurs under tuber-inducing and non-inducing conditions (8 and 2 % sucrose, respectively), demonstrating that ABF proteins mediate ABA signaling during tuberization even independently of sucrose-mediated induction. The fact that stolons from ABF4 plants are able to tuberize in non-inducing medium (MS plus 2 % sucrose) agrees well with this concept.

Leaves of ABF4 plants grown under non-inducing conditions (MS plus 2 % sucrose) accumulate lower ABA levels than control plants, probably as a result of their higher *StCYP707A1* transcriptional levels. This finding might reflect a mechanism that operates to balance the enhanced signaling through ABF4 transcription factor, which may have a negative effect on plant vegetative growth. The lower ABA levels could explain the absence of arrested growth in ABF4 plants. In contrast, endogenous ABA levels are higher in ABF4 stolons after high sucrose-induction, in agreement with the higher expression levels of *StZEP* and *StNCED1* genes, and equal or even lower transcriptional levels of *StCYP707A1*. Therefore, increased ABA sensitivity of the stolons of ABF4 plants under tuber-inducing conditions might be the result of an enhanced ABA signaling mediated by ABF4 transcription factor, as well as an increased endogenous ABA level. These findings suggest that ABF4 is activated in stolons induced to tuberize with high sucrose concentration and somehow regulates the expression of ABA biosynthetic enzymes genes to increase ABA levels.

The enhanced tuberization capacity of ABF transgenic plants may result either from increased effects of positive regulators and/or reduced effects of tuberization inhibitors. Exogenous application of GA reverts the short-stolon phenotype of ABF4 and ABF2 potato plants, indicating that higher tuberization capacity of these plants involves the alleviation of GA-mediated inhibition mechanism.

Transcriptional regulation of enzymes involved in GA metabolism provides a mechanism for GA homeostasis. Plants with reduced GA content show elevated transcript levels of *GA20ox* and *GA3ox* due to homeostatic regulation. Conversely, treatment of plants with bioactive GAs diminishes the levels of these transcripts (reviewed in Hedden and Phillips 2000). Overexpression of *GA2ox* genes in

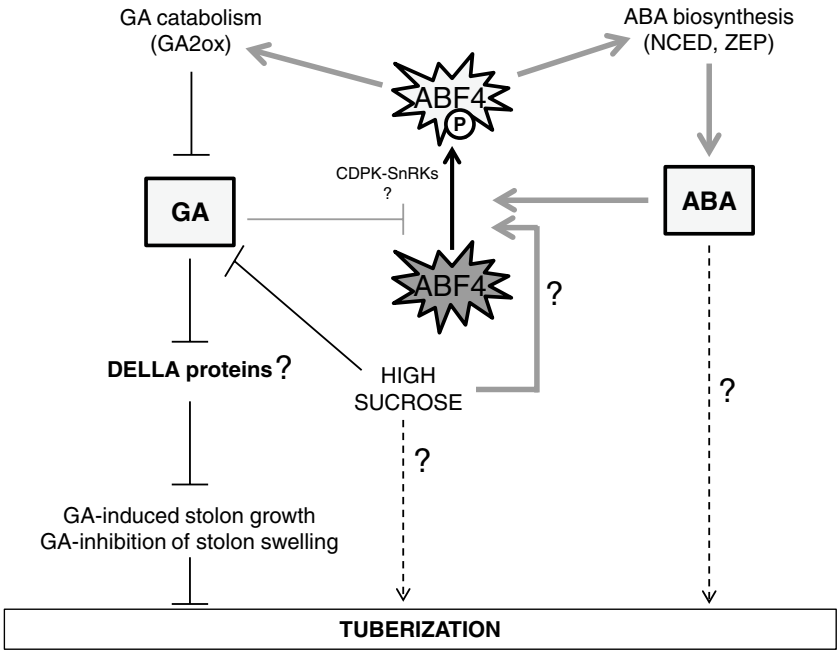

Fig. 10 Model for the regulation of tuberization in ABF4 transgenic potato plants. ABA and high sucrose are positive modulators of tuberization, while GA inhibits tuber formation. High sucrose promotes ABF4 activation (possibly by phosphorylation), which enhances *StGA2ox1*, *StNCED* and *StZEP1* expression, resulting in the decrease of GA levels and ABA accumulation. ABA, in turn, causes an enhanced activation of ABF4. The possibility that ABA or sucrose exert their tuber-promoting effects through other mechanisms cannot be excluded. The pathways addressed in this study are indicated by *thick lines*

different species results in increased rates of GAs deactivation (Appleford et al. 2007; Dijkstra et al. 2008; Lo et al. 2008). ABF4 potato plants show a transcriptional deregulation of GA metabolism enzymes, with higher levels of *StGA2ox1* transcripts as compared with control plants being associated with higher expression of *StGA20ox* and *StGA3ox* genes, probably due to the homeostatic control mentioned before. These results indicate that the enhanced tuberization capacity of the ABF4 plants is related to the transcriptional deregulation of GA metabolism genes that could result in lower levels of bioactive GAs and consequently, a stronger induction of tuberization.

The expression profile of GA metabolic genes in ABF2 leaves is similar to ABF4 leaves. This result suggests that differences in growth between ABF2 and ABF4 plants are not likely due to differences in GA levels. However, to confirm this hypothesis the levels of bioactive GA should be determined.

Analysis of ABA and GA contents along with the expression of their biosynthetic and catabolic genes demonstrates that both hormones mutually affect each other's metabolism (Oh et al. 2007; Seo et al. 2006; Zentella et al. 2007). Our results indicate that Arabidopsis ABF4 transcription factor enhances tuber formation in potato. Deregulation of GA metabolism gene expression in ABF4 plants places this transcription factor in a central point of ABA-GA crosstalk during tuberization (Fig. 10).

GA plays a negative role in the regulation of tuber formation, inducing stolon growth and inhibiting stolon swelling (Fig. 10). It is possible that these GA effects are repressed by DELLA proteins in cultivated potato (Thomas and Sun 2004; Griffiths et al. 2006). The existence of DELLA proteins in *S. tuberosum* has been reported (GenBank Accession Number AEI25530, Kang IH and Hannapel D), although they have not yet been characterized. Both ABA and sucrose are positive regulators of tuberization (Xu et al. 1998). According to our results, high sucrose somehow promotes ABF4 activation in ABF4 plants, which directly or indirectly increases *StZEP*, *StGA2ox1* and *StNCED1* transcriptional levels, resulting in ABA accumulation and, possibly, in the decrease of GA levels. ABA, in turn, causes an enhanced activation of ABF4, as a positive feedback mechanism (Fig. 10). It is worth mentioning that this is not the unique mechanism explaining enhanced tuberization of ABF4 lines, since both ABF4 and ABF2 lines exhibit an ABA-hypersensitive response and a GA-deficient phenotype, but the differences in tuberization rate between them are very clear.

Overall, our results demonstrate that heterologous expression of Arabidopsis *ABF4* gene in *S. tuberosum* increases potato tuber production in vitro without affecting plant growth and that ABF4 functions in potato as a mediator of the ABA-GA signaling crosstalk during tuberization. Since ABF transcription factors are key regulators of the adaptive response to abiotic stresses, these genes might be good candidates to increase potato crop yield, as they may improve tuber production and stress tolerance. The ABF4 plants were shown to be able to tuberize in soil (Fig. S6). Considering the promising results obtained in vitro, experiments will be conducted with mature tubers from field-grown plants to evaluate the performance of this genetically engineered crop.

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