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The protein phosphatase 2A catalytic subunit StPP2Ac2b acts as a positive regulator of tuberization induction in *Solanum tuberosum* L.

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Abstract

Key message **This study provides the first genetic evidence for the role of PP2A in tuberization, demonstrating that the catalytic subunit StPP2Ac2b positively modulates tuber induction, and that its function is related to the regulation of gibberellic acid metabolism. The results contribute to a better understanding of the molecular mechanism controlling tuberization induction, which remains largely unknown.**

Abstract The serine/threonine protein phosphatases type 2A (PP2A) are implicated in several physiological processes in plants, playing important roles in hormone responses. In cultivated potato (*Solanum tuberosum*), six PP2A catalytic subunits (StPP2Ac) were identified. The PP2Ac of the subfamily I (StPP2Ac1, 2a and 2b) were suggested to be involved in the tuberization signaling in leaves, where the environmental and hormonal signals are perceived and integrated. The aim of this study was to investigate the role of PP2A in the tuberization induction in stolons. We selected one of the catalytic subunits of the subfamily I, StPP2Ac2b, to develop transgenic plants overexpressing this gene (StPP2Ac2b-OE). Stolons from StPP2Ac2b-OE plants show higher tuber induction rates in vitro, as compared to wild type stolons, with no differences in the number of tubers obtained at the end of the process. This effect is accompanied by higher expression levels of the gibberellic acid (GA) catabolic enzyme *StGA2ox1*. GA up-regulates *StPP2Ac2b* expression in stolons, possibly as part of the feedback system by which the hormone regulates its own level. Sucrose, a tuber-promoting factor in vitro, increases *StPP2Ac2b* expression. We conclude that StPP2Ac2b acts in stolons as a positive regulator tuber induction, integrating different tuberization-related signals mainly though the modulation of GA metabolism.

Keywords PP2A · Phosphatase · Potato · *Solanum tuberosum* · Tuberization · Gibberellic acid · Gibberellin 2-oxidase · GA2ox · Abscisic acid

Introduction

Reversible protein phosphorylation represents a major form of post-translational modification that regulates many cellular processes (Hunter and Pawson 2012). Many serine/threonine protein phosphatases have been identified in plants: PP1, PP2A, PP2C, PP4, PP5, PP6, PP7 and protein phosphatases with kelch-repeat domains (Moorhead et al. 2009; Uhrig et al. 2013). Protein phosphatase 2A (PP2A) holoenzyme is a trimer, consisting of a catalytic (C), scaffolding (A), and regulatory (B) subunit (Shi 2009). Genes encoding PP2A subunits have been characterized in several plant species. *Arabidopsis thaliana* encodes five catalytic, three scaffolding and 17 regulatory subunits (Uhrig et al. 2013).

PP2A-mediated protein dephosphorylation has been implicated in several developmental programs and acclimation processes (País et al. 2009b), including seed

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germination (Chang et al. 1999), formative cell division regulation in roots (Yue et al. 2016), cytoskeleton control (Camilleri et al. 2002), cold acclimation (Monroy et al. 1998), drought tolerance (Xu et al. 2007), wounding (Rojo et al. 1998) and plant defense responses (He et al. 2004; Trotta et al. 2011). PP2A is an important regulator of PIN auxin transport proteins, contributing to the establishment of auxin gradients required for proper plant development (Michniewicz et al. 2007; Ballesteros et al. 2013). Accumulating genetic evidence reveals that PP2As play important roles in plant hormone responses. An Arabidopsis mutant with reduced levels of PP2A activity, *rcn1*, has been reported. RCN1 encodes an A subunit (PP2A-Aα) that was first characterized as a molecular component of auxin transport (Garbers et al. 1996; Rashotte et al. 2001), however two alleles of *rcn1* were identified as abscisic acid (ABA)-insensitive and ethylene-hypersensitive mutants, respectively (Kwak et al. 2002; Larsen and Cancel 2003). The characterization of a loss-of-function *pp2ac-2* mutant and transgenic plants overexpressing *PP2Ac-2* reveled that PP2Ac-2 catalytic subunit is a negative regulator of ABA signaling in Arabidopsis (Pernas et al. 2007). More recently, it has been shown that PP2A is involved in regulating intracellular responses to brassinosteroids (BRs). PP2A mediates the dephosphorylation and turnover of the BR receptor BRI1 (BR insensitive 1; Wu et al. 2011), and the dephosphorylation of the transcription factors BZR1 and BES1 (BZR2) (Tang et al. 2011).

In cultivated potato (*Solanum tuberosum*), six PP2Ac isoforms named *StPP2Ac1, 2a, 2b, 3, 4* and *5* were identified, which are clustered into two subfamilies (I and II; País et al. 2009a). Previous studies, based on expression profiles suggested that PP2As from the subfamily I (StPP2Ac1, 2a and 2b) could mediate the tuberization signaling in potato leaves, where the environmental and hormonal signals are perceived and integrated (País et al. 2010). The members of the subfamily II (StPP2Ac3, 4 and 5) are likely to be involved in housekeeping functions, since they show more constitutive and invariant expression patterns (País et al. 2009a, 2010).

Tuberization is a complex process that results in the differentiation of a specialized underground shoot, the stolon, into a storage organ, the tuber. Many environmental factors influence tuber formation, such as nitrogen levels, temperature and light (Jackson 1999). The environmental signals are hormone-mediated, thus phytohormones play a prominent role in the control of tuberization (for review, see Hannapel et al. 2004; Rodriguez-Falcon et al. 2006; Sarkar 2008). Gibberellic acid (GA) is a dominant negative regulator of tuberization (Smith and Rappaport 1969; Carrera et al. 2000). Exogenous application of GA promotes stolon elongation and inhibits tuber induction, whereas a drop in GA level precedes the first visible signs of swelling in the stolon tips (Xu et al. 1998a). A possible role for ABA in tuberization was suggested (Menzel 1980; Xu et al. 1998a). However, ABA is not an essential component of the tuberization induction, and its stimulatory action appears to be due to the antagonistic effect between ABA and GA (Xu et al. 1998a).

The aim of this study was to investigate the role of PP2A in the tuberization induction in stolons. For this purpose, transgenic plants overexpressing one of the catalytic subunits of the subfamily I, *StPP2Ac2b*, were developed and analyzed.

Materials and methods

Stolon growth conditions and in vitro tuberization

Tuberization can be studied in vitro, reproducing the process occurring in vivo with the advantages of generating tubers in a controlled environment (Xu et al. 1998a; Roumeliotis et al. 2012; Muñiz García et al. 2014; Kolachevskaya et al. 2015). Nodal segments from in vitro control and transgenic plants were grown on MS medium containing 20 g/L (2%) or 80 g/L (8%) sucrose (non-inducing and tuber-inducing conditions, respectively) solidified with 0.7% (w/v) agar. Stolons were grown in a growth chamber under darkness at 22 °C. 20 nodal segments were used for each condition evaluated.

Generation of StPP2Ac2b-OE transgenic potato plants

The *StPP2Ac2b* coding sequence was obtained by RT-PCR from RNA isolated from potato leaves using the primers FwC2bfullUTR: 5′-TGGAATTCGCAGATCG GTGAG-3′ and RvC2bfullUTR: 5′-CGATGAGCTAC AAACAACTAGTGC-3′. The PCR product was cloned into the EcoRV-digested pZErO-2 vector (Invitrogen, Carlsbad, CA). The resultant vector was used as template to amplify the *StPP2Ac2b* coding sequence using the primers StPP2Ac2bfw: 5′-CGGGATCCATGCCGTC GAACGCAG-3′ and StPP2Ac2brv: 5′-CCGGAATTCT CACAAGAAGTAATCAGGAG-3′ containing a *Bam*HI and *Eco*RI restriction site, respectively. The PCR product was ligated into the pHAP12 vector (Wirth et al. 2004) downstream of the cauliflower mosaic virus 35S promoter (35S CaMV) and the tobacco mosaic virus (TMV) Ω translation enhancer, and upstream the nopaline synthase terminator sequence (tNOS). The resultant vector was digested with *Hin*dIII to excise the fragment 35SCaMV-Ω-*StPP2Ac2b*-tNOS. This fragment was subcloned into the pPZP-Npt binary vector (Hajdukiewicz et al. 1994). Transformation of potato minituber discs was performed using *Agrobacterium tumefaciens*

strain EHA101. Transformation of *A. tumefaciens* was carried out by the 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 Muñiz García et al. (2014). For transformation, 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 (wild type). Eight lines were confirmed as transgenic and their phenotypes were examined for preliminary characterization. From these lines, three (L1, L4 and L7) were selected for detailed characterization in this study.

Transgenic and wild type plants were propagated in vitro from single-node cuttings on MS medium containing 20 g/L sucrose solidified with 0.7% (w/v) agar. Plants were grown in a growth chamber under a 16-h light photoperiod (4000 lx light intensity) at 22 °C.

PCR analysis of transgenic plants

Genomic DNA samples were prepared from leaves of transgenic and wild type plants using cetyltrimethylammonium bromide (CTAB; Doyle and Doyle 1987), and used as templates for PCR amplification and Southern blot analysis. PCR was performed using the primers forward: 5′-CACTGGATAACATACGATCATTGG-3′ and reverse: 5′-TGATAATCATCGCAAGACCG-3′ (annealing temperature: 50.5 °C, 35 cycles). These primers amplify the transgene but not the endogenous *StPP2Ac2b* gene. The forward primer corresponds to the *StPP2Ac2b* coding sequence and the reverse primer to the tNOS sequence. Reaction products were resolved by electrophoresis through 1.5% agarose gel.

Southern blot

Genomic DNA was isolated from leaves of transgenic and wild type plants using CTAB (Doyle and Doyle 1987). Aliquots of DNA (10 µg) were digested overnight at 37 °C with *Eco*RI (New England Biolabs, Beverly, MA), 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 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 65 °C for 16 h. Washes were carried out at 60 °C as follows: 2× SSC/0.1% SDS for 20 min, 1× SSC/0.1% SDS for 10 min and 0.5× SSC/0.1% SDS for 10 min (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate). Bands were visualized using a Storm PhosphorImager (GE Healthcare).

Semi-quantitative reverse transcription-PCR (RT-PCR)

Semi-quantitative RT-PCR was performed to determine the expression of the transgene, endogenous *StPP2Ac2b* and PP2Ac isoforms. Samples were collected, ground in liquid nitrogen and total RNA was extracted using the TRIzol Reagent (Invitrogen). M-MLV Reverse Transcriptase (Invitrogen) was used for cDNA synthesis according to the manufacturer's protocol, using a mix of oligo(dT) 12–18 and random primers (Invitrogen). The newly synthesized cDNA was used as template for PCR amplification using the primers, annealing temperature and cycle number shown in Supplementary Table S1. The conditions were chosen to ensure that the PCR reaction was terminated within the linear range of amplification. PCR products were separated on agarose gels. Ethidium bromide-stained gel images were inverted using Adobe Photoshop CS3 for easier visualization. RT-PCR bands were quantified relative to the internal control 18S rRNA or Elongation Factor 1-α (EF1-α). Quantifications were carried out using ImageJ software (http://rsb.info.nih.gov/ij/).

Preparation of protein extracts

Samples were harvested, ground in a mortar cooled with liquid nitrogen, and extracted with 50 mM Tris-HCl, pH 7.5, containing 2 mM β-mercaptoethanol, 1 mM EDTA, (v/v) glycerol and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 µg/mL soybean trypsin inhibitor and 25 units/mL aprotinin). The suspensions were centrifuged at 1000*g* for 10 min at 4 °C;

the supernatant fractions were centrifuged at 20,000*g* for 30 min at 4 °C, and the resulting supernatant fractions were used for western blot analysis or PP2A activity assays. Protein concentration was determined by the Bradford method.

Western blot analysis and PP2A activity

For western blot analysis, protein samples were separated on 12% polyacrylamide gels and transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare). The membranes were blocked with TBS buffer plus 0.05% (v/v) Tween 20 complemented with 5% (w/v) non-fat dried milk and blotted with commercial anti-PP2Ac 1D6 monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) diluted to 1 µg/mL in TBS—0.05% (v/v) Tween 20 buffer plus 1% (w/v) BSA for 1 h. After washing, the bound primary antibody was detected with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody, using the ECL technique according to the manufacturer's recommended procedures (GE Healthcare). Equal protein loading was checked by Ponceau S staining. PP2Ac bands were quantified relative to the Ponceau S staining of the Rubisco large subunit band, using the ImageJ software.

PP2A activity in protein extracts was assayed with and without 15 µM endothall (Sigma-Aldrich, St. Louis, MO), using 15 mM p-Nitrophenyl Phosphate (pNPP) as substrate in PP2A reaction buffer (20 mM $MgCl_2$, 50 mM Tris-HCl pH 8.5, 1 mM DTT) (DeGuzmán and Lee 1988). Enzyme assays were carried out at 30 °C, for 15 min, using 20 or 30 µg of protein extract in a final volume of 200 µL. Activities were linear with time and amount of protein extract. The PP2A activity was considered as the fraction of phosphatase activity inhibited by endothall.

Northern blot

Patatin transcripts were analyzed by northern blot. Total RNA was isolated as described above (see Semi-quantitative reverse transcription-PCR). Samples (10–20 µg RNA) were separated on formaldehyde 1.2% (w/v) agarose gels and blotted onto nylon membranes (Hybond N+, GE Healthcare). Membranes were hybridized with the *Patatin* probe (País et al. 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 65 °C), bands were visualized using a Storm PhosphorImager (GE Healthcare).

RT-qPCR analysis

RNA was isolated and cDNA synthesis was performed as described above (see Semi-quantitative Reverse Transcription-PCR). The newly synthesized cDNA was used as template for PCR amplification after dilution with sterile RNAse-free water. RT-qPCR reactions were carried out on a DNA Engine Opticon sequence detector (BioRad, Hercules, CA). The reactions were performed in a final volume of 20 µL using 5XHOT FIREPol EvaGreen qPCR Mix Plus (Solis-BioDyne, Tartu, Estonia). Potato EF1-α primers (Nicot et al. 2005) were used as a control. Accumulation of *StGA20ox1, StGA20ox3, StGA2ox1, StZE*P, *StNCED1* and *StCYP707A1* was determined as described in Kloosterman et al. (2007) and Destefano-Beltrán et al. (2006). The expression of *StAP6A* and *StBEL5* was determined according to Navarro et al. (2011) and Martin et al. (2009), respectively. *StPP2Ac2b, StPP2Ac2a* and *StPP2Ac1* expression levels were determined using the primers shown in Supplementary Table S1; the amount of cDNA used in each reaction was derived from 1 ng of total RNA; 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. Relative fold expression for each gene was calculated by the method of Pfaffl (2001).

Determination of endogenous ABA

To determine the ABA levels, in vitro-grown stolons obtained after 15 days of culture under tuber-inducing conditions (500 mg, approximately) were harvested in liquid nitrogen and freeze-dried. 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. The quantification of ABA by HPLC with UV detection has been validated in several other studies using different samples (e.g., Guinn et al. 1986; Tansupo et al. 2010; Ali et al. 2011).

Statistical analysis

Statistical analysis was carried out using the Student's *t* test. A p value below 0.05 was considered statistically significantly.

Results

Expression of *StPP2Ac2b, StPP2Ac2a* and *StPP2Ac1* in stolons

The expression of the three members of the subfamily I of *S. tuberosum* PP2Ac (*StPP2Ac2b, StPP2Ac2a* and

StPP2Ac1) was determined in stolons. *StPP2Ac2b* and *StPP2Ac2a* mRNA levels were higher in stolons cultured for 15 days under tuber-inducing conditions (MS plus 8% sucrose) with respect to non-inducing conditions (MS plus 2% sucrose), with no changes in the expression of *StPP2Ac1* (Fig. 1a). GA up-regulated the expression of all three isoforms of the subfamily I in stolons cultured under tuber-inducing conditions (Fig. 1b).

Generation of transgenic plants overexpressing *StPP2Ac2b* (StPP2Ac2b-OE)

StPP2Ac2b was selected for further analysis. Transgenic potato plants expressing the *StPP2Ac2b* gene under the control of the cauliflower mosaic virus 35S promoter were generated. Three independent lines (L1, L4 and L7) were selected for detailed examination. The presence of the transgene was confirmed by PCR amplification of genomic DNA (Supplementary Fig. S1a). Southern blot analysis showed that L1 and L4 contain three copies of the transgene, while L7 is a single-copy transformant. L1, L4 and L7 derived from independent integration events (Supplementary Fig. S1b). StPP2Ac2b-OE plants accumulated *StPP2Ac2b* transgene transcripts (Fig. 2a). Both wild type and transgenic lines exhibited similar expression levels of endogenous *StPP2Ac2b* (Fig. 2b). Overexpression of *StPP2Ac2b* did not significantly affect the expression of the other PP2Ac isoforms (Supplementary Fig. S2). The increase in *StPP2Ac2b* transcript level in the transgenic lines correlated with a higher content in the PP2Ac protein (Fig. 2c) and higher PP2A activity (Fig. 2d).

Overexpression of *StPP2Ac2b* increases tuber induction and impairs GA response in stolons

To evaluate the effect of *StPP2Ac2b* overexpression on tuber induction, single-node cuttings were cultured in darkness in tuber inducing medium (MS plus 8% sucrose) for 15 days, and the percentage of swollen stolons (stolons with visible sub-apical swelling) was determined. Stolon length was also measured, since cessation of stolon growth is a prerequisite for tuberization. StPP2Ac2b-OE lines exhibited a higher percentage of swollen stolons (Fig. 3a left, c), being the stolons shorter than those of wild type plants (Fig. 3b left, c). According to these results, StPP2Ac2b-OE stolons showed higher expression of the tuberization gene marker *Patatin* (Fig. 3d).

Since GA promotes stolon elongation and inhibits tuberization, the short-stolon/increased tuber induction phenotype of StPP2Ac2b-OE plants can be attributed to impaired GA responses. To tests this hypothesis, single-node cuttings were grown in tuber-inducing medium containing GA or the GA biosynthesis inhibitor (2-chloroethyl)-trimethylammonium chloride (CCC) for 15 days. As expected, exogenous application of 5 µM GA completely inhibited tuber induction, since no swelling was observed in stolons from wild type or transgenic plants (Fig. 3c). The same result was obtained using lower concentrations of the hormone (0.5 or 0.05 µM; not shown). GA increased stolon length in StPP2Ac2b-OE lines, however it only partially suppressed the short-stolon phenotype after applying 5 µM (Fig. 3b left, c), which is a saturating concentration of the hormone for this response (Supplementary Fig. S3). StPP2Ac2b-OE lines exhibited an increased sensitivity to 5 µM GA

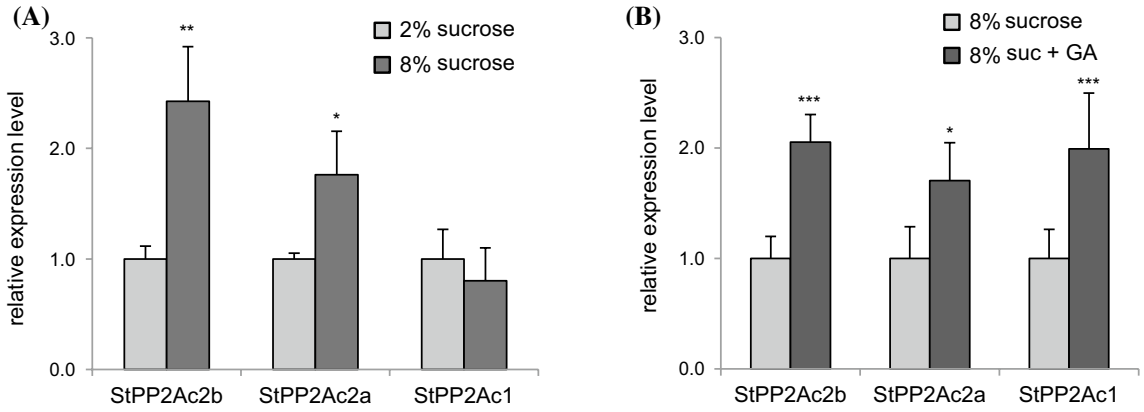


Fig. 1 Expression of *StPP2Ac* of the subfamily I in stolons. **a** Expression analysis (RT-qPCR) of *StPP2Ac2b, StPP2Ac2a* and *StPP2Ac1* in stolons cultured under non tuber-inducing conditions (MS plus 2% sucrose) or tuber-inducing conditions (MS plus 8% sucrose) for 15 days. Data are presented as expression level relative to 2% sucrose values, arbitrarily set as 1. **b** Expression analysis (RT-qPCR) of *StPP2Ac2b, StPP2Ac2a* and *StPP2Ac1* in stolons cultured under tuber-inducing conditions (MS plus 8% sucrose) in the absence or presence of 5 µM GA_3 (GA) for 15 days. Data are presented as expression level relative to 8% sucrose (without GA) values, arbitrarily set as 1. Quantitative data of three independent experiments (mean ± SD) are shown in the bar graphs. The *asterisks* indicate statistical significance: *p < 0.05, **p < 0.01, ***p < 0.005

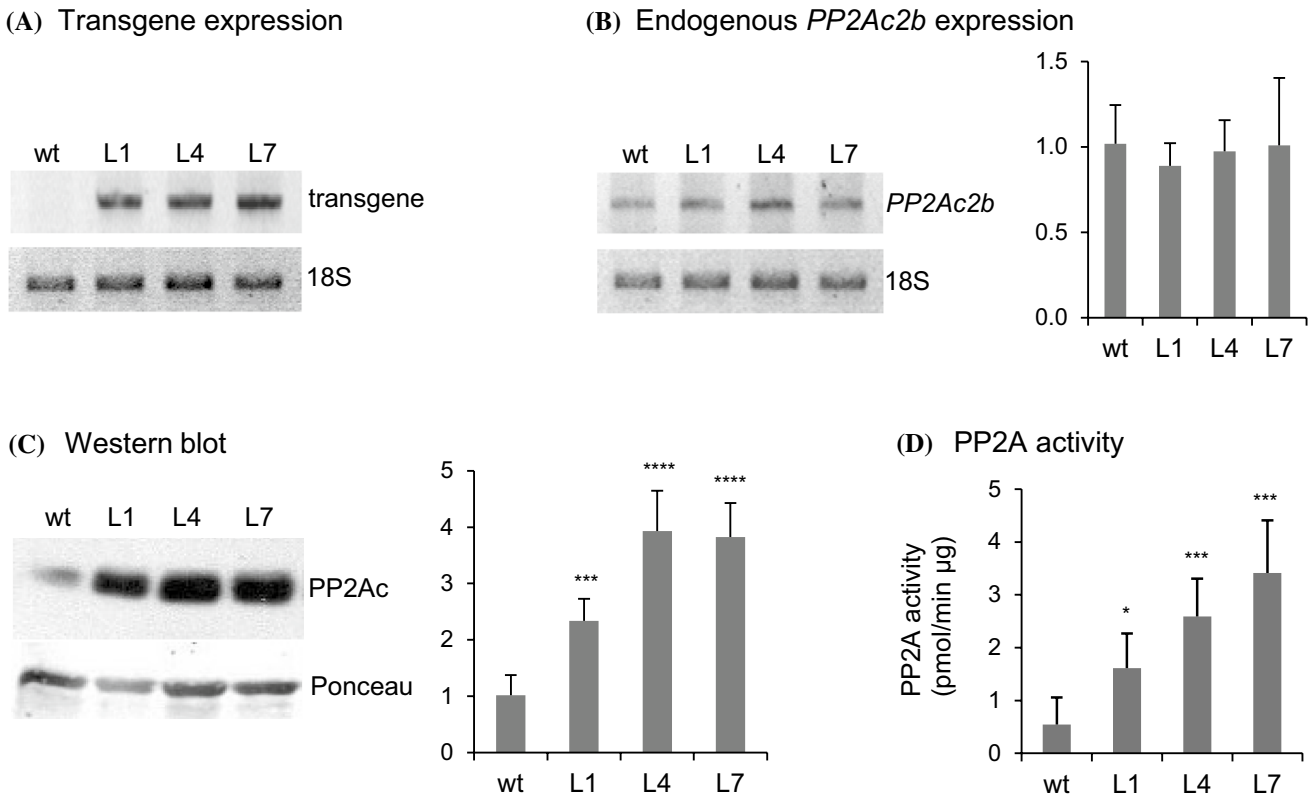


Fig. 2 Analysis of StPP2Ac2b-OE transgenic plants. **a, b** RT-PCR analysis of transgene and endogenous *StPP2Ac2b* expression, respectively, in wild type (*wt*) and transgenic lines (*L*). A representative result of three independent experiments is shown. Quantitative data of RT-PCR bands (mean ± SD, n = 3) are displayed in the bar graphs (arbitrary units). **c** Western blot analysis for PP2Ac. A representative blot of three independent experiments is shown. Quantitative data of western blot bands (mean ± SD, n = 3) are displayed in the bar graph (arbitrary units). **d** PP2A activity. Means ± SD of three independent experiments are shown. All measurements were performed on detached leaves of plants cultured in vitro. The *asterisks* in the bar graphs indicate statistical significance: *p < 0.05, ***p < 0.005, ****p < 0.001, with respect to wt

(Fig. 3b right). Larger differences in the effect of GA on stolon growth were observed at lower concentrations of the hormone (0.05 µM, Supplementary Fig. S4), confirming that PP2Ac-2b-OE stolons are more sensitive to GA than wild type stolons. Treatment with CCC significantly increased the percentage of swollen stolons and decreased stolon length in both wild type and StPP2Ac2b-OE lines (Fig. 3a left, b left, c), however the sensitivity to CCC was much higher in stolons from wild type plants (Fig. 3a right, b right). The same result was obtained when the GA biosynthesis inhibitor paclobutrazol was used (Supplementary Fig. S5).

Similar results were observed when the experiment was performed under non-inducing conditions (MS medium plus 2% sucrose). Stolons from StPP2Ac2b-OE plants became shorter than those of wild type plants, and GA application (5 µM) partially reversed the short-stolon phenotype of transgenic lines (Fig. 4a left, b). Stolons from StPP2Ac2b-OE lines grown under non-inducing conditions exhibited an increased sensitivity to GA treatment (Fig. 4a, right).

Time-course in vitro tuberization assays using single-node cuttings revealed that StPP2Ac2b-OE stolons present higher tuber induction rates at the beginning of the experiment (15 days), showing no significant differences with wild type stolons after 60 days (Fig. 5a; Supplementary S6). However, tubers developed from transgenic stolons in vitro showed an irregular shape, being more elongated than wild type tubers (Fig. 5b). This abnormal shape was not observed in the transgenic tubers obtained in soil (not shown), probably because the secondary tuber growth, caused by cell divisions randomly orientated in the perimedullary region, masks the initial elongated swelling phenotype.

The results obtained indicate that StPP2Ac2b act as a positive regulator of tuber induction and suggest that stolons from StPP2Ac2b-OE plants are impaired in GA response.

GA metabolism in StPP2Ac2b-OE stolons

GA levels are controlled through feedback and feedforward mechanisms acting on the expression of its biosynthetic and catabolic genes (Yamaguchi 2008). The expression of GA metabolism genes in StPP2Ac2b-OE stolons was analyzed. StPP2Ac2b-OE stolons showed higher levels of the catabolic gene *StGA2ox1* accompanied by higher levels of the biosynthetic gene *StGA20ox3*, as compared to wild type stolons, when cultivated under tuber-inducing or non-inducing conditions (Fig. 6a, b, respectively). No significant differences were observed in the expression of the biosynthetic gene *StGA20ox1* between transgenic and wild type stolons (Fig. 6a, b). This transcriptional profile of GA metabolism genes is consistent with reduced levels of bioactive GA in the transgenic stolons, due to the increased expression of *StGA2ox1*, with the consequent feedback up-regulation of *StGA20ox3*, as previously described by Kloosterman et al. (2007). A transcriptional up-regulation of *StGA2ox1* and *StGA20ox3* in StPP2Ac2b-OE could also be the result of higher accumulation of GA with respect to wild type stolons, caused by elevated *StGA20ox3* expression level, which secondary induces the expression of *StGA2ox1*. However, this possibility is excluded, since StPP2Ac2b-OE stolons present higher tuber induction rates than wild type stolons, which is consistent with lower, but not higher levels of bioactive GA. Treatment with CCC increased the expression of *StGA2ox1* and *StGA20ox3* (showing a more marked effect on *StGA20ox3*), while application of GA decreased the mRNA level of *StGA20ox1* (with no significant changes in *StGA20ox3* expression) in stolons cultured under tuber-inducing conditions (Supplementary Fig. S7). These results confirm that the expression of *StGA20ox1* and *StGA20ox3* is regulated by GA levels through a feedback mechanism in stolons. *StGA2ox1* expression was up-regulated by both CCC and GA (Supplementary Fig. S7), thus, the regulation of *StGA2ox1* in stolons under tuber-inducing conditions is more complex, and is possibly influenced by signals that regulates tuberization induction, such as sucrose.

ABA signaling and metabolism in StPP2Ac2b-OE stolons

The short-stolon/enhanced tuber induction phenotype of StPP2Ac2b-OE plants could also be explained by an increased response to ABA, due to an enhanced signaling, or augmented ABA levels. To determine ABA sensitivity, stolons from wild type and StPP2Ac2b-OE plants were grown on tuber-inducing medium in the presence of ABA for 15 days and stolon length was measured (Fig. 7a, left). No significant differences in ABA sensitivity were observed between wild type and transgenic stolons for 0.25 µM ABA, while StPP2Ac2b-OE stolons showed a reduced sensitivity to 1 µM ABA (Fig. 7a, right). No differences in ABA sensitivity were observed between wild type and transgenic stolons when the experiment was performed under tuber-inducing conditions for 40 days (Fig. 7b), or under non-inducing conditions for 15 days (Supplementary Fig. S8). The determination of ABA sensitivity through the measurement of stolon length was previously validated. This method has proven to be suitable to detect an increased sensitivity to ABA in transgenic potato plants over-expressing AREB/ABF transcription factors, which also present a short-stolon phenotype (Muñiz García et al. 2014). Exogenous application of 0.25 or 1 µM ABA had no significant effect on the tuberization induction neither in wild type nor in transgenic stolons (not shown), therefore, the sensitivity to ABA regarding this parameter could not be determined. Overall, these results indicate that StPP2Ac2b-OE stolons are not more sensitive to ABA than wild type stolons, excluding the possibility of an enhanced ABA signaling in StPP2Ac2b-OE stolons. Accordingly, ABA had no significant effect on *StPP2Ac2b* mRNA levels in stolons grown under tuber-inducing conditions (Fig. S9).

The endogenous ABA content was determined in StPP2Ac2b-OE stolons. ABA levels are controlled by the transcriptional regulation of ABA metabolism genes. The induction of the ABA biosynthetic genes *ZEP* and *NCED* leads to an increase in ABA content (Nambara and Marion-Poll 2005). Conversely, the expression of genes encoding degradative enzymes, such as *CYP70A*, negatively regulates ABA accumulation. Stolons from StPP2Ac2b-OE plants grown under either tuber-inducing or non-inducing conditions showed higher transcript levels of the ABA biosynthetic genes (*StZEP* and *StNCED1*), with no changes in the expression levels of *StCYP707A1*, as compared to wild type stolons (Fig. 7c, d). Accordingly, StPP2Ac2b-OE stolons grown under tuber-inducing conditions accumulated higher amounts of endogenous ABA (Fig. 7e).

If the short-stolon/enhanced tuber induction phenotype of StPP2Ac2b-OE plants is due primarily to the increased endogenous ABA levels, wild type stolons treated with exogenous ABA should mimic the stolon phenotype of StPP2Ac2b-OE plants. ABA treatment of wild type stolons inhibited growth, abolishing the differences in length between wild type and transgenic stolons (Fig. 8a, c). However, it failed to mimic the tuber induction capacity of StPP2Ac2b-OE stolons, even using high concentrations of the hormone (5 µM) (Fig. 8b, c).

Overexpression of *StPP2Ac2b* impairs GA-mediated vegetative growth

StPP2Ac2b-OE plants grown in vitro showed shorter stems than wild type plants (Fig. 9a left, c) as a consequence of reduced internode length (Fig. 9b left, c; Supplementary

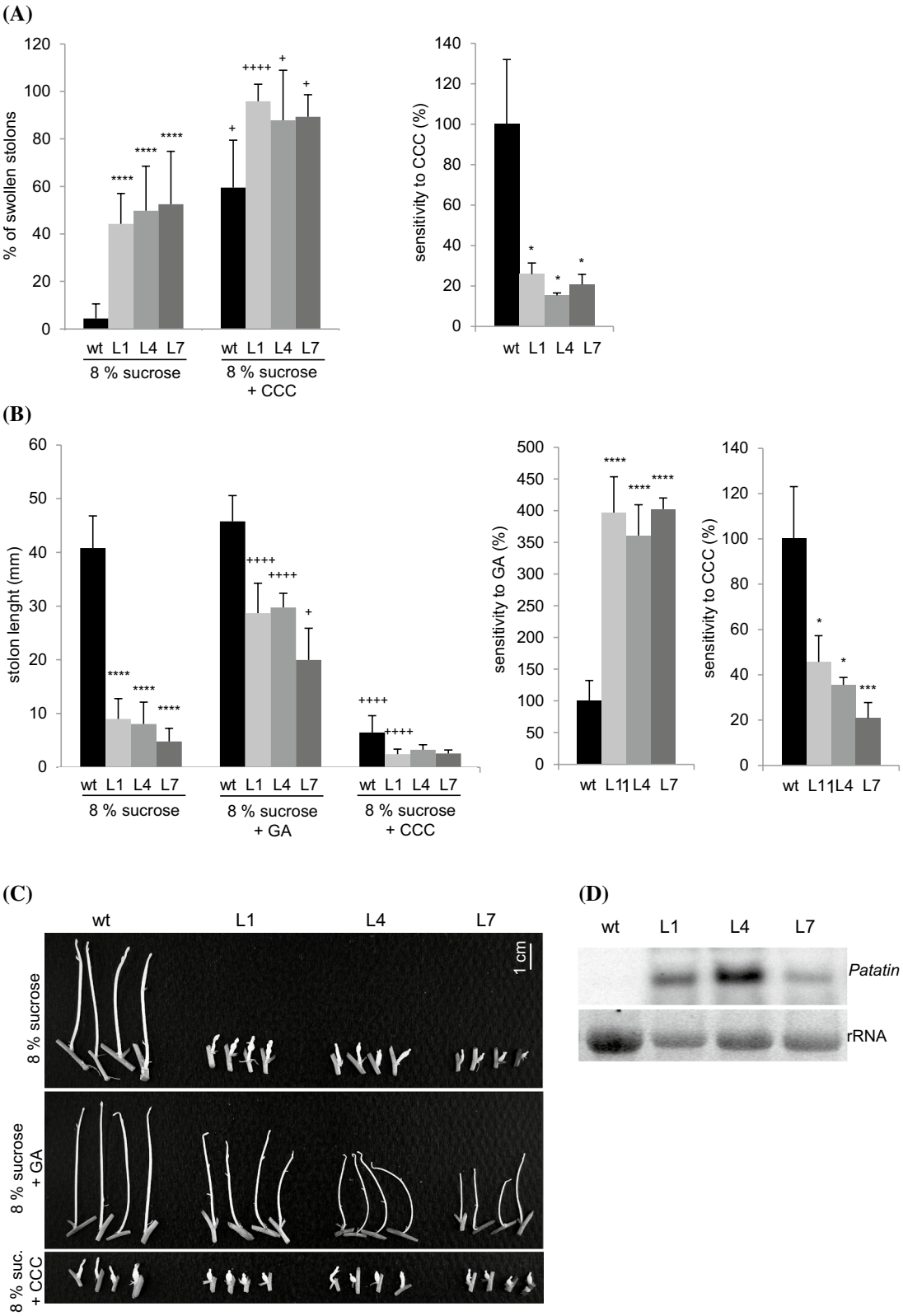


Fig. S10). Transgenic plants grown in soil were also shorter than wild type plants (Supplementary Fig. S11). Considering that overexpression of *StPP2Ac2b* affects GA metabolism in stolons, and that GA plays an important role in vegetative growth (Sun and Gubler 2004; Achard and Genschik 2009), the effects of GA or CCC were analyzed.

◂**Fig. 3** In vitro tuberization of stolons from StPP2Ac2b-OE plants. **a** *Left panel*, percentage of swollen stolons of single-node cuttings from wild type (*wt*) and transgenic lines (*L*) cultured under tuber-inducing conditions (MS medium plus 8% sucrose) for 15 days in the absence or presence of CCC (0.5 g/L). *Right panel*, sensitivity to CCC, calculated as the ratio of increase in the percentage of swollen stolons in response to CCC relative to the increase observed in wt stolons (100%). Quantitative data of five independent experiments (mean ± SD) are displayed in the bar graphs. **b** *Left panel*, length of stolons from wild type and transgenic lines cultured under tuber-inducing conditions for 15 days in the absence or presence of GA_3 (5.0 μM) or CCC (0.5 g/L). *Right panel*, sensitivity to GA and CCC, calculated as the ratio of the increase in stolon length in response to GA_3 or the inverted ratio of the reduction in length observed for stolons treated with CCC, respectively, relative to wt stolons (100%). Quantitative data of four independent experiments (mean ± SD) are displayed in the bar graphs. The *asterisks* and *crosses* in the bar graphs indicate statistical significance: $^{*}p < 0.05$, $^{***}p < 0.005$, $^{****}p < 0.001$, transgenic lines with respect to wt; $^{+}p < 0.05$, $^{++++}p < 0.001$, GA- or CCC-treated with respect to control (untreated). In **b**, there are significant differences between transgenic lines treated with 5.0 μM GA_3 and wt (untreated). **c** Representative images of stolons from control and transgenic plants. **d** Northern blot analysis for *Patatin* expression in stolons cultured under tuber-inducing conditions for 15 days. A representative blot from three independent experiments is shown

Exogenous application of 5 μM GA completely reversed the reduced height phenotype of transgenic plants (Fig. 9a left, c), increasing internode length (Fig. 9b left, c; Supplementary Fig. S10). GA also promoted stem elongation in wild type plants, however the sensitivity to the hormone was higher in transgenic plants (Fig. 9a right, b right). Treatment with CCC strongly reduced stem and internode length in wild type and transgenic plants (Fig. 9a left, b left, c; Supplementary Fig. S10). No significant differences in the sensitivity to CCC were observed between wild type and transgenic lines (Fig. 9a right, b right), except for line L7, that showed a reduced sensitivity to the inhibitor for stem length. It is worth noting that the differences in GA and CCC sensitivity with respect to vegetative growth between transgenic and wild type plants are smaller than those observed with respect to stolon length or percentage of swollen stolons (Figs. 3, 4).

The expression of GA and ABA metabolism genes was determined in leaves from StPP2Ac2b-OE plants. A slight, although no statistically significant increase in *StGA2ox1* and *StGA20ox3* expression was observed in leaves from transgenic lines (Fig. 9d). No differences in the expression of ABA metabolism genes were detected (Fig. 9e).

Overexpression of *StPP2Ac2b* up-regulates the expression of *StSP6A* and *StBEL5* in stolons

To better understand the molecular mechanism of action of StPP2Ac2b, the expression of *StSP6A* and *StBEL5* was determined in StPP2Ac2b-OE stolons, since both genes are involved in the up-regulation of *StGA2ox1* during tuberization induction. StSP6A is a FLOWERING LOCUS T (FT)-like protein, which induces tuber formation, at least in part, by the induction of *StGA2ox1* (Navarro et al. 2011). StBEL5 is a BEL1-like transcription factor that interacts with its Knox protein partner POTH1 to promote tuber initiation. The StBEL5/POTH1 complex is able to bind to the KN1-binding site present in the *StGA2ox1* promoter, and it was shown that StBEL5 up-regulates the expression of *StGA2ox1* (Lin et al. 2013; Sharma et al. 2016). StBEL5 also induces the transcription of *StSP6A* in stolons (Sharma et al. 2016). *StSP6A* and *StBEL5* expression was up-regulated in StPP2Ac2b-OE stolons cultured under tuber-inducing conditions (Fig. 10), suggesting that StPP2Ac2b acts upstream of both genes to increase *StGA2ox1* expression during tuber initiation. The expression of *StBEL5* was also high in leaves from transgenic plants grown under long-day conditions (not shown), therefore this phosphatase might act regulating tuberization signaling in leaves, where the hormonal and environmental signals are integrated and phloem-mobile factors are generated to induce tuber development in stolons.

Discussion

The PP2A catalytic subunits of the subfamily I (StPP2Ac1, 2a and 2b) were suggested to be involved in the tuberization signaling in potato leaves (país et al. 2010). Previous studies showed that GA down-regulates *StPP2Ac2b, StPP2Ac2a* and *StPP2Ac1* expression in leaves, while high sucrose/nitrogen ratio increases PP2A activity without affecting PP2Ac gene expression (país et al. 2010). In the present work, we studied the expression of the subfamily I members in stolons and found that sucrose up-regulates *StPP2Ac2b* and *StPP2Ac2a* expression, while GA up-regulates the expression of all three members of the subfamily I after 15 days of culture (Fig. 1a, b). These results suggest a role for the catalytic subunits of the subfamily I in the control of tuber induction in stolons, modulating the response to sucrose and GA. To confirm this hypothesis, transgenic potato plants overexpressing *StPP2Ac2b* (StPP2Ac2b-OE) were generated and analyzed.

After 15 days of culture under tuber-inducing conditions, StPP2Ac2b-OE stolons show higher tuber induction rates as compared to wild type stolons, with no significant differences in the number of tubers obtained after 60 days (Fig. 5a; Supplementary Fig. S6). Therefore, the differences between StPP2Ac2b-OE and wild type stolons are due to a different time on tuber initiation. These results indicate that StPP2Ac2b acts as a positive regulator of tuberization and its function is likely to be related to tuber induction at the early stages of tuber organogenesis. Previous studies have

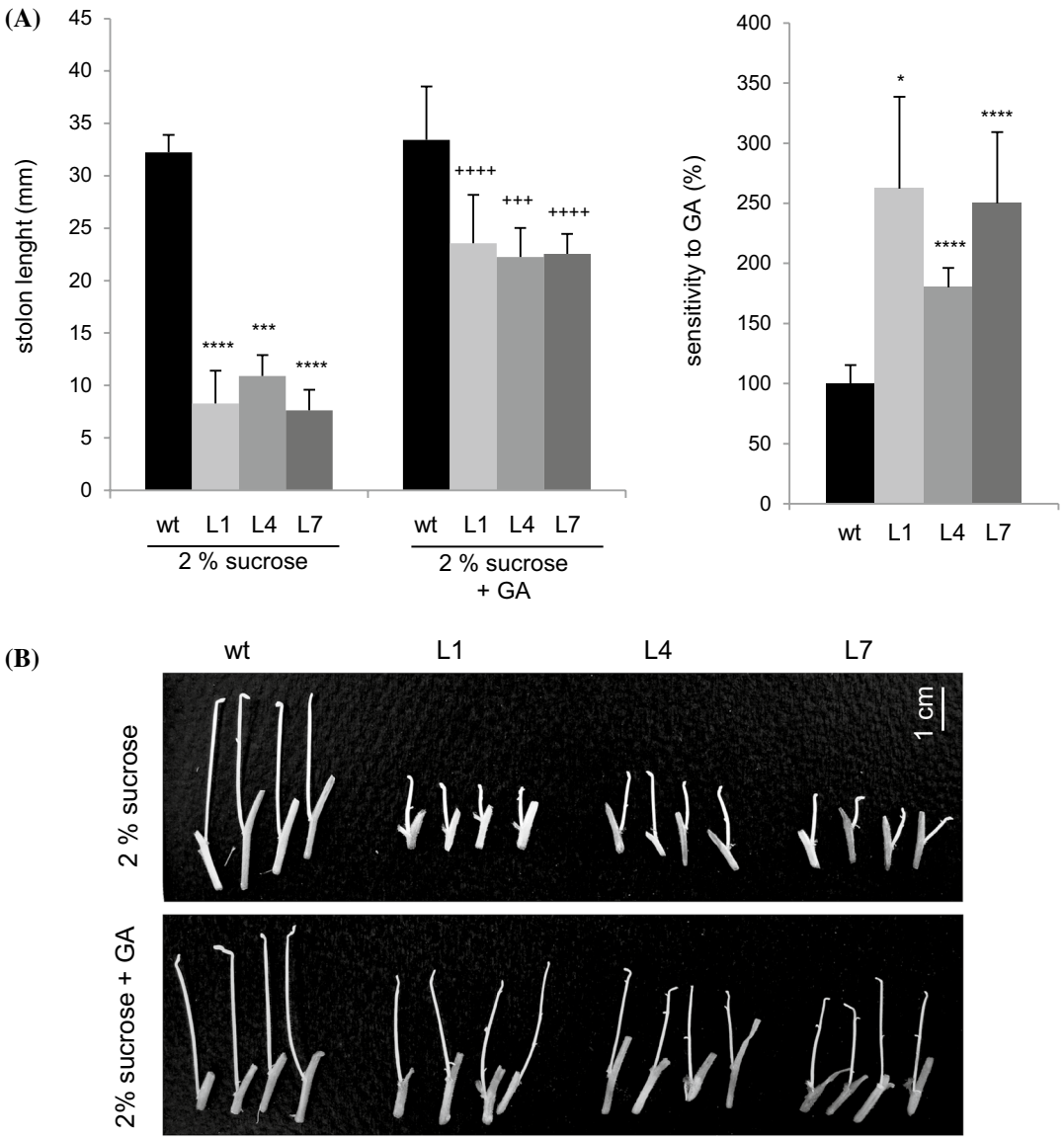


Fig. 4 Effect of GA on the length of StPP2Ac2b-OE stolons cultured under non-inducing conditions. **a** *Left panel*, length of stolons from wild type (*wt*) and transgenic lines (*L*) cultured under non-tuber inducing conditions (MS medium plus 2% sucrose) for 15 days, in the absence or presence of 5.0 μM GA_3. *Right panel*, sensitivity to GA, calculated as the ratio of the increase in stolon length in response to GA_3 relative to the increase observed in wt stolons (100%). Quantitative data of four independent experiments (mean ± SD) are displayed in the bar graphs. The *asterisks* and *crosses* indicate statistical significance: $^{*}p < 0.05$, $^{***}p < 0.005$, $^{****}p < 0.001$, transgenic lines with respect to wt; $^{+++}p < 0.005$, $^{++++}p < 0.001$, GA-treated with respect to control (untreated). There are significant differences between transgenic lines treated with 0.5 μM GA_3 and wt (untreated). **b** Representative images of stolons from wild type and transgenic plants

provided only indirect evidence suggesting the participation of protein phosphatases in tuberization (MacIntosh et al. 1996; Raíces et al. 2003; País et al. 2010). Our data provide the first genetic evidence for the role of a PP2Ac isoform in tuberization.

Stolons from StPP2Ac2b-OE plants show an increased sensitivity to GA and a decreased sensitivity to the GA biosynthesis inhibitors CCC and PAC, indicative of a role of StPP2Ac2b in the GA response (Fig. 3a right, b right, Fig. 4a right; Supplementary Fig. S5). Exogenous application of GA to StPP2Ac2b-OE stolons suppresses, at least partially, the short stolon phenotype (Fig. 3b left, Fig. 4a left), suggesting that StPP2Ac2b acts as a negative regulator of GA responses in stolons.

Levels of bioactive GAs are controlled by a homeostatic mechanism based on the transcriptional feedback regulation of GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox), involved in the synthesis of bioactive GAs, and GA 2-oxidases (GA2ox), responsible for inactivating bioactive forms of GAs (Yamaguchi 2008). During the early

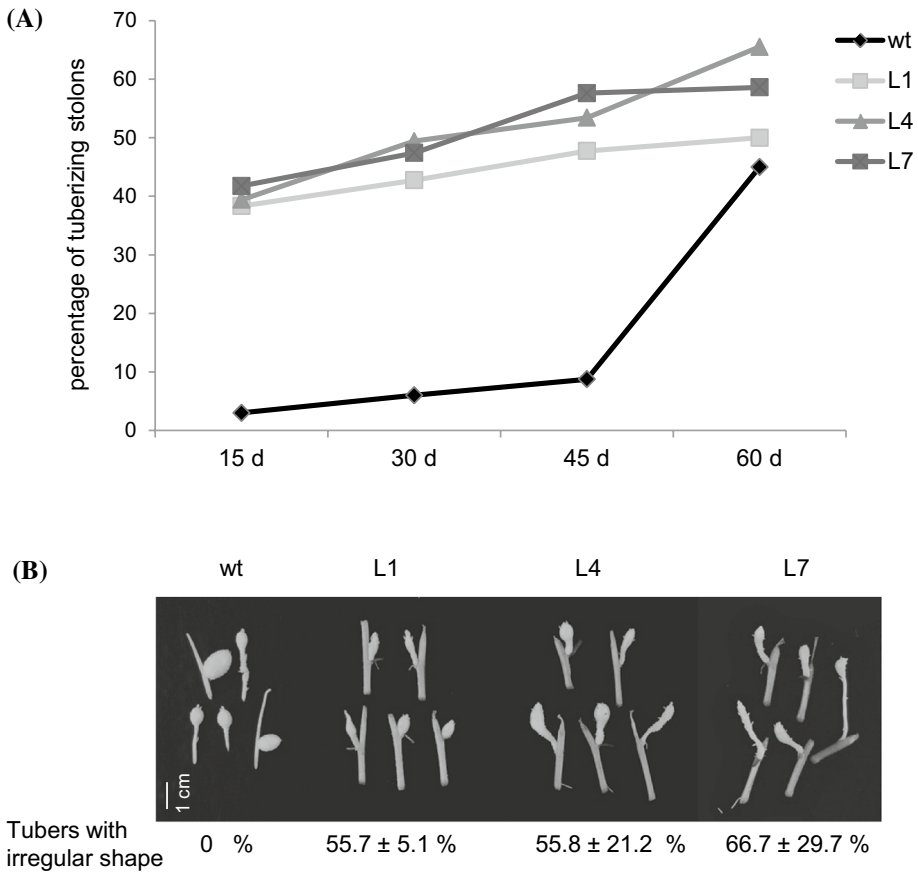


Fig. 5 Time-course tuberization rate of StPP2Ac2b-OE stolons. **a** The percentage of tuberizing stolons from wild type (*wt*) and transgenic lines (*L*) was determined at the indicated times of culture under tuber-inducing conditions (MS medium plus 8% sucrose). The results are shown in a *line graph* for better visual comparison. Values are means of five independent experiments (statistical analysis is shown in Supplementary Fig. S6). **b** Images of tubers obtained after 60 days of culture, representative of five independent experiments (20 nodal segments obtained from different plants for each *line* were used in each experiment). The percentage of tubers presenting irregular shape is indicated as mean ± SD of five independent experiments

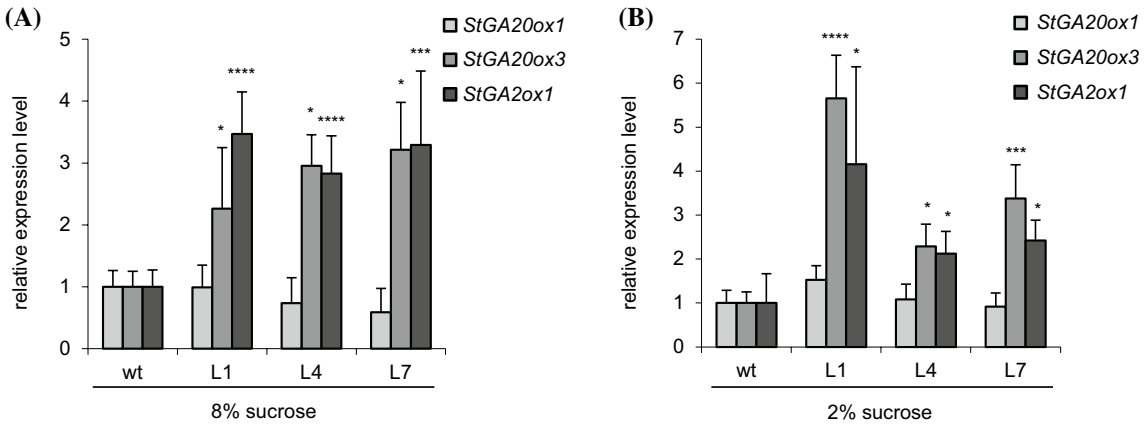
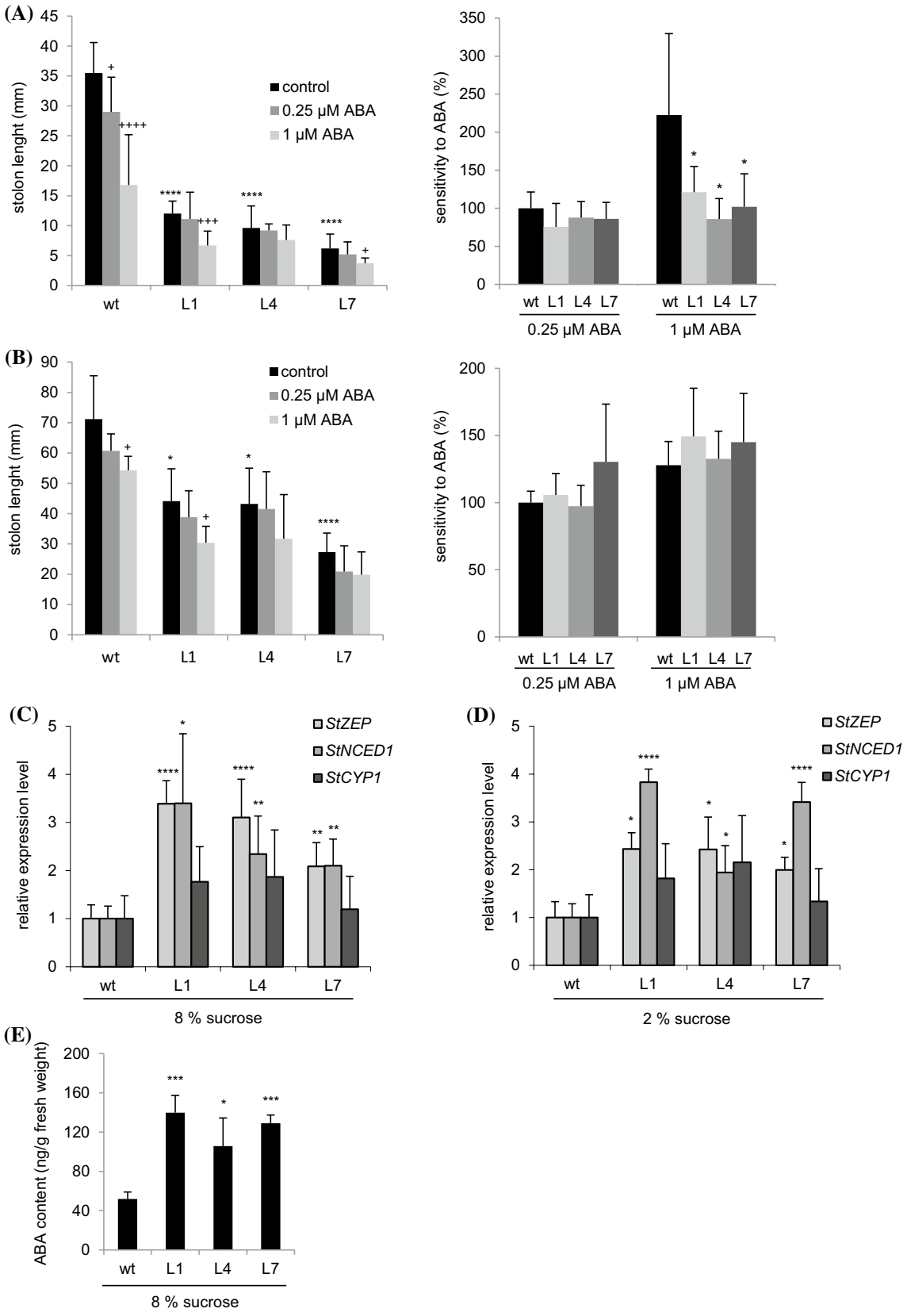


Fig. 6 Expression of GA metabolism genes in StPP2Ac2b-OE stolons. Expression analysis (RT-qPCR) of GA metabolism genes in stolons from wild type (*wt*) and transgenic plants (*L*), cultured under tuber inducing conditions (**a**, MS medium plus 8% sucrose) or non-inducing conditions (**b**, MS medium plus 2% sucrose) for 15 days. Data are presented as expression level relative to wt stolons values, arbitrarily set as 1. Quantitative data of three independent experiments (mean ± SD) are shown in the bar graphs. The *asterisks* indicate statistical significance: *$p < 0.05$, ***$p < 0.005$, ****$p < 0.001$, with respect to wt

stages of potato tuberization the expression of *StGA2ox1* is up-regulated in the subapical stolon region, leading to a decrease in the levels of bioactive GA, thereby facilitating tuber development (Kloosterman et al. 2007; Xu et al. 1998a). Concomitantly, the expression of the biosynthetic genes *StGA20ox3* and *StGA20ox1* is increased as a result of the transcriptional feedback regulation (Kloosterman et al. 2007). StPP2Ac2b-OE stolons cultured under tuber- or non tuber-inducing conditions show higher levels of *StGA2ox1* expression than wild type stolons, consistent with reduced


(A)
stolon lenght (mm)
control
0.25 µM ABA
1 µM ABA
wt L1 L4 L7
sensitivity to ABA (%)
0.25 µM ABA
1 µM ABA
(B)
stolon lenght (mm)
control
0.25 µM ABA
1 µM ABA
wt L1 L4 L7
sensitivity to ABA (%)
0.25 µM ABA
1 µM ABA
(C)
relative expression level
StZEP
StNCED1
StCYP1
wt L1 L4 L7
8 % sucrose
(D)
relative expression level
StZEP
StNCED1
StCYP1
wt L1 L4 L7
2 % sucrose
(E)
ABA content (ng/g fresh weight)
wt L1 L4 L7
8 % sucrose

◂**Fig. 7** **a, b** ABA sensitivity of StPP2Ac2b-OE stolons. Effect of exogenous ABA (0.25 or 1.0 µM) on the length of stolons from wild type (*wt*) and transgenic plants (*L*) cultured under tuber-inducing conditions (MS medium plus 8% sucrose) for 15 days (**a**) or 40 days (**b**). Sensitivity to ABA was determined as the inverted ratio of the reduction in length observed for stolons in response of ABA, relative to the reduction observed in wt stolons (100%). Quantitative data of four independent experiments (mean ± SD) are displayed in the bar graphs. **c, d** Quantitative expression analysis (RT-qPCR) of ABA metabolism genes in stolons from wild type and transgenic plants cultured under tuber-inducing (**c**, 8% sucrose) or non-inducing conditions (**d**, 2% sucrose). Data are presented as expression level relative to wt stolons values, arbitrarily set as 1. Quantitative data of three independent experiments (mean ± SD) are shown in the bar graph. **e** ABA content in stolons from wild type and transgenic plants cultured under tuber-inducing conditions for 15 days. Data are presented as means ± SD of three independent experiments. The *asterisks* and *crosses* in the bar graphs indicate statistical significance: *$p < 0.05$, **$p < 0.01$ ***$p < 0.005$, ****$p < 0.001$, transgenic lines with respect to wt; $^{+}p < 0.05$, $^{+++}p < 0.005$, $^{++++}p < 0.001$, ABA-treated with respect to control (untreated)

levels of bioactive GA, associated with increased levels of *StGA20ox3* transcripts due to the feedback mechanism (Fig. 6a, b). The expression of the *StGA20ox1* is not affected in StPP2Ac2b-OE stolons. This could be due to differences in the feedback response between both biosynthetic genes; in fact, CCC treatment has a more marked effect on *StGA20ox3* than *StGA20ox1* in stolons cultured under tuber-inducing conditions (Supplementary Fig. S7). These results indicate that the effect of StPP2Ac2b on tuber initiation is accompanied by a differential transcriptional regulation of GA metabolism genes that could result in lower levels of bioactive GA and consequently, an enhanced induction of tuberization and shorter stolons.

The enhanced tuber induction/short stolon phenotype of StPP2Ac2b-OE plants could also be explained by an impaired GA signaling, being StPP2Ac2b a negative modulator. However GA increases the expression of this PP2Ac isoform in stolons (Fig. 1b), therefore it is more likely that StPP2Ac2b regulates GA metabolism (through induction of *StGA2ox1*), rather than its signaling. In this context, StPP2Ac2b could be part of the feedback system by which GA regulates its own level. Sucrose also increases *StPP2Ac2b* expression in stolons (Fig. 1a), suggesting that this phosphatase may integrate different

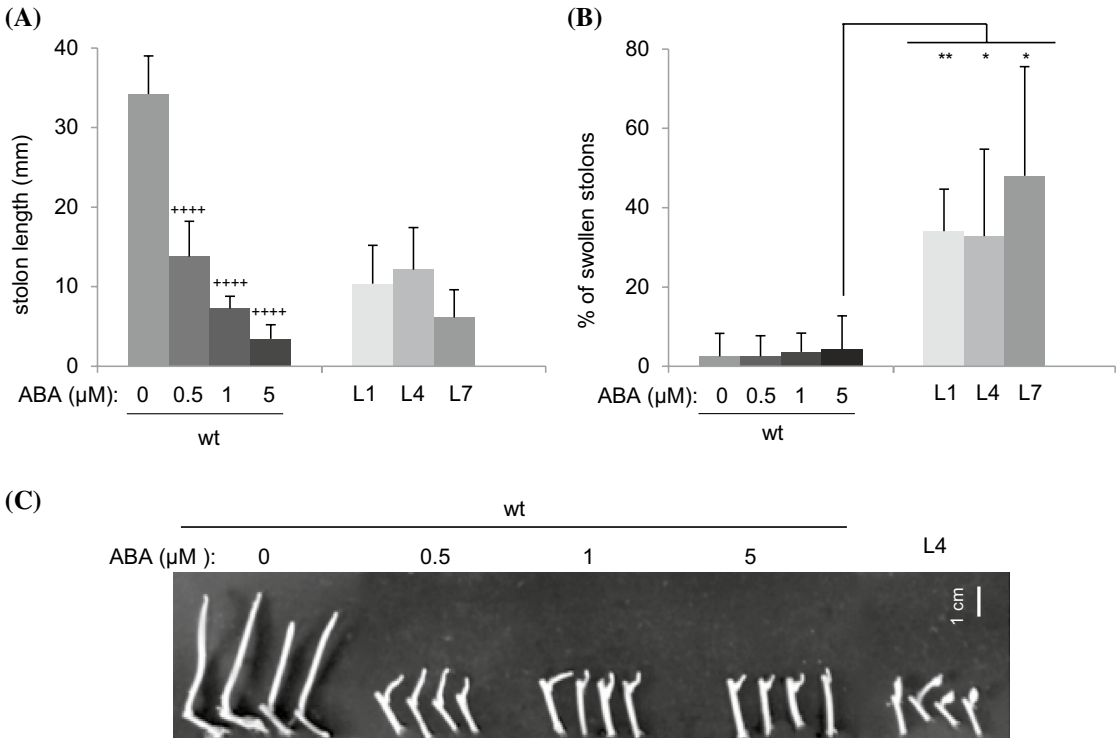
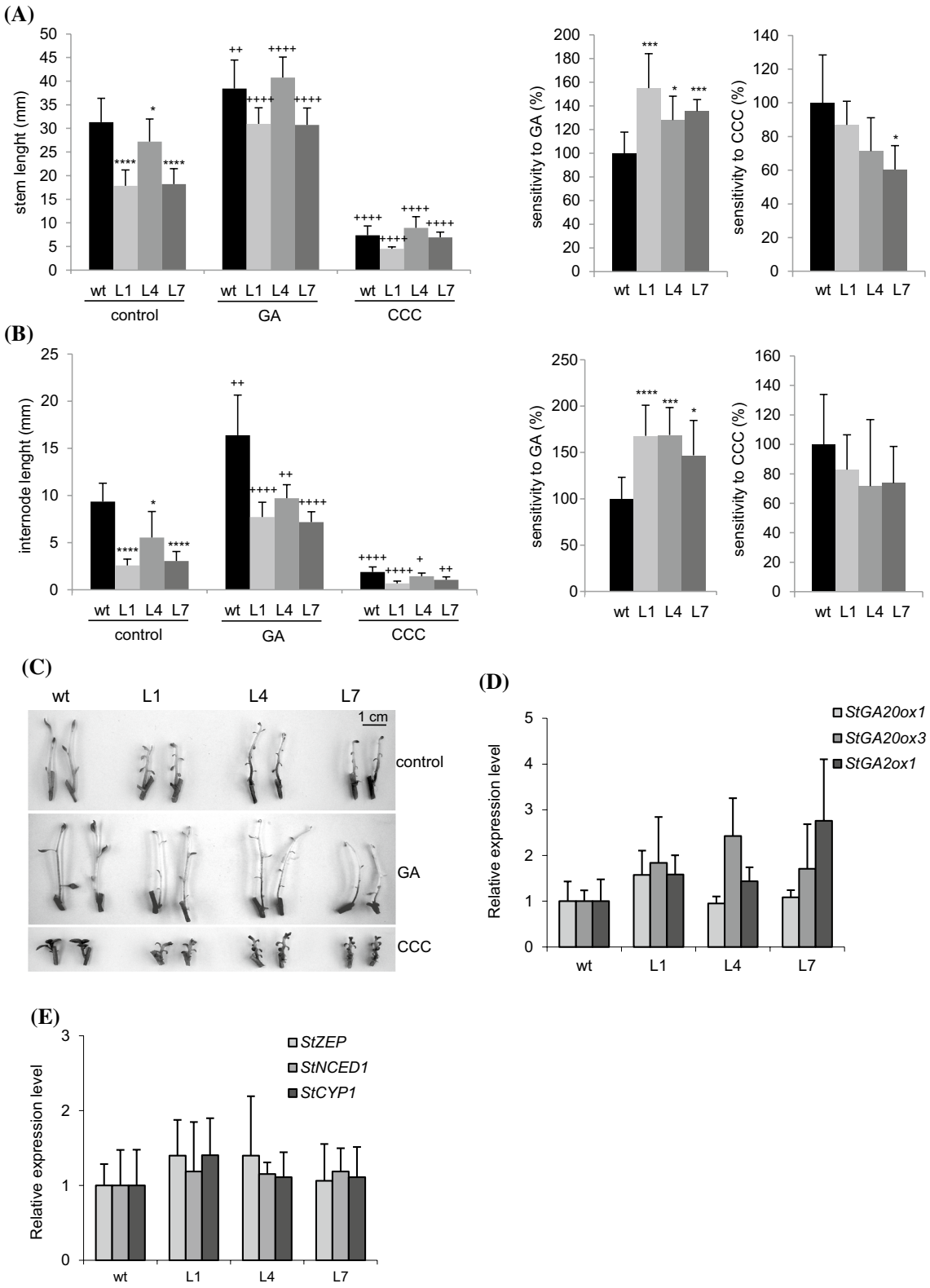


Fig. 8 Effect of ABA application to wild type stolons on the length and tuberization induction rate. Stolons from wild type (*wt*) and StPP2Ac2b-OE plants (*L*) were cultured under tuber-inducing conditions (MS medium plus 8% sucrose) for 15 days. Additionally, wild type stolons were treated with 0.5, 1.0 or 5.0 µM ABA. The stolon length (**a**) and percentage of swollen stolons (**b**) were determined. Quantitative data of four independent experiments (mean ± SD) are displayed in the bar graphs. The *asterisks* and *crosses* indicate statistical significance: *$p < 0.05$, **$p < 0.01$, ABA-treated wt with respect to transgenic lines; $^{++++}p < 0.001$, ABA-treated with respect to control (untreated). In **a**, there are no significant differences between wt stolons treated with ABA and transgenic lines (L1, L4 and L7, untreated). **c** Representative images of stolons from wild type plants (with or without ABA treatment) and StPP2Ac2b-OE line L4 (untreated) after 15 days of culture



signals that control tuber induction by regulating GA metabolism. GA only partially reverses the short stolon phenotype of transgenic plants (Fig. 3b left, c), indicating that StPP2Ac2b might regulate other tuberization related signals in stolons that are independent of GA metabolism.

The elongated shape of the tubers obtained in vitro from StPP2Ac2b-OE stolons (Fig. 5b) is consistent with a failure

◂**Fig. 9** Vegetative growth of StPP2Ac2b-OE plants. **a, b** *Left panels*, stem and first internode length, respectively, of wild type (*wt*) and transgenic lines (*L*) grown on MS medium plus 2% sucrose for 15 days, in the absence or presence of GA_3 (5.0 μM) or CCC (0.5 g/L). *Right panels*, sensitivity to GA and CCC, calculated as the ratio of the increase in stem (**a**) or first internode (**b**) length in response to GA_3, or the inverted ratio of the reduction in stem (**a**), or first internode (**b**) length in response to CCC, respectively, relative to wt plants (100%). Quantitative data of five independent experiments (mean ± SD) are displayed in the bar graphs. The *asterisks* and *crosses* indicate statistical significance: *$p < 0.05$, ***$p < 0.005$, ****$p < 0.001$, transgenic lines with respect to wt; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{++++}p < 0.001$, GA- or CCC-treated with respect to control (untreated). **a, b** There are no significant differences between transgenic lines treated with 0.5 μM GA and wt (untreated). **c** Representative images of wild type and transgenic plants grown on MS medium plus 2% sucrose for 15 days in the absence or presence of GA_3 (5.0 μM) or CCC (0.5 g/L). **d, e** Expression of GA and ABA metabolism genes in StPP2Ac2b-OE leaves. Expression analysis (RT-qPCR) of GA (**d**) and ABA (**e**) biosynthetic and catabolic genes in leaves from wild type and transgenic plants grown in MS medium plus 2% sucrose for 15 days. Data are presented as expression level relative to wild type plants values, arbitrarily set as 1. Quantitative data of three independent experiments (mean ± SD) are shown in the bar graph. There are no statistically significant differences between transgenic and wt plants

to restrict tuber transition to the subapical region, leading to an unrestricted differentiation throughout the stolon. When stolons are induced to tuberize, the reduction in the bioactive GA levels in the subapical region, caused by the up-regulated expression of *StGA2ox1*, leads to a change in the plane of cell division, from transversal to longitudinal, allowing lateral cell expansion and division (Shibaoka 1994; Fujino et al. 1995; Xu et al. 1998b). A possible explanation for the elongated shape of the StPP2Ac2b-OE tubers could be a reduction of bioactive GA levels occurring throughout the stolon, instead of being restricted to the subapical region. This could be a consequence of the constitutive expression of *StPP2Ac2b* that leads to a constitutive up-regulation of *StGA2ox1* all along the stolon length, expanding the domain of tuber differentiation to the whole stolon.

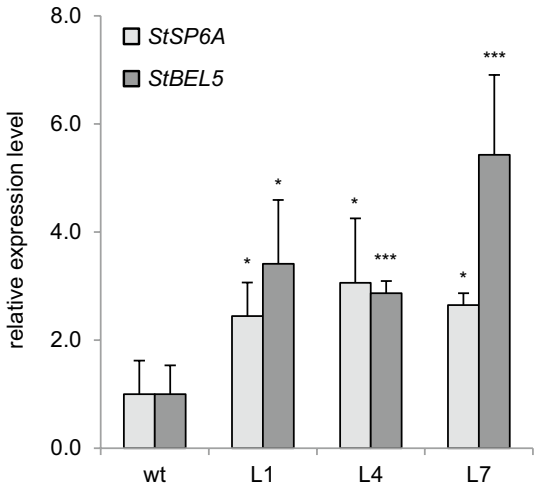


Fig. 10 Expression of *StSP6A* and *StBEL5* in stolons of StPP2Ac2b-OE plants. RT-qPCR analysis of *StSP6A* and *StBEL5* in stolons cultured under tuber-inducing conditions (MS plus 8% sucrose) for 15 days. Data are presented as expression level relative to wt values, arbitrarily set as 1. Quantitative data of three independent experiments (mean ± SD) are shown in the bar graphs. The asterisks indicate statistical significance: *$p < 0.05$, ***$p < 0.005$

ABA has been shown to have a promoting effect on tuberization, although it is not an essential regulator of the process (Menzel 1980; Xu et al. 1998a). ABA synthesis is not required for tuber induction, since the ABA-deficient mutant *Droopy* is able to tuberize (Quarrie 1982). The effect of ABA on tuberization is proposed to be due to the antagonism between ABA and GA regarding stolon growth. ABA might inhibit stolon elongation, which is required for tuberization (Vreugdenhil and Struik 1989), while GA promotes longitudinal growth of stolons. Supporting this hypothesis, it has been shown that ABA and GA have antagonistic effects on the orientation of cortical microtubules, which determines the cell division plane (Shibaoka 1994). Stolons from StPP2Ac2b-OE plants do not show an increased sensitivity to ABA (Fig. 7a, b; Supplementary Fig. S8), therefore the short-stolon/enhanced tuber induction phenotype is not likely due to an enhanced ABA signaling. ABA and GA antagonistically affect each other's metabolism by regulating the transcription of the corresponding metabolic genes (Seo et al. 2006; Oh et al. 2007; Zentella et al. 2007). Reduced levels of bioactive GA trigger an increase in ABA content by up-regulation of ABA biosynthetic genes (Oh et al. 2007). ABA levels are increased in StPP2Ac2b-OE stolons (Fig. 7e). This is an expected result since transgenic stolons have higher levels of *StGA2ox1* expression that may result in lower levels of bioactive GA.

It is difficult to determine whether StPP2Ac2b enhances tuber induction by inducing GA inactivation or ABA biosynthesis, since both hormones are simultaneously affected regardless of the direct target hormone. However, it seems more likely that StPP2Ac2b primarily affects GA metabolism in stolons, increasing *StGA2ox1* expression, and that stimulation of ABA biosynthesis is merely a consequence of the change in GA content. This hypothesis is supported by the observation that inhibition of GA biosynthesis in wild type stolons mimics the phenotype of StPP2Ac2b-OE plants (Fig. 3a–c), while ABA application fails to do so (Fig. 8). Furthermore, *StPP2Ac2b* expression in stolons is regulated by GA, but not by ABA (Fig. 1b; Supplementary Fig. S9). These results further reinforce the concept that GA is the main regulator of tuberization induction, while ABA plays a secondary role, acting as an antagonist of GA.

Our results indicate that StPP2AC2b affects the GA:ABA balance in stolons to enhance tuber induction.

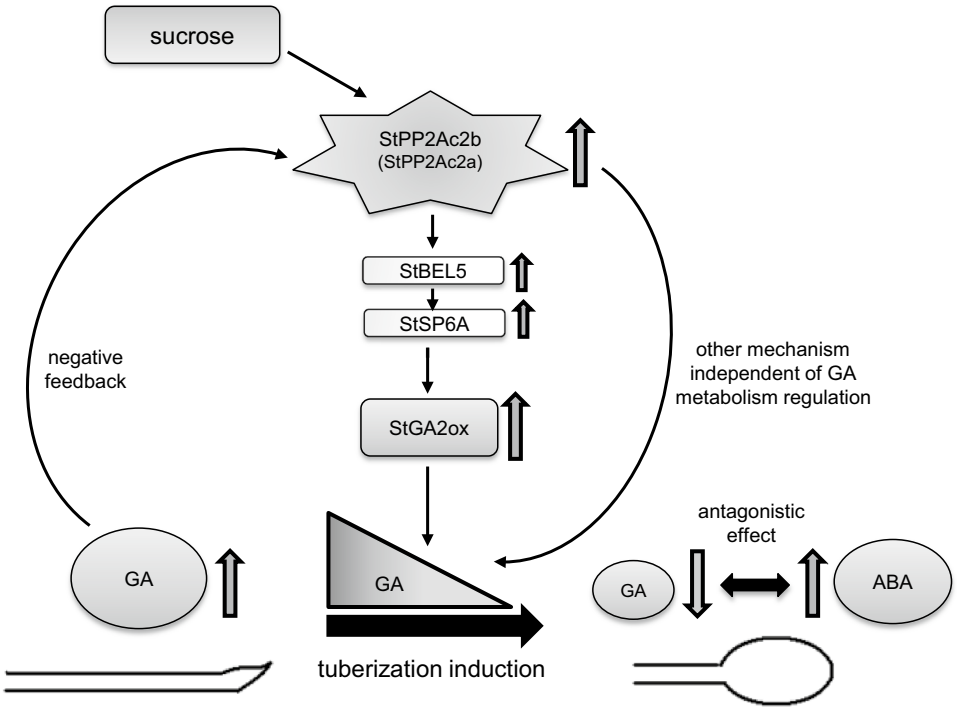


Fig. 11 Model for StPP2Ac2b action in stolons based on the results obtained in this study (see text for details)

This raises the question of whether StPP2Ac2b directly targets the GA:ABA balance, or exerts its action at another level of the tuberization regulatory network, being the altered GA:ABA balance a consequence of an enhanced tuber induction. The first scenario is more likely than the second, since the regulation of GA and ABA metabolic gene expression by StPP2Ac2b occurs in stolons even in the absence of the tuberization stimulus. StPP2Ac2b-OE stolons show higher *StGA2ox1, StZEP* and *StNCED1* expression than wild type stolons when cultivated under non-tuber inducing conditions, as well as under tuber-inducing conditions (Figs. 6a, b, 7c, d).

StPP2Ac2b-OE plants show reduced height, short internodes and increased sensitivity to GA, as compared to wild type plants (Fig. 9), indicating that StPP2Ac2b also impairs GA responses in vegetative tissues. However, this effect is much lower than that observed in stolons, suggesting that a different mechanism regulating GA:ABA balance operates in the aerial part of the plant. This mechanism would prevent a decrease in the GA:ABA ratio that may negatively affect plant growth and development. In fact, only a slight, but not statistically significant increase in *StGA2ox1* and *StGA20ox3* expression was observed in leaves from transgenic lines, with no differences in the expression of ABA metabolism genes (Fig. 9d, e).

The results obtained in this study are consistent with the following model for StPP2Ac2b action in stolons (Fig. 11): StPP2Ac2b acts as a positive regulator of tuber induction at the early stages of tuber organogenesis mainly by up-regulating the expression of *StGA2ox1*, which decreases the levels of bioactive GA, thereby facilitating tuber initiation. The mechanism by which StPP2Ac2b increases *StGA2ox1* expression probably involves the transcription factor StBEL5 and the FT-like protein StSP6A. GA up-regulates *StPP2Ac2b* expression, possibly as part of the feedback mechanism by which GA regulates its own level (accordingly, the expression of *StGA2ox1* in stolons cultured under tuber-inducing conditions is up-regulated by GA application; Supplementary Fig. S7). Sucrose, a tuber-promoting factor in vitro, increases *StPP2Ac2b* expression, with the consequent up-regulation of *StGA2ox1* and tuber initiation. In this scenario, ABA levels decrease as a consequence of the antagonistic transcriptional regulation of ABA metabolism genes by GA. StPP2Ac2b might also regulate other tuberization related signals that are independent of the regulation of GA levels. *StPP2Ac2a* expression is also increased in stolons in response to sucrose, therefore, it is possible that StPP2Ac2a and StPP2Ac2b have redundant functions regarding tuberization induction.

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Author contributions MNMG, MCM characterization of the phenotype of StPP2Ac2b-OE plants. LCM molecular analysis of StPP2Ac2b-OE plants and preliminary determination of the phenotype. SMP generation of StPP2Ac2b-OE plants. MS determination of

StPP2Ac2b, StPP2Ac2a and *StPP2Ac1* expression and accumulation of *StSP6A* and *StBEL5*. MS determination of the expression of GA metabolic genes in stolons treated with CCC or GA; determination of GA sensitivity at low concentrations of the hormone. DAC design, direction and coordination of the study; manuscript writing.

References

Achard P, Genschik P (2009) Releasing the brakes of plant growth: how GAs shutdown DELLA proteins. J Exp Bot 60:1085–1092. doi:10.1093/jxb/ern301

Ali K, Gujjar RS, Niwas R, Gopal M, Tyagi A (2011) A rapid method for estimation of abscisic acid and characterization of ABA regulated gene in response to water deficit stress from rice. Am J Plant Physiol 6:144–156. doi:10.3923/ajpp.2011.144.156

Ballesteros I, Domínguez T, Sauer M, Paredes P, Duprat A, Rojo E, Sanmartín M, Sánchez-Serrano JJ (2013) Specialized functions of the PP2A subfamily II catalytic subunits PP2A-C3 and PP2A-C4 in the distribution of auxin fluxes and development in Arabidopsis. Plant J 73:862–872. doi:10.1111/tpj.12078

Camilleri C, Azimzadeh J, Pastuglia M, Bellini C, Grandjean O, Bouchez D (2002) The Arabidopsis TONNEAU2 gene encodes a putative novel protein phosphatase 2 A regulatory subunit essential for the control of the cortical cytoskeleton. Plant Cell 14:833–845. doi:10.1105/tpc.010402

Carrera E, Bou J, Garcia-Martinez JL, Prat S (2000) Changes in GA 20-oxidase gene expression strongly affect stem length, tuber induction and tuber yield of potato plants. Plant J 22:247–256. doi:10.1046/j.1365-313x.2000.00736.x

Chang M, Wang B, Chen X, Wu R (1999) Molecular characterization of catalytic-subunit cDNA sequences encoding protein phosphatases 1 and 2A and study of their roles in the gibberellin-dependent Osamy-c expression in rice. Plant Mol Biol 39:105–115

DeGuzmán A, Lee EY (1988) Preparation of low-molecular-weight forms of rabbit muscle protein phosphatase. Methods Enzymol 159:356–368. doi:10.1016/0076-6879(88)59036-5

Destefano-Beltrán L, Knauber D, Huckle L, Suttle JC (2006) Effects of postharvest storage and dormancy status on ABA content, metabolism, and expression of genes involved in ABA biosynthesis and metabolism in potato tuber tissues. Plant Mol Biol 61:687–697. doi:10.1007/s11103-006-0042-7

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Fujino K, Koda Y, Kikuta Y (1995) Reorientation of cortical microtubules in the sub-apical region during tuberization in single-node stem segments of potato in culture. Plant Cell Physiol 36:891–895

Garbers C, DeLong A, Deruére J, Bernasconi P, Söll D (1996) A mutation in protein phosphatase 2A regulatory subunit A affects auxin transport in Arabidopsis. EMBO J 15:2115–2124

Guinn G, Brummett DL, Beier RC (1986) Purification and measurement of abscisic acid and indoleacetic acid by high performance liquid chromatography. Plant Physiol 81:997–1002. doi:10.1104/pp.81.4.997

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol 25:989–994. doi:10.1007/BF00014672

Hannapel DJ, Chen H, Rosin FM, Banerjee AK, Davies PJ (2004) Molecular controls of tuberization. Am J Potato Res 81:263–274. doi:10.1007/BF02871768

He X, Anderson JC, del Pozo O, Gu YQ, Tang X, Martin GB (2004) Silencing of subfamily I of protein phosphatase 2A catalytic subunits results in activation of plant defense responses and localized cell death. Plant J 38:563–577. doi:10.1111/j.1365-313X.2004.02073.x

Holsters M, DeWarle D, Depicker A, Messens E, Van Montague M, Schell J (1978) Transfection and transformation of *Agrobacterium tumefaciens*. Mol Gen Genet 163:181–187. doi:10.1007/BF00267408

Hunter T, Pawson T (2012) The evolution of protein phosphorylation. Preface. Philos Trans R Soc Lond B Biol Sci 367:2512. doi:10.1098/rstb.2012.0374

Iriti M, Picchi V, Rossoni M, Gomarasca S, Ludwig N, Gargano M, Faoro F (2009) Chitosan antitranspirant activity is due to abscisic acid-dependent stomatal closure. Environ Exp Bot 66:493–500. doi:10.1016/j.envexpbot.2009.01.004

Jackson, S.D (1999) Multiple signalling pathways control tuber induction in potato. Plant Physiol 119:1–8. doi:10.1104/pp.119.1.1

Kelen M, Cubek Demiralay E, Sen S, Ozkan G (2004) Separation of abscisic acid, indole-3-acetic acid, gibberellic acid in 99 R (*Vitis berlandieri* ×*Vitis rupestris*) and rose oil (*Rosa damascena* Mill.) by reversed phase liquid chromatography. Turk J Chem 28:603–610. doi:10.1155/2012/103575

Kloosterman B, Navarro C, Bijsterbosch G, Lange T, Prat S, Visser RG, Bachem CW (2007) StGA2ox1 is induced prior to stolon swelling and controls GA levels during potato tuber development. Plant J 52:362–373. doi:10.1111/j.1365-313X.2007.03245.x

Kolachevskaya OO, Alekseeva VV, Sergeeva LI, Rukavtsova EB, Getman IA, Vreugdenhil D, Buryanov YI, Romanov GA (2015) Expression of auxin synthesis gene tms1 under control of tuber-specific promoter enhances potato tuberization in vitro. J Integr Plant Biol 57:734–744. doi:10.1111/jipb.12314

Kwak JM, Moon J-H, Murata Y, Kuchitsu K, Leonhardt N, DeLong A, Schroeder JI (2002) Disruption of a guard cell-expressed protein phosphatase 2A regulatory subunit, RCN1, confers abscisic acid insensitivity in Arabidopsis. Plant Cell 14:2849–2861. doi:10.1105/tpc.003335

Larsen PB, Cancel JD (2003) Enhanced ethylene responsiveness in the Arabidopsis eer1 mutant results from a loss-of-function mutation in the protein phosphatase 2A A regulatory subunit, RCN1. Plant J 34:709–718. doi:10.1046/j.1365-313X.2003.01762.x

Lin T, Sharma P, Gonzalez DH, Viola IL, Hannapel DJ (2013) The impact of the long-distance transport of a BEL1-like messenger RNA on development. Plant Physiol 161:760–772. doi:10.1104/pp.112.209429

MacIntosh GC, Ulloa RM, Raices M, Tellez-Inon MT (1996) Changes in calcium-dependent protein kinase activity during in vitro tuberization in potato. Plant Physiol 112:1541–1550. doi:10.1104/pp.112.4.1541

Martin A, Adam H, Díaz-Mendoza M, Zurczak M, González-Schain ND, Suárez-López P (2009) Graft-transmissible induction of potato tuberization by the microRNA miR172. Development 136:2873–2881. doi:10.1242/dev.031658

Menzel BM (1980) Tuberization in potato (*Solanum tuberosum*) cultivar Sebago at high temperatures: responses to gibberellins and growth inhibitors. Ann Bot 46:259–266

Michniewicz M, Zago MK, Abas L, Weijers D, Schweighofer A, Meskiene I, Heisler MG, Ohno C, Zhang J, Huang F, Schwab R, Weigel D, Meyerowitz EM, Luschnig C, Offringa R, Friml J (2007) Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. Cell 130:1044–1056. doi:10.1016/j.cell.2007.07.033

Monroy AF, Sangwan V, Dhindsa RS (1998) Low temperature signal transduction during cold acclimation: protein phosphatase 2A as an early target for cold-inactivation. Plant J 13:653–660. doi:10.1046/j.1365-313X.1998.00070.x

Moorhead GB, De Wever V, Templeton G, Kerk D (2009) Evolution of protein phosphatases in plants and animals. Biochem J 417:401–409. doi:10.1042/BJ20081986

Muñiz García MN, Stritzler M, Capiati DA (2014) Heterologous expression of Arabidopsis ABF4 gene in potato enhances tuberization through ABA-GA crosstalk regulation. Planta 239:615–631. doi:10.1007/s00425-013-2001-2

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 56:165–185. doi:10.1146/annurev.arplant.56.032604.144046

Navarro C, Abelenda JA, Cruz-Oró E, Cuéllar CA, Tamaki S, Silva J, Shimamoto K, Prat S (2011) Control of flowering and storage organ formation in potato by FLOWERING LOCUS T. Nature 478:119–122. doi:10.1038/nature10431

Nicot N, Hausman JF, Hoffmann L, Evers D (2005) Housekeeping gene selection for real-time RTPCR normalization in potato during biotic and abiotic stress. J Exp Bot 56:2907–2914. doi:10.1093/jxb/eri285

Oh E, Yamaguchi S, Hu J, Yusuke J, Jung B, Paik I, Lee HS, Sun TP, Kamiya Y, Choi G (2007) PIL5, a phytochrome-interacting bHLH protein, regulates gibberellin responsiveness by binding directly to the GAI and RGA promoters in Arabidopsis seeds. Plant Cell 19:1192–1208. doi:10.1105/tpc.107.050153

País SM, González MA, Téllez-Iñón MT, Capiati DA (2009a) Characterization of potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) protein phosphatases type 2A catalytic subunits and their involvement in stress responses. Planta 230:13–25. doi:10.1007/s00425-009-0923-5

País SM, Téllez-Iñón MT, Capiati DA (2009b) Serine/threonine protein phosphatases type 2A and their roles in stress signaling. Plant Signal Behav 4:1013–1015. doi:10.4161/psb.4.11.9783

País SM, García MN, Téllez-Iñón MT, Capiati DA (2010) Protein phosphatases type 2A mediate tuberization signaling in *Solanum tuberosum* L. leaves. Planta 232:37–49. doi:10.1007/s00425-010-1150-9

Pernas M, García-Casado G, Rojo E, Solano R, Sánchez-Serrano JJ (2007) A protein phosphatase 2A catalytic subunit is a negative regulator of abscisic acid signalling. Plant J 51:763–778. doi:10.1111/j.1365-313X.2007.03179.x

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45. doi:10.1093/nar/29.9.e45

Quarrie SA (1982) Droopy: a wilty mutant of potato deficient in abscisic-acid. Plant Cell Environ 5:23–26. doi:10.1111/1365-3040.ep11587567

Raíces M, Ulloa RM, MacIntosh GC, Crespi M, Téllez-Iñón MT (2003) StCDPK1 is expressed in potato stolon tips and is induced by high sucrose concentration. J Exp Bot 54:2589–2591. doi:10.1093/jxb/erg282

Rashotte AM, DeLong A, Muday GK (2001) Genetic and chemical reductions in protein phosphatase activity alter auxin transport, gravity response, and lateral root growth. Plant Cell 13:1683–1697. doi:10.1105/TPC.010158

Rodriguez-Falcon M, Bou J, Prat S (2006) Seasonal control of tuberization in potato: conserved elements with the flowering response. Annu Rev Plant Biol 57:151–180. doi:10.1146/annurev.arplant.57.032905.105224

Rojo E, Titarenko E, León J, Berger S, Vancanneyt G, Sánchez-Serrano JJ (1998) Reversible protein phosphorylation regulates jasmonic acid-dependent and -independent wound signal transduction pathways in Arabidopsis thaliana. Plant J 13:153–165. doi:10.1046/j.1365-313X.1998.00020.x

Roumeliotis E, Kloosterman B, Oortwijn M, Kohlen W, Bouwmeester HJ, Visser RG, Bachem CW (2012) The effects of auxin and strigolactones on tuber initiation and stolon architecture in potato. J Exp Bot 63:4539–4547. doi:10.1093/jxb/ers132

Sarkar D (2008) The signal transduction pathways controlling in planta tuberization in potato: an emerging synthesis. Plant Cell Rep 27:1–8. doi:10.1007/s00299-007-0457-x

Seo M, Hanada A, Kuwahara A, Endo A, Okamoto M, Yamauchi Y, North H, Marion-Poll A, Sun TP, Koshiba T, Kamiya Y, Yamaguchi S, Nambara E (2006) Regulation of hormone metabolism in Arabidopsis seeds: phytochrome regulation of abscisic acid metabolism and abscisic acid regulation of gibberellin metabolism. Plant J 48:354–366. doi:10.1111/j.1365-313X.2006.02881.x

Sharma P, Lin T, Hannapel DJ (2016) Targets of the StBEL5 transcription factor include the FT ortholog StSP6A. Plant Physiol 170:310–324. doi:10.1104/pp.15.01314

Shi Y (2009) Serine/threonine phosphatases: mechanism through structure. Cell 139:468–484. doi:10.1016/j.cell.2009.10.006

Shibaoka H (1994) Plant hormone-induced changes in the orientation of cortical microtubules: alterations in the cross-linking between microtubules and the plasma membrane. Annu Rev Plant Physiol Plant Mol Biol 45:527–544. doi:10.1146/annurev.pp.45.060194.002523

Smith OE, Rappaport L (1969) Gibberellins, inhibitors, and tuber formation in the potato (*Solanum tuberosum*). Am Potato J 46:185–191. doi:10.1007/BF02863046

Sun TP, Gubler F (2004) Molecular mechanism of gibberellin signaling in plants. Annu Rev Plant Biol 55:197–223. doi:10.1146/annurev.arplant.55.031903.141753

Tang W, Yuan M, Wang R, Yang Y, Wang C, Oses-Prieto JA, Kim TW, Zhou HW, Deng Z, Gampala SS, Gendron JM, Jonassen EM, Lillo C, DeLong A, Burlingame AL, Sun Y, Wang ZY (2011) PP2A activates brassinosteroid-responsive gene expression and plant growth by dephosphorylating BZR1. Nat Cell Biol 13:124–131. doi:10.1038/ncb2151

Tansupo P, Suwannasom P, Luthria DL, Chanthai S, Ruangviriyachai C (2010) Optimised separation procedures for the simultaneous assay of three plant hormones in liquid biofertilisers. Phytochem Anal 21:157–162. doi:10.1002/pca.1172

Trotta A, Wrzaczek M, Scharte J, Tikkanen M, Konert G, Rahikainen M, Holmström M, Hiltunen HM, Rips S, Sipari N, Mulo P, Weis E, von Schaewen A, Aro EM, Kangasjärvi S (2011) Regulatory subunit B'gamma of protein phosphatase 2A prevents unnecessary defense reactions under low light in Arabidopsis. Plant Physiol 156:1464–1480. doi:10.1104/pp.111.178442

Uhrig RG, Labandera AM, Moorhead GB (2013) Arabidopsis PPP family of serine/threonine protein phosphatases: many targets but few engines. Trends Plant Sci 18:505–513. doi:10.1016/j.tplants.2013.05.004

Vreugdenhil D, Struik PC (1989) An integrated view of the hormonal regulation of tuber formation in potato (*Solanum tuberosum*). Physiol Plant 75:525–531. doi:10.1111/j.1399-3054.1989.tb05619.x

Wirth S, Calamante G, Mentaberry A, Bussmann L, Lattanzi M, Barañao L, Bravo-Almonacid F (2004) Expression of active human epidermal growth factor (hEGF) in tobacco plants by integrative and non-integrative system. Mol Breed 13:23–35. doi:10.1023/B:MOLB.0000012329.74067.ca

Wu G, Wang X, Li X, Kamiya Y, Otegui MS, Chory J (2011) Methylation of a phosphatase specifies dephosphorylation and degradation of activated brassinosteroid receptors. Sci Signal 4:ra29. doi:10.1126/scisignal.2001258

Xu X, van Lammeren AAM, Vermeer E, Vreugdenhil D (1998a) The role of gibberellin, abscisic acid, and sucrose in the regulation of potato tuber formation in vitro. Plant Physiol 117:575–584. doi:10.1104/pp.117.2.575

Xu X, Vreugdenhil D, van Lammeren AMM (1998b) Cell division and cell enlargement during potato tuber formation. J Exp Bot 49:573–582. doi:10.1093/jxb/49.320.573

Xu C, Jing R, Mao X, Jia X, Chang X (2007) A wheat (*Triticum aestivum*) protein phosphatase 2A catalytic subunit gene provides enhanced drought tolerance in tobacco. Ann Bot 99:439–450. doi:10.1093/aob/mcl285

Yamaguchi S (2008) Gibberellin metabolism and its regulation. Annu Rev Plant Biol 59:225–251. doi:10.1146/annurev.arplant.59.032607.092804

Yue K, Sandal P, Williams EL et al (2016) PP2A-3 interacts with ACR4 and regulates formative cell division in the Arabidopsis root. Proc Natl Acad Sci USA 113:1447–1452. doi:10.1073/pnas.1525122113

Zentella R, Zhang ZL, Park M, Thomas SG, Endo A, Murase K, Fleet CM, Jikumaru Y, Nambara E, Kamiya Y, Sun TP (2007) Global analysis of della direct targets in early gibberellin signaling in Arabidopsis. Plant Cell 19:3037–3057. doi:10.1105/tpc.107.054999