Plant Mol Biol (2007) 63:719–730
DOI 10.1007/s11103-006-9120-0

ci21A/Asr1 expression influences glucose accumulation in potato tubers

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Received: 17 June 2006 / Accepted: 24 November 2006 / Published online: 9 January 2007

Abstract *Asr* genes are exclusively found in the genomes of higher plants. In many species, this gene family is expressed under abiotic stress conditions and during fruit ripening. The encoded proteins have nuclear localisation and consequently a transcription factor function has been suggested. Interestingly, yeast-one-hybrid experiments revealed that a grape ASR binds to the promoter of a hexose transporter gene (*VvHT1*). However, the role of these proteins *in planta* is still elusive. By using a reverse genetics approach in potato we found that modification of *Asr1* expression has no incidence on the aerial phenotype of the plant but exerts a dramatic effect in tuber. *Asr1* antisense potatoes displayed decreased tuber fresh weight whereas *Asr1* overexpressors had a diminished number of tubers. Moreover, overexpression lines showed lower transcript levels of a plasma membrane hexose transporter and a concomitant decrease in glucose content in parenchyma cells of potato tubers. On the same hand glucose uptake rate was also reduced in one of the overexpressing lines. It thus seems likely that *Asr1* is involved in the control of hexose uptake in heterotrophic organs. In addition, the transgenic plants were characterized by several other changes in steady state metabolite levels. Results presented here support a role for *ci21A/Asr1* in glucose metabolism of potato tuber.

Keywords Tomato · *Asr* genes · Hexose metabolism · Potato tuber

Electronic Supplementary Material The online version of this article (doi:10.1007/s11103-006-9120-0) contains supplementary material, which is available to authorized users.

IB-INTA is partner group of the Max Planck Institute for Molecular Plant Physiology (Potsdam-Golm, Germany).

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Introduction

Asr genes were first described in cultivated tomato (Iusem et al. 1993; Amitai-Zeigerson et al. 1994; Rossi and Iusem 1994). Since then, several homologs were isolated from different plant species including grape, potato, pine, lily and maize (Cakir et al. 2003; Schneider et al. 1997; Chang et al. 1996; Yang et al. 2005; Janneau et al. 2002). However, despite intensive analysis of its genome sequence no *Asr*-like gene has been identified in *Arabidopsis* (Carrari et al. 2004). Targeted experiments have revealed that members of the *Asr* gene family are induced by abscisic acid (ABA), various abiotic stresses and during the process of fruit ripening (Carrari et al. 2004). Moreover, recent data indicate that tomato *Asr1* (the orthologue in potato was named *ci21A* by Schneider et al. 1997) localizes to the nucleus where it binds to a specific DNA sequence (Kalifa et al. 2004a). Interestingly, yeast-one-hybrid experiments, published by Cakir et al. (2003), revealed

that a grape ASR binds to the promoter of a hexose transporter gene (*VvHT1*). The above data are in close agreement with the proposed role of ASR as a transcription factor (Carrari et al. 2004; Kalifa et al. 2004b). Furthermore, evidence reported by Yang et al. (2005) suggests a dual role for an ASR from *Lilium longiflorum* (LLA23) uncovering an independent function as a protective molecule against water loss.

The regulation of hexose transport is of high interest since hexoses are both essential intermediates and precursors of central metabolic pathways and display signaling functions (Sherson 2003, Roitsch 2004), appearing to regulate the expression of a large spectrum of genes (Koch 1996; Price et al. 2004). Relatively little is known about sugar sensing mechanisms and their associated signaling cascades in higher plants, however, a substantial level of evidence has accumulated that hexokinases play a fundamental role within this process in *Arabidopsis* (Moore et al. 2003). Hexose transport in plants is mediated by two major classes of proteins christened either ''hexose transporters'' (HT) or ''sugar transport proteins'' (STP). Those present in the plastid envelope or the vacuolar membrane that allow intracellular mobilization of hexoses (Weber et al. 2000) and those localized in the plasma membrane. The later are hexose/H+ symporters involved in the uptake of glucose or fructose (products of sucrose hydrolysis by cell wall invertases; Sauer et al. 1994; Kühn et al. 2003; Baxter et al. 2005; Fridman et al. 2004). Whilst these transporters are relatively poorly characterized in comparison to the sucrose transporter family (Hackel et al. 2006; Sauer et al. 2004; Lalonde et al. 2003; Delrot et al. 2000; Boorer et al. 1996), it is known that the genes encoding HTs are regulated by abiotic stresses, pathogen infection and wounding and their expression is generally restricted to sink tissues (Buttner and Sauer 2000). Although the importance of apoplastic unloading of hexose into cells is now well accepted, its regulation remains poorly understood. In potato, transition from tuber initiation to enlargement involves a shift from apoplastic to predominantly symplastic unloading (Viola et al. 2001; Fernie and Willmitzer 2001). In tomato (Patrick and Offler 1996), grape berry (Patrick 1997) and apple (Zhang et al. 2004) fruits an apoplastic pathway has been suggested to predominate. However, recent results render this suggestion contentious in the case of tomato (Hackel et al. 2006; Baxter et al. 2005; Fridman et al. 2004). The case for grape has solid evidence with the expression of the functionally characterized plasma membrane hexose transporter *VvHT1* (Vignault et al. 2005) being restricted to young leaves and berries in this species (Fillion et al. 1999).

Whilst attention has focused on both the structure and functionality of hexose transporters (Lalonde et al. 2004), their regulation remains poorly explored. In this direction, the connection between ASRs and hexose transport requires further analysis. By following a reverse genetics approach, we report here a phenotypic, biochemical and molecular analysis of transgenic potato plants in which *ci21A*/*Asr1* gene expression was modified by overexpression or antisense inhibition. Our results provide insight into the ASR to HT relationship with transgenic plants being characterized by modified tuber glucose levels and tuber uptake coincident with changes in the expression level of plasma membrane HTs.

Materials and methods

Plant material

Solanum tuberosum L. cv. Desiree was obtained from Saatzucht Lange AG (Bad Schwartau. Germany). Plants were maintained in tissue culture with a 16-h light. 8-h dark regime on MS medium (Murashige and Skoog 1962), which contained 2% sucrose. In the greenhouse, plants were grown under the same light regime with a minimum photon flux density (PFD) of 250 μmol m^{-2} s^{-1} at 22°C. Samples for phenotypic and biochemical characterization were taken from 10-week-old plants frozen immediately in liquid N_2 and stored at –80°C until use.

Preparation and selection of potato transgenic lines

The 348 bp coding region of the tomato *Asr1* gene (GeneBank # U86130) was cloned into the multiple cloning site of the pBINAR vector (Liu et al. 1990) with *Xba*I and *KpN*I enzymes, between the CaMV 35S promoter and the *ocs* terminator. This construct was transferred into potato leaf discs via *Agrobacterium* (Rocha-Sosa et al. 1989). Emerging shoots were excised and selected on MS media containing kanamycin (100 mg l^{-1}). Initial screening of around 40 overexpressors and 40 antisense lines was determined at the *Asr*1 mRNA level in leaves of plants grown in 2 l pots, under greenhouse conditions. Total RNA was isolated from 1 g of leaf tissue by Trizol (Gibco) following manufacturer instructions. Standard conditions were used to transfer RNA to membranes (Sambrook et al. 1989). Loading was standardized relative to total RNA levels. Blots containing RNA from overexpressing lines were hybridized overnight with a probe generated by random primer labeling (rediprime kit, Amersham),

washed once for 20 min at 65°C in 2× SSC, 0.1% SDS, once for 20 min at 65°C in 0.5× SSC, 0.1% SDS and finally for 20 min at 65°C in 0.1× SSC, 0.1% SDS. Filters were exposed overnight to X-ray films (Xomat, Kodak). In order to specifically detect the *ci21A* mRNA from the endogenous gene in the antisense lines, blots were hybridized with a riboprobe generated by an in vitro transcription reaction of a pBlueScript clone carrying the tomato *Asr*1 gene in antisense orientation with respect to the T3 promoter by using the RiboScribe RNA Probe Synthesis kit (Epicentre Technologies Corp). Filters were hybridized overnight at 42°C in buffer containing 50% formamide, 0.25 M $NaPO_4$ pH 7.2, 0.25 M NaCl, 1 mM EDTA and 7% SDS, washed once for 5 min at room temperature in 2× SSC, 0.1% SDS, twice for 15 min at 60°C in 0.25 M $NaPO_4$ pH 7.2, 2% SDS and 1 mM EDTA, twice for 15 min at 60°C in 0.04 M $NaPO_4$ pH 7.2, 1% SDS and 1 mM EDTA, once for 15 min at room temperature in 2× SSC, 0.1% SDS with 1 μg ml^{-1} of RNAse A. Finally filters were washed once for 30 min at 50°C in 0.1× SSC, 0.1% SDS and exposed to X-ray films for 4–6 days. Films were scanned with a UMAX scanner and bands intensity was quantified densitometrically with NHI image software. From this initial study, 6 lines (3 overexpressing -S- and 3 antisense -AS-) were selected and amplified. Six replicates of each line were then transferred to the greenhouse for biochemical and phenotypic characterization.

Real time RT-PCR

Total RNA from tuber of 10 week-old transgenic and wild type plants was extracted as detailed in Logemann et al. (1987). To test ABA effect on HTs, total RNA was extracted from tuber discs of wild type plants incubated for 4 h in 100 ml erlenmeyer flasks with constant shaking in MES-KOH 10 mM pH 6.5 with or without the addition of 50 μM ABA (Sigma Aldrich). RT reactions were performed with 3 μg of total RNA following the instructions manual in SuperScript II Reverse Transcriptase (Invitrogen). The 20 μl RT reactions were ½ diluted and 1 μl was mixed with 9 μl of Applied Biosystems SYBR Green PCR Master Mix. Three technical replicates for each gene were run in an ABI HT7300 (Applied Biosystems). A standard curve was constructed for each gene with purified PCR products. Quantities were extrapolated from the curves and normalized by comparison to EF-1α expression (housekeeping gene). All samples were within 1 Ct when EF-1α expression was inspected. No primer dimerisation was detected. The technical replicates showed very low variance allowing us to work with the average of the three. The primer sequences used for each gene were as follows:

TC96617 (HT barley)
FW: 5′ACAgAgTTCTTTgCCgTTggAg 3′
RV: 5′gCATgAATCgCCTCgAgTCAC 3′
TC97054 (HT2 tomato)
FW 5′CAgACAACCTTAACCACggTTgg 3′
RV 5′gTCCggCTgAACgAgTTTCC 3′
EF-1α (GenBank # 29892962)
FW 5′ TGGTGTGATCAAGCCTGGTA 3′
RV 5′ AAGAGCTTCGTGGTGCATCT 3′
ci21B (GenBank #4098249)
FW 5′ AGTAGCTGCAGTTGGTGCTG 3′
RV: 5′ACAACATGGAGTCCATGGGTG 3′

The Tentative Consensus sequences (TC) were retrieved from TIGR (http://www.tigr.org/tdb/tgi/plant.shtml)

Incubation of tuber disks with [U-14C]glucose

Developing tubers were removed from 10-week-old plants and a 10-mm diameter longitudinal core was taken. The core was then sliced into 1-mm thick discs and washed three times in fresh incubation medium (10 mM MES-KOH. pH 6.5). Tuber disks (1 cm^2) were incubated in 8 ml of MES (in 100 ml Erlenmeyer flasks) containing 20 mM cold glucose and 2 μCi (3 mCi $mmol^{-1}$) of ^{14}C-glucose. Two disks per plant were taken and frozen at each incubation timepoint. Frozen tissue was extracted successively for 10 min in 2 ml 80% (v/v) ethanol, 50% (v/v) ethanol, 20% (v/v) ethanol, H_2O and finally 80% (v/v) ethanol. The pellet was quickly resuspended in water and radioactivity measured by liquid scintillation counting. The supernatants were combined and dried down under vacuum; the ethanol-soluble components were resuspended in 2 ml H_2O and separated into neutral, anionic and basic fractions as in Fernie et al. (2001a). The $^{14}CO_2$ liberated was captured in a KOH trap and the amount of released radiolabel was again quantified by liquid scintillation counting. Fluxes were estimated using the approach and assumptions detailed in Geigenberger et al. (1997, 2000).

Metabolite analysis

Tuber samples were taken at the time point indicated, immediately frozen in liquid nitrogen and stored at –80°C until further analysis. Extraction was performed by rapid grinding of tissue in liquid nitrogen and immediate addition of the appropriate extraction

buffer. Metabolites from six replicates were quantified by GC-MS as described in Roessner et al. (2001). Data are presented normalized to wild type as detailed by Roessner et al. (2001). The levels of starch, Suc and Glc were determined exactly as described previously (Fernie et al. 2001b).

Phothosynthetic parameters

Gas exchange parameters from six plants per line (two technical replicates per plant) were performed in a special custom-designed open system (Muschak et al. 1997). The Diagas software package (Walz) was used to calculate the assimilation rates, transpiration rates, leaf conductances, intercellular CO_2-concentrations and air-to-leaf vapour pressure deficits as detailed in Lytovchenko et al (2002).

Statistical analysis

Student's *t* tests were performed using the algorithm embedded into Microsoft Excel (Microsoft Corporation. Seattle). The term significant is used in the text only when the change in question has been confirmed to be significant ($P < 0.05$) with the Student's *t* test.

Results

Preparation and selection of potato transgenic plants with altered levels of ci21A/Asr1 mRNA

Asr1 complete coding sequence was cloned, either in sense or antisense orientation, into the transformation vector pBINAR (Liu et al. 1990), between the cauliflower mosaic virus (CaMV) promoter and the OCS terminator (Fig. 1). We then transferred approximately 80 transgenic potato plants obtained by *Agrobacterium tumefaciens*-mediated transformation to the greenhouse. Screening of the lines for overexpression or reduction of the mRNA levels was carried out by Northern hybridization (Fig. 1A and B). Given the use of a full length coding region for the antisense approach, in order to verify the reduction of the potato *ci21A* (tomato *Asr1* orthologue) expression, the blot containing the total RNA from the antisense plants was hybridized with a riboprobe produced by an in vitro transcription reaction of the antisense cDNA chain. Moreover, given that the expression of *ci21A* was revealed to be relatively low in the wild type during analysis of the transcript levels, the hybridization conditions were modified in order to allow better quantification of the reduction of transcript in the antisense lines. From this first screening we selected 3 overexpressing (S4, S6, S8) and 3 antisense (AS29, AS31, AS36) lines that we deemed suitable for further studies.

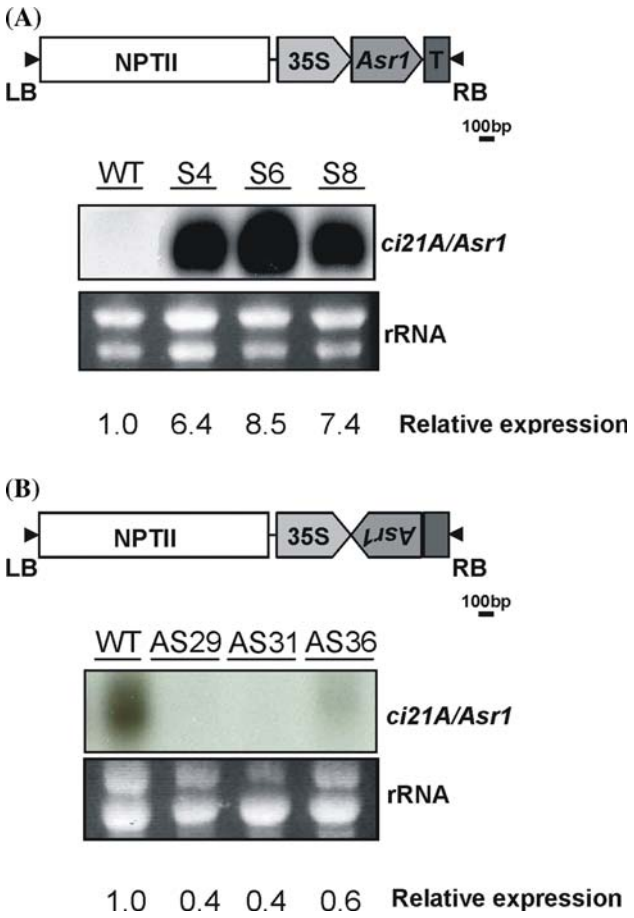

Fig. 1 Expression of *ci21A/Asr1* gene in potato transgenic plants. Northern blot analysis of leaves from T0 potato sense (**A**) and antisense (**B**) plants. Total RNA was extracted from leaves of 6-week-old plants and probed with the entire *Asr*1 cDNA clone radiolabelled by a random prime reaction (for the overexpressing plants) or with a specific riboprobe produced by an in vitro transcription reaction (for the antisense plants). A scheme of the constructs used to transform plants is presented above panels **A** and **B**. Number bellow panels indicate *ci21A/Asr1* expression levels relative to those of rRNA

Since *ci21A*/*Asr1* is part of a gene family of 3 and 4 members in potato and tomato, respectively (Frankel et al. 2006; Doczi et al. 2005), we next tested whether mRNA levels of the member of the potato family closest in sequence identity was affected in the transgenic lines. Tomato *Asr2* is orthologue to *ci21B* from potato (Schneider et al 1997). With a pair of specific primers we measured the levels of *ci21B/Asr2* in the

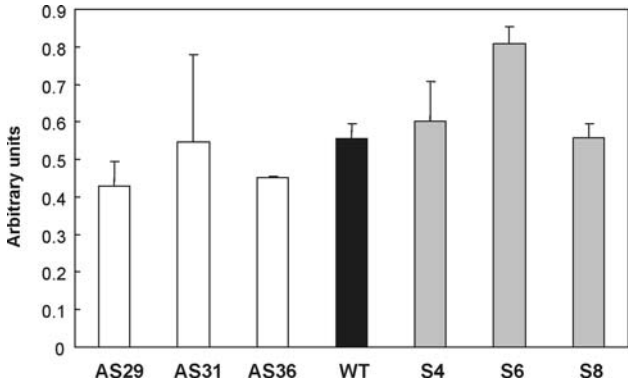

Fig. 2 mRNA steady state levels of *ci21B/Asr2* in tubers of ten-week-old plants measured by real time RT-PCR as detailed in Material and Methods

selected lines by real time RT-PCR. Results shown in Fig. 2 suggest that except for a mild transcript reduction in line AS36, the levels of *ci21B* are unaltered in these lines. As this gene is the most similar in sequence to *ci21A* and presents unaltered mRNA levels, we rule out expression changes in DS2, the remaining and divergent member of the family.

Altered expression of *ci21A/Asr1* gene affects tuber phenotype

Selected overexpressing and antisense lines were grown in 2L pots alongside wild type controls. With the exception of a mild increment in the total stem fresh weight in lines AS36 and S6 the transfomants were invariant from the wild type with respect to aerial phenotype (Fig. 3A, B and C). Moreover, gas-exchange analysis of the transgenic plants revealed assimilation, transpiration rates and a stomatal conductance similar to those exhibited by the wild type (see the Supplementary material). However, both the number and the fresh weight of the tubers were dramatically altered in the transformants. Lines AS31 and AS36 displayed a decreased tuber yield (Fig. 3D) and a consequent reduction of the harvest index (Fig. 3F). In addition, all overexpression lines showed a significant reduction in the number of tubers per plant (Fig. 3E) but no differences in total tuber fresh weight (Fig. 3D). From these data it can be deduced that the tubers of the overexpressors were clearly larger (not shown). Conversely, in the antisense lines AS31 and AS36 tubers were smaller than those from wild type (not shown).

Carbohydrate content and partitioning in the transformants

Poolsizes of carbohydrates were measured in leaves at three different time points during the diurnal cycle: beginning (BL) and end (EL) of the light period, and end of the dark period (ED). Neither glucose nor sucrose levels showed significant differences in the transgenics with respect to the wild type control (Fig. 4A). There were neither significant differences nor conserved trends in the level of starch in the transformants. The levels of the major carbohydrates were also measured in tubers of the same plants (Fig. 4B); whilst starch and sucrose (with the exception

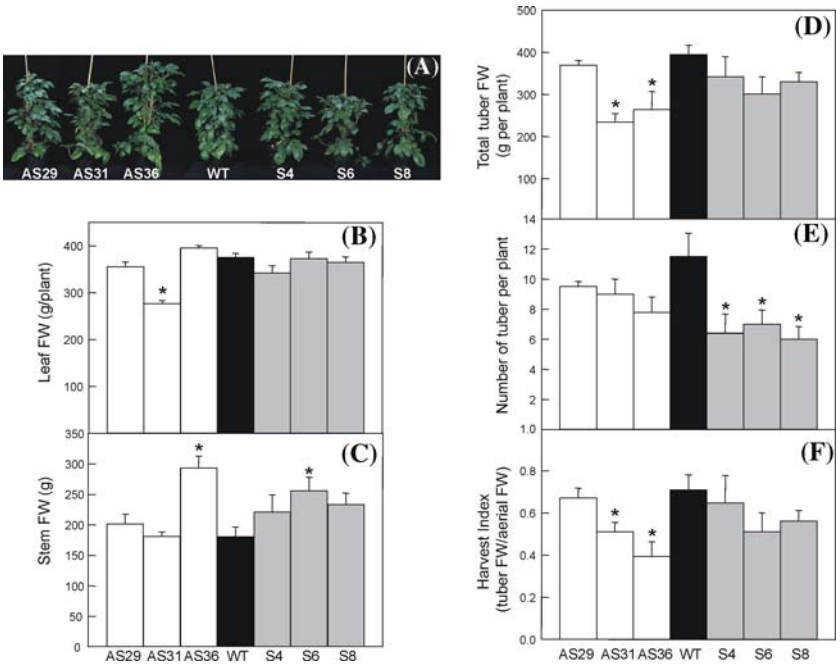

Fig. 3 Phenotype of 10-week-old potato selected lines. Growth and aerial phenotype (**A**). Fresh matter accumulated in leaves (**B**), stems (**C**), tubers (**D**). Total number of tubers per plant (**E**) and harvest index (**D**). Values are means ± SE of six plants. *denotes significant differences ($P < 0.05$) with respect to the wild type controls

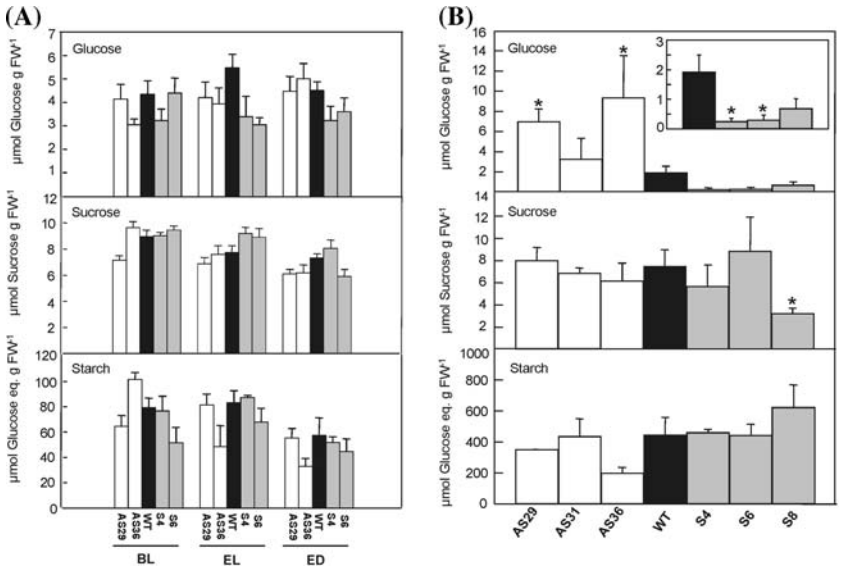

Fig. 4 Carbohydrate contents in leaves (**A**) and tubers (**B**) of 10-week-old transgenic plants. Glc, Suc and starch were determined spectrophotometrically in potato leaves harvested (A) at the beginning (BL -7 AM-) and at the end (EL -8 PM-) of the light period and at the end of the dark period (ED -5 AM -). Sugars in potato tubers (**B**) were determined from the same plants harvested at the middle of the light period. Values are means of six determinations ± SE. * denotes significant differences ($P < 0.05$) with respect to the wild type controls

of line S8) were unaltered in the transformants, glucose levels were dramatically affected. Levels of this hexose were generally depleted in the overexpressing plants and elevated in antisense plants (significantly so in lines AS29, AS36, S4 and S6; Fig. 4B). These results led us to investigate whether uptake and partitioning of glucose were altered. For this purpose we incubated tuber slices (harvested from developing tubers of wild type, two antisense and two overexpressing lines) in [U-^{14}C]glucose for 2 h and determined the redistribution of radiolabel that occurs during this timeperiod. The results of this experiment, presented in Fig. 5, reveal that the total uptake of glucose was markedly different between the lines. Line AS36 showed a higher rate of uptake (significantly so following incubation for a period of 4–6 h, data not shown) whereas S6 showed a significant lower uptake (Fig. 5A). The changes found in the neutral fraction (mainly soluble sugars) presented a similar trend (Fig. 5B) while the amount of label accumulated in the basic fraction (mainly amino acids) remained unchanged (Fig. 5C). In contrast, antisense lines showed increased label in the acidic fraction (mainly organic acids) (significant in the case of AS29 line; Fig. 5D) what is consistent with the metabolic analysis (see below). The label found in starch (Fig. 5E), protein (Fig. 5F), cell wall (Fig. 5G) and $^{14}CO_2$ evolution (Fig. 5H) was similar among lines. Estimated specific activity of the hexose phosphate pool was then used to calculate specific fluxes. With the exception of a mild increment in glycolytic flux in line S4, there were no changes in metabolic fluxes within the transformants (data not shown).

Transcript levels of hexose transporter genes

To gain insight into the molecular changes observed in the potato lines with altered expression levels of *ci21A/Asr1*, the mRNA steady-state level of three genes encoding plasma membrane hexose transporters was evaluated. For this analysis we selected HTs whose homologs had been functionally characterized and were known to be expressed in sink organs in other plant species; namely the HvHT homologue (*Hordeum vulgare* STP1, Weschke et al. 2003, TC96617) and two LeHT2 homologues (*Lycopersicum esculentum* HT2, Gear et al. 2000; TC97054 and TC111123). Figure 6A and B shows the transcript levels of these plasma membrane hexose transporters in tubers of 10 week-old plants. The HvHT-homologue mRNA was only significantly increased in line AS36 (Fig. 6A). However, the mRNA of the tomato hexose transporter homologue (LeHT2; TC97054) shows significantly decreased levels in the tubers of the overexpressing lines S4 and S6 compared to WT tubers (Fig. 6B). The other tomato homologue (TC111123) was barely expressed, rendering accurate quantification of its expression level impossible (data not shown).

In order to test whether the hexose transporters are themselves regulated by ABA, the mRNA steady-state levels of both HvHT and LeHT2 homologues were measured by Real Time RT-PCR in WT tubers incubated for four hours with 50 µM ABA. None of the HTs showed significant differences in their expression level compared to the untreated controls under these experimental conditions (Figure S2).

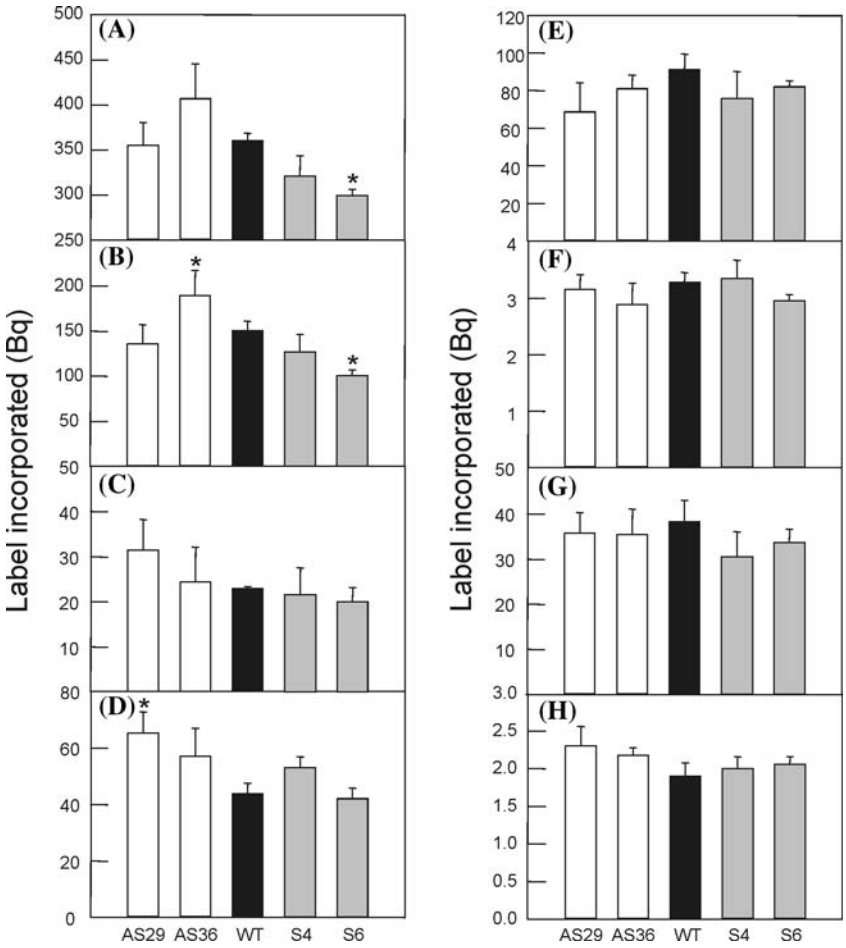

Fig. 5 Uptake and metabolism of ^{14}C-glucose by potato tuber slices. Freshly cut slices of growing potato tubers of wild type and transformants were incubated for 2 h in the presence of 10 mM Mes-KOH (pH 6.5) and 2 μCi of [U-^{14}C]glucose. (**A**) [U-^{14}C] glucose absorbed by the tissue. Incorporation of ^{14}C into neutral fraction (**B**), basic fraction (**C**), acidic fraction (**D**), starch (**E**), protein (**F**), cell wall (**G**) and CO_2 (**H**). Results are means ± SE (n = 4). * denotes significant differences ($P < 0.05$) with respect to the wild type controls

Metabolite poolsize in the transformants

Given that the results of the above-described experiments suggest alterations in uptake and steady state levels of glucose in the transgenic lines, we next chose to investigate the consequences of these changes on the steady state metabolite levels of the transgenic tubers. An established gas chromatography (GC)-mass spectrometry (MS) protocol (Fernie et al., 2004) was used for this purpose. Fructose and many sugars derived from fructose and glucose (including manose, galactose and mannitol) show significantly higher levels in antisense lines, whilst fructose is also reduced in the overexpressors (see Table 1). In contrast, both antisense and overexpressing lines displayed significant elevations in the levels of the phosphorylated intermediates glucose-6-phosphate, and fructose-6-phosphate. Whilst this is at first glance counter-intuitive hexose-phosphates are at the juncture of a diverse range of metabolic pathways (Tauberger et al., 2000), and these increases could therefore quite conceivably be caused by independent mechanisms. Antisense lines, furthermore, displayed increased levels of the Krebs cycle intermediates (namely isocitrate, citrate, malate and fumarate) suggesting an increase in tuber respiration. However, with the exception of succinate, which showed low quantities, there were no significant changes in the Krebs intermediates of the *Asr1* overexpressors and the lack of change in glycolytic flux suggests that a direct link between *Asr1* expression and respiration is unlikely. Regarding organic acids not directly linked to the Krebs cycle, there was no clear trend when comparing wild type tubers either with antisense or overexpressing lines. In the same chromatograms we also evaluated the relative sizes of the individual amino acid pools. Interestingly, in both antisense and overexpressing lines many amino acids, including phenylalanine, aspartic, methionine, threonine and serine, were decreased. A clear exception to this finding was the levels of proline which displayed a 14- and an 8-fold increase in lines AS29 and AS31, respectively.

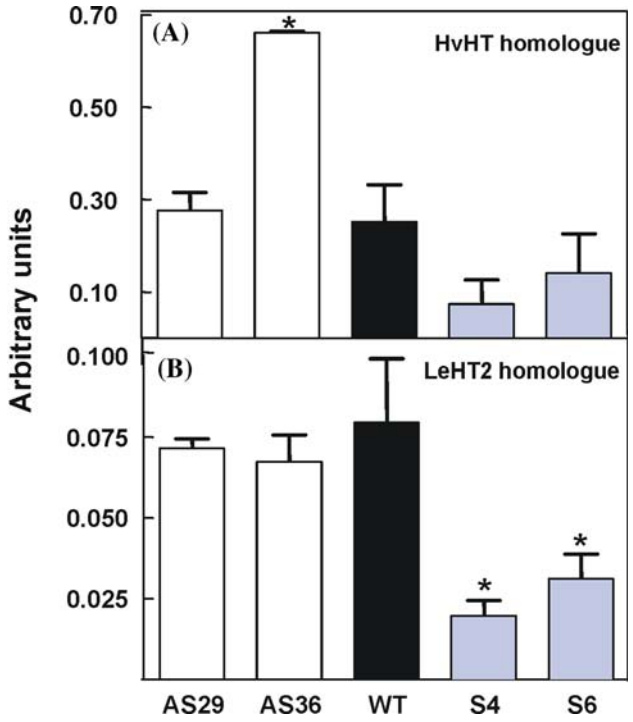

Fig. 6 Steady state levels of mRNA hexose transporters as determined by Real Time RT-PCR. (**A**) HvHT homologue (**B**) LeHT2 homologue. Values represent mean ± SE ($n = 3$). * denotes significant differences ($P < 0.05$) with respect to the wild type controls

Discussion

We have analyzed the effect of modulating *ci21A/Asr1* expression from a whole plant perspective utilizing both antisense inhibition and overexpression strategies. In general, the aerial phenotype of the transgenic lines was similar to that of controls with very few significant changes in growth pattern. In addition, the level of soluble sugars and starch in leaves was not different from wild type plants during a diurnal period and CO_2 assimilation rate, transpiration and stomatal conductance are comparable between transgenic and wild type plants suggesting that leaf metabolism was little affected by this genetic perturbation. Furthermore, estimation of the rate of carbon export from the leaves (data not shown), using the method proposed by Strand et al. (2000), suggests that the transformants are not greatly affected in phloem loading. There are at least two possible explanations for the lack of effect of the modulation of *ci21A/Asr1* in the aerial part of the plant. First, there is relatively little evidence of a role for plasma membrane hexose transporters in leaves of plants. When the plasma membrane transporters themselves are considered, the majority of work is focused on heterotrophic tissues (Sherson et al. 2003; Weshke et al. 2003) and there is very little known regarding their function in leaves with the exception of their role in glucose uptake for stomatal functioning (Stadler et al. 2003). The results of the present study suggest that stomatal function is independent of *ci21A/Asr1* expression in potato plants not subjected to water stress. The lack of changes in leaf properties can, however, also be explained merely on the basis of the normal localisation of CI21A/ASR1 *in planta*. Schneider et al (1997) showed that this protein is only found in tubers under normal conditions. It is therefore conceivable that cofactors necessary for normal CI21A/ASR1 function are absent in leaf but present in tuber under normal conditions. This explanation could be postulated for both overexpression and antisense under normal conditions and suggests that future studies should concentrate on characterization of leaf metabolism of the *Asr* plants following exposure to stresses, such as water stress, that induce its expression (Schneider et al. 1997).

In contrast to the situation in the leaves, clear effects were observed in the tubers of transgenic plants. Two antisense lines showed significantly decreased tuber fresh weight, which led to a diminished harvest index. The overexpressing lines developed less tubers but did not display an altered tuber yield. This low number of tubers is probably related to impaired apoplastic unloading during tuberization. In contrast, the reason for the decreased tuber yield in the antisense lines remains unclear from the present study. Despite these changes in morphology, the transformants did not display gross alterations in tuber metabolism, with no clear changes observable in the major metabolic fluxes. When taken together with the fact that the metabolite profiles of these lines reveal few changes that correlate with the expression of *Asr1* suggesting that the function of this gene, under normal growth conditions, is largely confined to modulating the cellular glucose content. High glucose in antisense plants is, however, also accompanied by a general increase in soluble sugars such as fructose, galactose and mannose as well as the hexose phosphates. Whilst low quantities of amino acids were found in *ci21A/Asr1* antisense plants (a fact that has previously been reported in plants with high sucrose; Roessner-Tunali et al. 2003), surprisingly, a similar trend was observed in sense plants suggesting that this is likely a pleiotropic effect. One change in the metabolite profiles that stands out is the high accumulation of proline in the antisense plants. The fact that this is in close agreement with the recent report by Kalifa et al. (2004b) stating that tobacco *Asr1*-overexpressing plants accumulated less proline in leaf under salt stress.

Table 1 Metabolite levels in tubers of 10-week-old plants of potato plants expressing tomato *Asr1* gene both in antisense (AS29, AS31 and AS36) and sense (S4, S6 and S8) orientation

	AS29	AS31	AS36	WT	S4	S6	S8
Gluconic	0.52 ± 0.05	1.84 ± 0.48	1.21 ± 0.16	1.00 ± 0.03	**1.52 ± 0.12**	**1.30 ± 0.12**	**1.21 ± 0.08**
Quinic	0.77 ± 0.13	**0.50 ± 0.05**	0.85 ± 0.10	1.00 ± 0.05	1.24 ± 0.12	1.24 ± 0.10	1.48 ± 0.03
Dehydroascorbic	0.87 ± 0.15	0.80 ± 0.10	1.03 ± 0.08	1.00 ± 0.09	1.00 ± 0.03	0.87 ± 0.05	1.07 ± 0.06
Isocitric	1.23 ± 0.41	**2.85 ± 0.67**	2.12 ± 0.91	1.00 ± 0.37	1.71 ± 0.28	1.76 ± 0.49	2.11 ± 0.51
Citric	1.29 ± 0.16	1.70 ± 0.42	**2.27 ± 0.42**	1.00 ± 0.06	1.75 ± 0.23	1.11 ± 0.12	0.99 ± 0.13
Threonic	0.96 ± 0.03	**1.51 ± 0.14**	1.12 ± 0.09	1.00 ± 0.05	0.89 ± 0.04	1.07 ± 0.07	0.99 ± 0.04
Malic	**2.22 ± 0.35**	**2.73 ± 0.62**	**2.57 ± 0.48**	1.00 ± 0.07	1.21 ± 0.35	1.27 ± 0.02	1.53 ± 0.27
Fumaric	**3.43 ± 1.60**	1.80 ± 0.47	2.11 ± 0.62	1.00 ± 0.12	0.87 ± 0.15	0.84 ± 0.16	0.86 ± 0.09
Succinic	0.15 ± 0.05	2.03 ± 0.61	0.48 ± 0.34	1.00 ± 0.35	0.16 ± 0.05	0.63 ± 0.13	**0.23 ± 0.09**
Phosphoric	1.08 ± 0.04	**1.19 ± 0.07**	**1.28 ± 0.11**	1.00 ± 0.04	1.05 ± 0.04	0.88 ± 0.11	1.06 ± 0.03
Glutamine	**0.26 ± 0.03**	**0.81 ± 0.17**	0.64 ± 0.27	1.00 ± 0.21	1.24 ± 0.34	**3.74 ± 1.24**	1.31 ± 0.40
Tyrosine	3.40 ± 1.19	nd	0.44 ± 0.35	1.00 ± 0.52	0.54 ± 0.22	4.91 ± 2.10	0.82 ± 0.39
Arginine	nd	1.44 ± 0.10	0.76 ± 0.39	1.00 ± 0.40	1.30 ± 0.57	2.43 ± 1.12	0.88 ± 0.37
Asparagine	0.82 ± 0.77	0.51 ± 0.39	0.57 ± 0.48	1.00 ± 0.63	1.29 ± 0.65	1.98 ± 1.09	1.17 ± 0.67
Phenylalanine	0.65 ± 0.16	**0.18 ± 0.03**	**0.35 ± 0.07**	1.00 ± 0.04	**0.33 ± 0.05**	1.13 ± 0.31	**0.53 ± 0.06**
Glutamic	1.38 ± 0.48	0.93 ± 0.29	0.82 ± 0.27	1.00 ± 0.24	2.06 ± 0.65	1.66 ± 0.59	1.76 ± 0.47
GABA	**0.40 ± 0.08**	1.25 ± 0.18	1.03 ± 0.27	1.00 ± 0.05	**0.74 ± 0.07**	0.91 ± 0.06	**0.85 ± 0.04**
Aspartic	**0.70 ± 0.08**	0.95 ± 0.31	0.75 ± 0.13	1.00 ± 0.10	0.83 ± 0.08	0.81 ± 0.10	0.88 ± 0.06
Methionine	0.77 ± 0.14	0.53 ± 0.26	**0.58 ± 0.13**	1.00 ± 0.10	**0.57 ± 0.06**	0.94 ± 0.20	0.80 ± 0.04
B-alanine	**0.51 ± 0.06**	0.68 ± 0.16	**0.74 ± 0.07**	1.00 ± 0.07	0.46 ± 0.12	1.02 ± 0.07	0.78 ± 0.11
Threonine	0.72 ± 0.17	**0.44 ± 0.10**	**0.54 ± 0.11**	1.00 ± 0.06	**0.59 ± 0.04**	**0.82 ± 0.06**	**0.71 ± 0.03**
Serine	**0.51 ± 0.13**	0.68 ± 0.17	**0.57 ± 0.12**	1.00 ± 0.04	**0.45 ± 0.05**	0.90 ± 0.03	**0.76 ± 0.07**
Glycine	0.77 ± 0.38	0.94 ± 0.18	0.96 ± 0.13	1.00 ± 0.26	0.99 ± 0.10	1.02 ± 0.20	1.28 ± 0.17
Proline	**14.58 ± 8.43**	**8.68 ± 5.19**	0.53 ± 0.08	1.00 ± 0.17	1.69 ± 0.31	0.83 ± 0.13	0.76 ± 0.09
Valine	1.14 ± 0.23	**0.65 ± 0.09**	**0.70 ± 0.06**	1.00 ± 0.05	**0.76 ± 0.07**	1.08 ± 0.12	1.05 ± 0.03
Sorbitol/galactitol	**1.10 ± 0.03**	1.13 ± 0.08	**1.44 ± 0.14**	1.00 ± 0.02	1.14 ± 0.07	1.09 ± 0.04	1.18 ± 0.06
Mannitol	**2.19 ± 0.20**	1.39 ± 0.17	**1.63 ± 0.11**	1.00 ± 0.10	0.83 ± 0.04	1.06 ± 0.14	1.15 ± 0.12
Galactose	2.11 ± 0.66	1.04 ± 0.02	**1.73 ± 0.29**	1.00 ± 0.05	0.89 ± 0.15	1.03 ± 0.22	0.70 ± 0.14
Manose	**4.11 ± 0.10**	**1.27 ± 0.01**	**2.30 ± 0.38**	1.00 ± 0.08	0.78 ± 0.09	1.09 ± 0.20	0.82 ± 0.12
Fructose	**7.19 ± 3.26**	**1.80 ± 0.35**	**2.34 ± 0.30**	1.00 ± 0.17	**0.59 ± 0.10**	0.88 ± 0.19	**0.42 ± 0.03**
Myo-inositol-1-P	**0.59 ± 0.07**	**1.46 ± 0.17**	0.93 ± 0.12	1.00 ± 0.12	**0.64 ± 0.07**	0.93 ± 0.07	**0.68 ± 0.07**
Glucose-6-P	**1.76 ± 0.13**	**1.89 ± 0.19**	**1.85 ± 0.31**	1.00 ± 0.04	**1.46 ± 0.21**	0.98 ± 0.09	**1.54 ± 0.21**
Fructose-6-P	**1.85 ± 0.15**	**1.96 ± 0.33**	**1.90 ± 0.37**	1.00 ± 0.04	**1.53 ± 0.23**	1.01 ± 0.12	**1.67 ± 0.25**
Glycerol-l-P	0.92 ± 0.13	**1.40 ± 0.17**	1.12 ± 0.14	1.00 ± 0.03	0.69 ± 0.06	0.91 ± 0.05	0.94 ± 0.07

Tuber discs were harvested from 10 week-old plants and processed as described in the Material and Methods. Values presented are the normalized mean ± SE of measurements from six plants per genotype. Bold types indicate those that were determined by the *t*-test to be significantly different from the WT genotype ($P < 0.05$). N.d.: not detectable

The question remains: what is the relationship between *ci21A/Asr1* and hexoses? Feeding experiments reveal that tubers overexpressing this gene have lower uptake of glucose (significant in one line). Consistently with this observation, these lines display reduced expression levels of a plasma membrane HT mRNAs. Furthermore, glucose uptake is augmented in an antisense line, which displays extremely high expression of one of the plasma membrane HT mRNAs and a dramatically elevated glucose content. It thus seems likely that *ci21A/Asr1* is involved in the control of hexose uptake in heterotrophic organs. Another possibility that we cannot formally exclude is that the gene may be involved in the transduction of a sugar signal cascade and that on its overexpression the cell mistakenly experiences a sensation of high hexose and downregulated hexose transport accordingly. In this vein, Conde et al. (2006) have recently published a model for grape cells that propose regulation of glucose uptake at transcriptional (mediated by hexokinase) and at protein levels (triggering inactivation, mistargeting and/or proteolysis of transport proteins).

The presence of signal transduction cascades responding to cues sensed in the apoplast have been often proposed (Lalonde et al. 2004; Fernie et al. 2000, 2001b; Roitsch et al. 2003), however, whilst attractive in theory, little direct evidence is available to support such theories. Furthermore given that the results of Cakir et al (2003) demonstrated that a grape ASR is able to bind the promoter of a hexose transporter gene, suggest that *ci21A/Asr1* might be involved in the reg-

ulation of glucose accumulation in tuber (by control of hexose transporter expression). This proposed function is in accordance with *Asr1* expression patterns: the mRNA is primarily observed in phloem companion cells—a cell type related to sugar mobilization (Maskin et al., unpublished results), whilst in sugarcane, expression is also detected in bundle sheath cells (Sugiharto et al. 2002). Interestingly, the expression of one of its target genes, *VvHT1* was also detected in phloem companion cells (Vignault et al. 2005) giving further, albeit indirect, support to our proposed functional role of *ci21A/Asr1*.

In conclusion, in this work we have demonstrated that modification of the expression of *ci21A/Asr1* alters the expression of plasma membrane glucose transporters, the rate of glucose uptake and content in tuber parenchyma cells. This observation is in agreement with the negative correlation between *Asr* and putative HT genes expression found during tomato fruit development and ripening in microarray experiments (Carrari et al. 2006 and http://bti.cornell.edu/CGEP/CGEP.html). The transgenic plants were additionally characterized by several other changes in steady state metabolite levels. This could be explained either by *ci21A*/*Asr1* being implicated in the regulation of other metabolic processes or by multiple effects caused by abnormal glucose levels. One such example is the strong accumulation of proline in the antisense transformants. However, it is unlikely that the observed changes in these plants are only due to the modified expression of the hexose transporter genes and further work is required in order to elucidate this. Microarray experiments could help to find more candidate genes subjected to CI21A/ASR1 regulation.

In the last years, several interesting studies have been published that address the crosstalk between glucose and ABA signaling in *Arabidopsis* (Li et al. 2006; Huijser et al. 2000), suggesting a high degree of interconnection between the two pathways. At least under our experimental conditions, the two HTs were not regulated by ABA at transcriptional level. Therefore, the results presented here do not allow us to propose a relation between CI21A/ASR1, glucose and ABA within potato tubers.

Acknowledgements We are grateful to Prof. Lothar Willmitzer for support and helpful discussion. We acknowledge the excellent care of greenhouse plants to Helga Kulka. We acknowledge financial support from Max Planck Society (ARF, AN, and FC), DAAD (NF and DC), EMBO (NF and FC), University of Buenos Aires (NDI), CONICET (NF, FC and NDI) and INTA (FC).

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