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RESEARCH PAPER

Allelic differences in a vacuolar invertase affect Arabidopsis growth at early plant development

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Abstract

Improving carbon fixation in order to enhance crop yield is a major goal in plant sciences. By quantitative trait locus (QTL) mapping, it has been demonstrated that a vacuolar invertase (vac-Inv) plays a key role in determining the radical length in Arabidopsis. In this model, variation in vac-Inv activity was detected in a near isogenic line (NIL) population derived from a cross between two divergent accessions: Landsberg *erecta* (Ler) and Cape Verde Island (CVI), with the CVI allele conferring both higher Inv activity and longer radicles. The aim of the current work is to understand the mechanism(s) underlying this QTL by analyzing structural and functional differences of vac-Inv from both accessions. Relative transcript abundance analyzed by quantitative real-time PCR (qRT-PCR) showed similar expression patterns in both accessions; however, DNA sequence analyses revealed several polymorphisms that lead to changes in the corresponding protein sequence. Moreover, activity assays revealed higher vac-Inv activity in genotypes carrying the CVI allele than in those carrying the Ler allele. Analyses of purified recombinant proteins showed a similar K_m for both alleles and a slightly higher V_{max} for that of Ler. Treatment of plant extracts with foaming to release possible interacting Inv inhibitory protein(s) led to a large increase in activity for the Ler allele, but no changes for genotypes carrying the CVI allele. qRT-PCR analyses of two vac-Inv inhibitors in seedlings from parental and NIL genotypes revealed different expression patterns. Taken together, these results demonstrate that the vac-Inv QTL affects root biomass accumulation and also carbon partitioning through a differential regulation of vac-Inv inhibitors at the mRNA level.

Key words: Arabidopsis, biomass, inhibitor, near isogenic line, quantitative trait loci, vacuolar invertase.

Introduction

The use of sucrose as a carbon and energy source depends on its conversion to hexose-phosphates. In plants, sucrose synthases (Susys; EC 2.4.1.13) and invertases (Invs; EC 3.2.1.26) can catalyze the first reaction in this process. Susy is a glycosyl transferase which converts sucrose, in the presence of UDP, into UDP-glucose and fructose in the cytoplasm. In most plants, Susy exists as two or more closely related isoforms (Barratt *et al.*, 2009). Invs are hydrolases cleaving sucrose into glucose and fructose and exist as several distinct isoforms with different biochemical properties and subcellular locations (Tymowska-Lalanne and Kreis, 1998; Sturm, 1999). Although both enzymes cleave sucrose, the Inv reaction produces twice as much hexose (one fructose+one glucose) as the reversible Susy reaction (one fructose+one UDP-glucose). This difference marks an important distinction, given that hexose formation can impact on subsequent sugar-sensing networks (Huang, 2006).

The specific functions of the different isoforms of Inv are currently unclear, but it seems that they are involved in regulating the entry of sucrose in different pathways. As sugars in plants are not only nutrients but also important regulators of gene expression and signaling (Koch, 1996; Chiou and Bush, 1998), it has been postulated that Invs may be indirectly involved in controlling cell differentiation and plant development, and a wealth of evidence from mutant and transgenic plants in many different species including carrot, maize, *Arabidopsis thaliana*, and tomato supports this hypothesis (Cheng *et al.*, 1996; Klann *et al.*, 1996; Tang *et al.*, 1999; Barratt *et al.*, 2009). In Arabidopsis, the loss of two cytosolic isoforms of Inv (*civn1*/*cinv2* mutant) results in a severe growth inhibition (Barratt *et al.*, 2009). This was in contrast to Susy, for which in the same study a quadruple mutant showed no obvious growth phenotype, and only SUS5 and SUS6, which are specifically expressed in phloem cells, were shown to be essential for normal development and growth of the plant.

Most plant species contain at least two isoforms of vacuolar Inv (vac-Inv), which accumulate as soluble proteins in the lumen of this compartment. Also, many Inv isoforms have been found in the extracellular space attached to the cell wall (cw-Inv) by ionic bonds. Vac- and cw-Invs share some properties such as the pH at which they act (between 4.5 and 5.0), the K_m values for sucrose that ranged from 5 mM to 12 mM, and maximal activity at 45 °C (Goetz and Roitsch, 1999). Plants additionally have at least two isoforms of cytoplasmic Inv (cyt-Inv) that work at neutral pH (≥7). Morever, recent findings demonstrated that alkaline/neutral Invs also play an important role in developmental energy-demanding processes within the mitochondrion (Martín *et al.*, 2013). The mature acid Invs are *N*-glycosylated at multiple sites and their molecular weights range from 55 kDa to 70 kDa (Tymowska-Lalanne *et al.*, 1998). Although glycosylation does not seem to be essential for the enzyme activity or stability, it is required for protein transport across either the plasma membrane (for cw-Invs) or the tonoplast (for vac-Invs) (as reviewed in Tymowska-Lalanne and Kreis, 1998). Functional studies on the role of these enzymes in the metabolism of non-photosynthetic organs suggest that they participate in the regulation of complex phenotypes such as crop quality and yield (Cheng *et al.*, 1996; Klann *et al.*, 1996; Tang *et al.*, 1999; Andersen *et al.*, 2002; Koch, 2004; Fridman *et al.*, 2004; Draffehn *et al.*, 2015).

It has also been reported that the activity of acid Inv is inhibited by a polypeptide of ~17 kDa in a pH-dependent way (Weil *et al.*, 1994) forming an inactive complex (Pressey, 1967; Bracho and Whitaker, 1990a, b; Rausch and Greiner, 2004; Jin *et al.*, 2009; Hothorn *et al.*, 2010; Tauzin *et al.*, 2014). Inv inhibitors are closely related to pectin methylesterase inhibitor proteins (Hothorn *et al.*, 2004) and are found in both apoplasts and vacuoles. The binding of the inhibitor to the protein is inhibited by low concentrations of sucrose, and the expression of both proteins is not always co-ordinated (Greiner *et al.*, 1998). In potato tubers, for example, hybrid mRNAs from a single gene encoding an acid Inv inhibitor peptide (INH2) accumulate in response to low temperatures, and this seems to be a plausible mechanism of resistance to cold-induced sweetening (Brummell *et al.*, 2011; McKenzie *et al.*, 2012).

Quantitative trait loci (QTL) studies revealed that several loci affecting Inv activity co-localize with loci affecting radicle and hypocotyl lengths in Arabidopsis plants (Sergeeva *et al.*, 2006). Root length correlates positively with acid Inv activity, particularly in the apical regions of the radicles. The role of acid Inv in root growth was verified by analyzing a knockout mutant for the *At1g12240* gene, which is co-located in the genomic region spanning the mentioned QTLs. Acid Inv activity was significantly lower in these mutants, which in turn show shorter radicles than the wild-type genotype. In the same mapping population, a paralog to *At1g12240*, namely *At1g62660*, did not co-locate with any of the QTLs detected for root length, indicating that not all loci related to Inv activity affect biomass accumulation in roots.

These studies by Sergeeva *et al.* (2006) and Barratt *et al.* (2009) clearly demonstrate the significance of vac- and cyt-Invs for normal growth of Arabidopsis plants. However, the mechanism(s) underlying the above-mentioned Inv QTLs are not yet clear. The aim of the current work was to shed light on such mechanisms. The results presented here from detailed analyses of the structural and functional variations found in the *At1g12240* locus within homogeneous genetic backgrounds suggest that mechanisms regulating biomass accumulation in seedling roots operate via post-translational regulation at the enzyme activity level. Kinetic analyses of purified proteins from the two parental loci showed differences in activity compared with those measured in crude extracts, suggesting that other factor(s) might be operating in regulating vac-Inv *in vivo*. Activity assays in crude extracts from the near isogenic lines (NILs) used here and analyses of vac-Inv inhibitor mRNA suggest that the interaction of the vac-Inv–inhibitor complex is one of the main mechanisms behind the QTLs reported by Sergeeva *et al.* (2006).

Materials and methods

Plant material and phenotypic analyses

Seeds from the four genotypes used here (Ler, CVI, NIL-14, and NIL-15) were obtained from the Plant Physiology Laboratory of Dr M. Koornneef in Wageningen (The Netherlands). They were grown in culture chambers (Conviron) according to standard conditions for *Arabidopsis thaliana* [16 h/8 h light/dark, 22 °C/23 °C, 220 photosynthetic active radiation (PAR) µmol m^{-2} s^{-1}, and 55% humidity] and samples were taken at the indicated stages for DNA, RNA, and protein extractions, and immediately frozen in liquid nitrogen and kept at –80 °C until use.

For germination assays, 50 disinfected seeds were sown in Petri dishes with filter paper and kept at 4 ºC for 7 d. After this time, germinated seeds were recorded daily. The algorithm proposed by Reddy *et al.* (1985) was used to calculate the germination index.

For phenotypic characterization of adult plants, between seven and 10 plants of each genotype were grown as described above. During plant development, the number of leaves appearing in the rosette and in the inflorescence was recorded separately, and 40 d post-emergence measurements on final height (in cm), and number of branches, pods, and total flowers were performed. Fresh and dry weight of roots, rosettes, and inflorescences were also recorded after dissection. Phenotypic analyses of seedlings were done with 50–100 disinfected seeds sown as described above and all measurements were performed 7 d after germination. Radicle length was measured in 20 seedlings of each genotype by using a binocular magnification optic with a millimetric slide. Each experiment was repeated two or three times, and data analyses are described in the Statistical analysis section below.

Sequence analyses of At1g12240 *genomic clones*

Total DNA of each genotype was extracted according to Dellaporta *et al.* (1983). In order to amplify the entire genomic clone of the *At1g12240* locus, two pairs of oligonucleotides (ORF 1F/2R, 5'TCTCCGCCAGA GAAGAAGAA3'/5'TGAAATG GGTCAAGTTCCC3'; and ORF 3F/4R, 5'TGAAATGGGTCA AGTTCCC3'/5'TACTCTGACGAAGC TGTCCG3') were designed based on the Col-0 sequence and used for PCR amplification. Fragments of ~2000 bp and 1600 bp, corresponding to the first and second half of the gene were amplified, respectively. PCR conditions were as follows: 94 ºC for 2 min, 30 cycles at 94 ºC for 60 s, 50 ºC for 30 s, and 68 ºC for 90 s, with a final extension at 68 ºC for 2 min. Fragments obtained by PCR were ligated in TOPO-TA vector (Invitrogen, Carlsbad, CA, USA) and used to transform competent *Escherichia coli* cells. Several positive clones were sequenced with an ABI3130XL sequencer (Applied Biosytems, Foster City, CA, USA), and chromatograms and the resulting reads were evaluated by alignments using the ClustalX 1.8 software. Contig assemblies for each allele were performed with the Bioedit 5.0.9 software. Sequence uncertainties were evaluated manually by analyzing each site in the corresponding chromatograms. Exonic and intronic regions were predicted *in silico* by alignments with the Col-0 reference sequence. Proportions of single nucleotide polymorphisms (SNPs), insertions/deletions (InDels) and synonymous (ds) and non-synonymous (dn) substitutions were analyzed with the DNAsp software, and significance was evaluated by a Fisher test.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from roots and leaves of 7-day-old seedlings and from roots and rosettes of adult plants at different developmental stages. A 1 µg aliquot of total RNA was used to synthesize cDNA with SuperscriptIII Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Oligonucleotides designed on the CVI and Ler cDNA sequence were used to amplify a 156 bp product from the vac-Inv cDNA (invRT F/invRT R, 5'CATCAAGAGT CTATCCAACAAC3'/5'AGA GAGCACGGACA GCTTCGTC3') and 166 bp and 152 bp products from the Inv-inh1 (*At1g47960*) (RT INH1 F, 5'CTAAAACGGGC TTTGGATGA3'/R, 5'TCCCATTAA ACCCTCCTTGA3') and Inv-inh2 (*At5g64620*) (RT INH2 F,5'TATGCCTC CATGCATGTGTT3'/R, 5'TCAAGAATCCC GGAGACAAC3') cDNA, respectively, in qRT-PCRs by using 1× Quantitect SYBR Green PCR Master Mix (Qiagen) according to the manufacturer's instructions. PCR conditions were as follows: 95 ºC for 10 min, 40 cycles at 95 ºC for 15 s, 48 ºC for 40 s, and a final extension at 72 ºC for 40 s in an ABI Prism 7500 Real Time PCR system, and data were analyzed with the 7500 system software (Applied Biosystems, Foster City, CA, USA). Two independent extractions of RNA from two biological replicates were made, and three technical replicates of each biological sample were performed.

Invertase activity assays

Proteins were extracted from roots and leaves of 7-day-old seedlings which were homogenized in extraction buffer [50 mM HEPES KOH pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100, and 5 mM DTT] and 20 mg of PVP. The protein concentration was determined using the protein–dye binding method, employing BSA as standard. Vac-Inv activity was measured by incubating 4 µg of protein at 30 ºC in reaction buffer [25 mM citrate-phosphate buffer pH 5.2, 25 mM ultrapure sucrose or indicated concentrations for each case] 1 h without shaking. Tubes containing boiled extracts (95 ºC for 7 min) and without sucrose were used as controls. Reactions were stopped at 95 ºC for 5 min. Glucose was quantified as described by Fernie *et al.* (2001).

The calibration curve, necessary to calculate the initial velocity of the reaction to determine the enzyme activity, was made by incubating 1 µg of purified vac-Inv-Ler and vac-Inv-CVI recombinant proteins with 100 mM acetic/sodium acetate buffer, pH 5.2, and different sucrose concentrations (1, 5, 10, 20, 40, 60, 80, 100, and 160 mM) for various incubation times (4, 7, 10, 13, 15, 17, 20, 25, and 30 s). K_m and V_{max} parameters were measured by incubating 1 µg of purified vac-Inv-Ler and vac-Inv-CVI recombinant proteins as mentioned before at 30 °C for 30 s. Reactions were stopped by adding 400 mM Tris pH 8 and heating to 95 ºC for 5 min. Control reactions for each sample were performed by heating the reaction aliquots of protein extract to 95 ºC for 7 min before addition of substrate and buffer. Glucose was quantified as described by Fernie *et al.* (2001). K_m and V_{max} parameters were measured using Graph Path Prism 5.0 program.

Protein gel blot analyses

Denatured protein samples were separated on 12% SDS–polyacrylamide gels and transferred to a Hybond ECL Nitrocellulose Membrane (GE Healthcare, Amersham). Thereafter, the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS), pH 7.2, plus 0.1% Tween-20 and then probed with either a polyclonal anti-vac-Inv antibody raised in mice (Rojo *et al.*, 2003) or with a vac-Inv antibodies raised in rabbit against a synthetic peptide of sequence RNDGLWKLSGDRNTC derived from the N-terminal end of the AT1G12240 vac-Inv (Genscript) and also with a monoclonal anti-myc antibody raised in mice for detection of the recombinant proteins expressed in the *Pichia pastoris* system. Western blot signals were detected by using goat anti-mouse or anti-rabbit IgG conjugated with alkaline phosphatase. The anti-vac-Inv antibodies detected a polypeptide of 64 kDa or 81 kDa which matched with the predicted sizes of native vac-Inv from *A. thaliana* and *P. pastoris* recombinant proteins, respectively.

Heterologous expression construct and expression in Pichia pastoris

The entire ORF of the AT1G12240 vac-Inv was amplified with PstIFow/NotIRev primers (5'TACTGCAGCACAC GACGGAT CTAGATCCAA CG3'/5'GAGATGCGGCC GCGGTACGGGAG

AGAGCACGG3') to introduce appropriate restrictions enzyme sites. PCR conditions were: 94 ºC for 5 min, 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 2 min, followed by one cycle of 68 °C for 10 min using DNA polymerase (Roche, Pleasanton, CA, USA). PCR products were cloned and sequenced to verify that no errors occurred during amplification. The vac-Inv fragment was restricted using *Pst*I and *Not*I restriction enzymes (NEB, Ipswich, MA, USA) and ligated into the pPICZαB expression vector (Invitrogen) in-frame with the α-factor sequence from *Saccharomyces cerevisiae* to enable secretion of the recombinant protein for further isolation. The *P. pastoris* wild-type strain X-33 was transformed by electroporation with 10 µg of *Pme*I-linearized pPICZαB-vac-Inv (Ler or CVI) or empty pPICZαB, and plated on selection YPDS Zeocin plates (500 µg ml^{-1}) following the manufacturer's instructions (Invitrogen). To produce recombinant vac-Inv enzyme for functional characterization, single colonies of transformed *P. pastoris* were inoculated into 5 ml of pre-culture medium [buffered glycerol complex medium (BMGY): 1% yeast extract; 2% peptone; 100 mM potassium-phosphate buffer, pH 6; 1.34% yeast nitrogen base (YNB); 4×10^{-5}% biotin; 1% glycerol] and incubated at 28–30 °C overnight under vigorous shaking. Cells were harvested by centrifugation and resuspended to an OD_{600}=1–2 in induction medium [buffered methanol complex medium (BMMY): 1% yeast extract; 2% peptone; 100 mM potassium-phosphate buffer, pH 6; 1.34% YNB; 4×10^{-5}% biotin; 1% methanol]. Methanol was replenished every 24 h to a final concentration of 1%. For secreted expression, the culture medium aliquots were transferred to a new tube at 72 h, centrifuged, and the supernatant was concentrated and equilibrated with 25 mM MES buffer, by ultrafiltration on Amicon Centriplus concentrators YM30 (cut-off at 30 000 Da; Millipore, Danvers, MA, USA). The concentrate was purified using Ni-NTA columns for 6×His-tagged proteins (Ni-NTA Spin Kit; Qiagen, Germany). Purified protein extracts were used for further kinetic measurements.

Inhibitor assay

Extracts from 7-day-old seedlings from Ler, CVI, NIL-14, and NIL-15 genotypes were shaken at 37 °C for 30 min rapidly enough to generate foaming, to minimize the activity of endogenous Inv inhibitor proteins (Pressey, 1966). Vac-Inv activity was measured with 4 µg of protein extract, 100 mM acetic/sodium buffer, pH 5.2, and 100 mM sucrose. Reactions were carried out at 30 °C for 30 min, and were stopped by heating at 95 °C for 5 min. Control reactions for each sample were performed by heating the reaction aliquots of protein extract to 95 °C for 7 min before addition of substrate and buffer. Glucose formed was determined employing a standard curve. Activity was expressed as mmol of glucose formed h^{-1} mg^{-1}protein.

Statistical analyses

The values were averaged in each independent experiment and then used for calculations of the SD and SE using Excel software. Significance was evaluated through the Student's *t*-test (except when otherwise indicated) considering *P*-values <0.05 (compared against the Ler genotype) as statistically significant.

Results

Structural analysis of genomic introgressed regions

The genetic analyses reported by Sergeeva *et al.* (2006) uncovered two NILs with significant effects on both radicle length and vac-Inv activity: NIL-14 and NIL-15 derived from recombination events in a cross between CVI and Ler genotypes (Keurentjes, 2007). Molecular marker analyses of the introgressed genomic regions defined a 1.8 Mb and 1.1 Mb CVI introgression into the Ler genetic background in NIL-14 and NIL-15, respectively (assuming that no internal recombination occurred; Keurentjes, 2007). Further bioinformatic analyses identified 551 and 361 annotated genes in the genomic regions in NIL-14 and NIL-15, respectively, whose allelic variants correspond to the CVI genotype. As described by Sergeeva *et al.* (2006) and Keurentjes (2007), the vac-Inv-encoding gene (*At1g12240*) was found within the introgressed fragment in both of these NILs.

To confirm the identity of the allelic forms of the vac-Inv gene harbored in the two NILs, the complete genomic sequences of the *At1g12240* gene for both NILs and the two parents were obtained. A detailed comparative analysis of the consensus sequences obtained from multiple readings of the same allele allowed the detection of several SNPs and InDels between the Ler and CVI alleles (Fig. 1A). Moreover, this analysis allowed us to determine that the CVI and NIL alleles are 100% identical to each other. This result indicates that the two segregating genotypes NIL-14 and NIL-15 did not exhibit any recombination at this specific locus.

Nucleotide changes in the coding region were examined in terms of the exchange rate, the position of each involved codon, and the frequency of synonymous and non-synonymous substitutions. The synonymous substitution rate was significantly higher than the non-synonymous rate (ds=0.0477, dn=0.0056, $P<0.05$) (Supplementary Table S1 at *JXB* online). A comparative alignment of the two alleles allowed the prediction of proteins consisting of 664 and 663 amino acid residues for the Ler and CVI genotypes, respectively (Supplementary Fig. S1A). This length is consistent with those reported by Qi *et al.* (2007) and with our own results from western blot experiments with an anti-vac-Inv antiserum (AT1G12240) using crude extracts from leaves and roots of Arabidopsis (see below). Non-synonymous polymorphisms resulted in eight amino acid substitutions (SAS) between the allelic forms, resulting in a 98.7% (655/664) amino acid identity (Supplementary Fig. S1A) None of these SAS was found within the catalytic sites previously described for different Invs (Fridman *et al.*, 2004; Le Roy *et al.*, 2007), but two of them (I/S^{75} and E/–^{77}) are proximal to the N-terminal of the mature protein region involved in vacuolar trafficking (Jung *et al.*, 2011) (Supplementary Fig. S1A). Using the crystal structure of the Arabidopsis cell wall invertase 1 (INV1), in complex with a protein inhibitor as a template (PDBid2XQR; Hothorn *et al.*, 2010), we mapped most of the mentioned SAS. As shown in Fig. 1B, five of them, namely A/D^{336}, S/T^{387}, F/L^{433}, T/K^{549}, and I/V^{578}, are suggested to be found on the protein surface and on the opposite side from the active and the inhibitor binding sites. Moreover, most of these SAS are rather conserved in terms of the physiochemical properties of the residues. Since the N-terminal domain is missing in the INV1 structure, the I/S^{75} SAS and the E/–^{77} deletion which are facing the inhibitor-binding region could not be structurally mapped with precision.

Detailed phenotypic analyses of parental and recombinant lines

In order to assess the effect of the allelic substitutions of the *At1g12240* locus, we first performed a detailed phenotypic analysis of both NILs and the two parents. In line with the

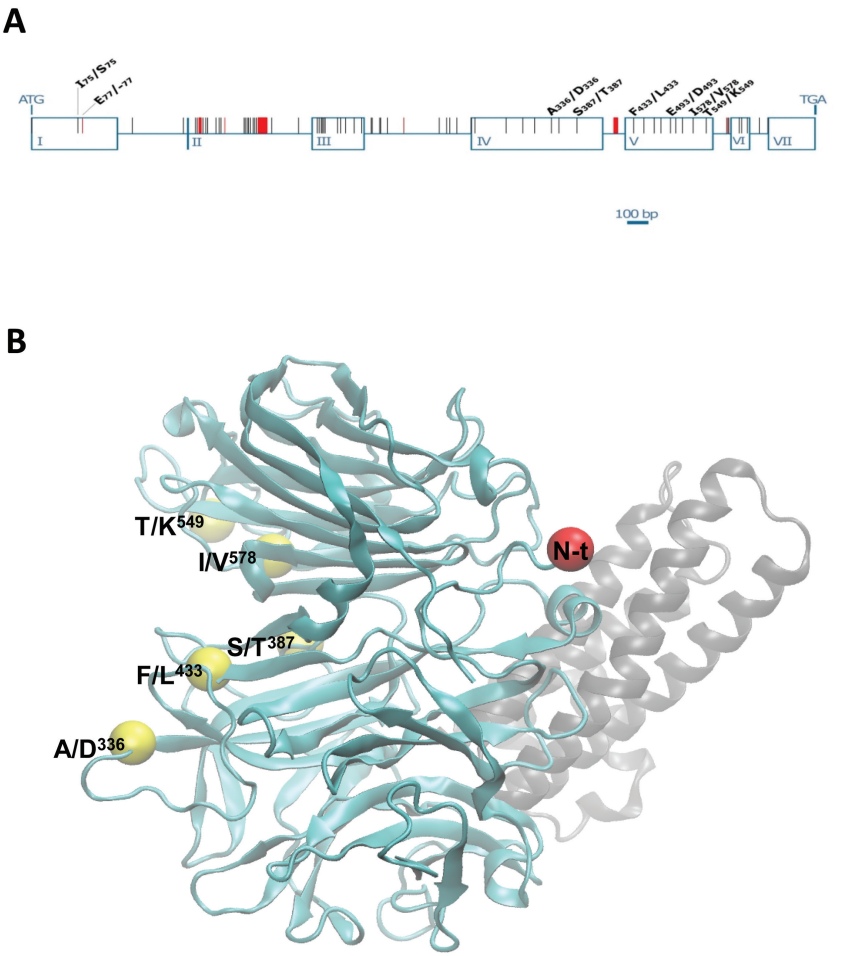

Fig. 1. vac-Inv gene (*At1g12240*) and protein modeling. (A) Scheme of the *At1g12240* genomic clone showing the polymorphisims found in the two analyzed alleles (CVI and Ler). Blocks represent sequences corresponding to exons (indicated with roman numbers) and lines indicate introns. SNPs and InDels are depicted by gray bars. Amino acid changes (Ler/CVI) are shown over the corresponding nucleotide. (B) Structure of Arabidopsis cell wall invertase 1 (INV1), in complex with a protein inhibitor, pdbid2XQR in ribbon diagram.The INV1 structure is shown in dark, and the protein inhibitor (Nt-CIF) is shown in gray. Spheres on the link denote the alpha carbon positions of the INV1 residues corresponding to the observed amino acid substitutions (SAS) between Ler and CVI proteins as determined by the corresponding sequence alignment. The sphere on the right shows the location of the INV1 N-terminus (This figure is available in colour at *JXB* online.)

results reported by Sergeeva *et al.* (2006), the radicle length of 7-day-old seedlings was significantly longer in the genotypes harboring the CVI allele of the vac-Inv gene (Fig. 2A). This increase in the length of the radicles ranged between 25% and 35% for CVI, NIL-14, and NIL-15 genotypes. To test whether these differences could be related to an accelerated dormancy exit of the seeds, experiments were performed to characterize the germination of the different genotypes, and germination indexes were calculated as previously described (Carrari *et al.*, 2001). As no differences in germination rates were observed between the genotypes (Fig. 2B), we conclude that the observed differences in radicle length are more related to post-germinative growth events than to differences in the dormancy exit of seeds.

Aerial fresh weight accumulation in 7-day-old seedlings was also significantly higher in lines harboring the CVI allele of the vac-Inv gene (Fig. 2C), rising by ~80–170% compared with Ler which, with the lower increase in radicle length, resulted in a higher aerial/root ratio in the genotype harboring the CVI allele of vac-Inv (Fig. 2D).

In adult plants (40 d after germination), neither rosette nor root fresh matter is affected by introgression of the vac-Inv CVI allele into the Ler genetic background (Fig. 3A, B). However, biomass accumulated in inflorescences was significantly reduced in the genotypes harboring the CVI allele of the vac-Inv gene (Fig. 3C).When the whole aerial biomass is considered together (rosettes plus inflorescences), only NIL-14 showed a significant reduction with respect to the Ler genotype (Fig. 3D). However, the total aerial biomass/root ratio was significantly reduced in CVI and NIL-15 genotypes, compared with Ler (Fig. 3E). The total number of branches, flowers, and pods as well as plant height at maturity and daily leaf appearance were also recorded, but no clear differences were detected between the genotypes (data not shown).

Taken together, the phenotypic data presented above reveal that the presence of the CVI allele of the vac-Inv affects not only radicle length but also carbon partitioning at the beginning of seedling development. In adult plants, the presence of this allele affected carbon partitioning to inflorescences

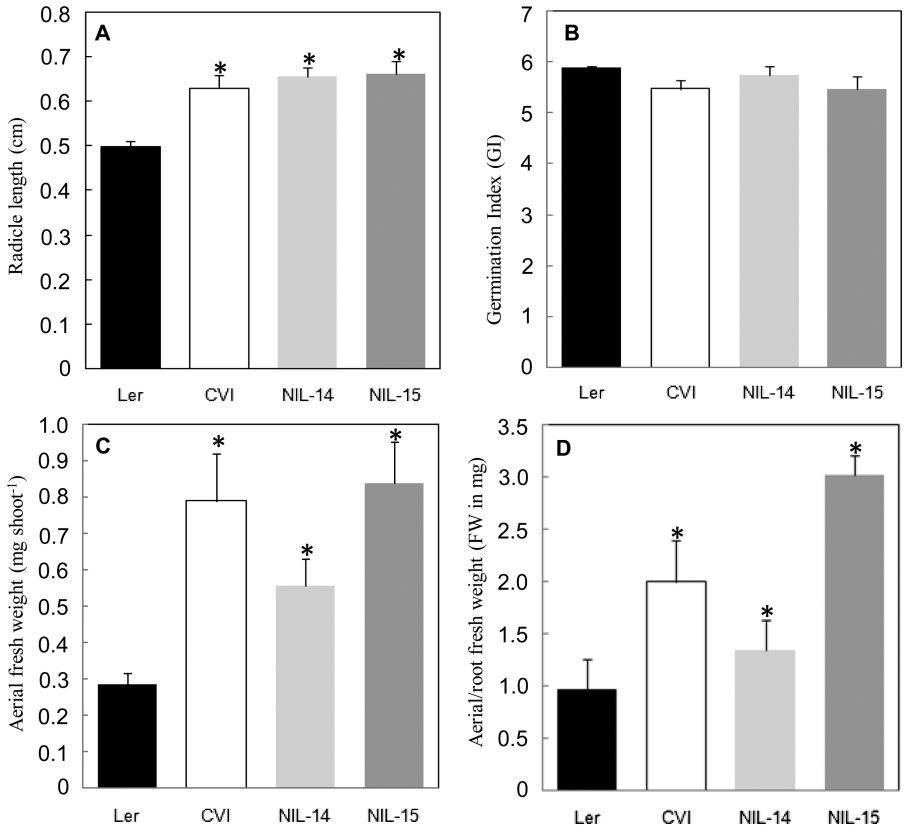

Fig. 2. Radicle length (A) and seed germination (B). Fresh matter accumulation in aerial organs (C) of seedlings 7 d after germination. Aerial to root ratios (D). Accessions: Ler, Landsberg *erecta*; CVI, Cape Verde Islands; NIL-14 and NIL-15 correspond to near isogenic lines harboring 1.8 Mb and 1.1 Mb CVI introgressions into the Ler genetic background. Bars indicate averages ±SE from three independent experiments with 100–150 individuals. An asterisk denotes a statistically significant difference in comparison with the Ler accession (*t*-test $P<0.01$).

but has no effect in other vegetative organs when introgressed into the Ler genetic background.

Analysis of At1g12240 *gene expression in different organs and developmental stages of the recombinant and parental genotypes*

In order to explore whether the phenotypes described above associated with the presence of the CVI allele are in turn related to a differential regulation of the vac-Inv gene at the mRNA levels in these tissues, we evaluated the relative abundance of *At1g12240* transcripts by qPCR in radicles and shoots from 7-day-old seedlings. There were no significant differences in the corresponding mRNA levels among the genotypes in the analyzed tissues (Fig. 4A). Moreover, *At1g12240* mRNA levels were also analyzed in different tissues from adult plants at different stages of development. Similarly to what was observed in the analysis of expression in seedlings, the expression levels of the *At1g12240* gene remained constant throughout the studied period in both roots and leaves and showed no differences between the lines (data not shown).

Vac-Inv activity in extracts from radicles and aerial parts

Having established that during seedling development the relative mRNA contents of *At1g12240* do not differ among the genotypes, we next analyzed the levels of enzymatic activity in tissue extracts. The results showed that acid Inv activity was higher in extracts from CVI, NIL-14, and NIL-15 than in extracts from Ler, for both the radicle and the aerial part of the plant. The increase was statistically significant in all cases except for NIL-14 in the radicle (Fig. 4B). In further experiments, we assayed acid Inv activity in extracts from roots and from rosette leaves harvested from adult plants 10, 20, 30, and 40 d after sowing. The CVI genotype displayed a significantly higher activity in comparison with Ler and with both analyzed NIL genotypes (data not shown).

We next examined the AT1G12240 protein levels in protein extracts from radicles and aerial tissue of 7-day-old seedlings by a western blot analysis hybridized with an anti-vac-Inv antibody raised against 20-mer and 15-mer peptides of the N-terminal portion of the AT1G12240 protein (see the Materials and methods) (Fig. 4C). This analysis revealed a single ~70 kDa band of comparable intensity in radicles of the four analyzed genotypes. In extracts from the aerial part of the seedlings, the immunoblot also showed comparable levels among the genotypes of the vac-Inv protein. However, a double band, compatible with the size of the pre-processed and mature peptides, was observed in this tissue. Also, the levels of total vac-Inv protein detected by immunoblots in extracts obtained from adult plants (roots and rosette leaves) were similar among the analyzed genotypes (data not shown). These analyses were also performed with a second

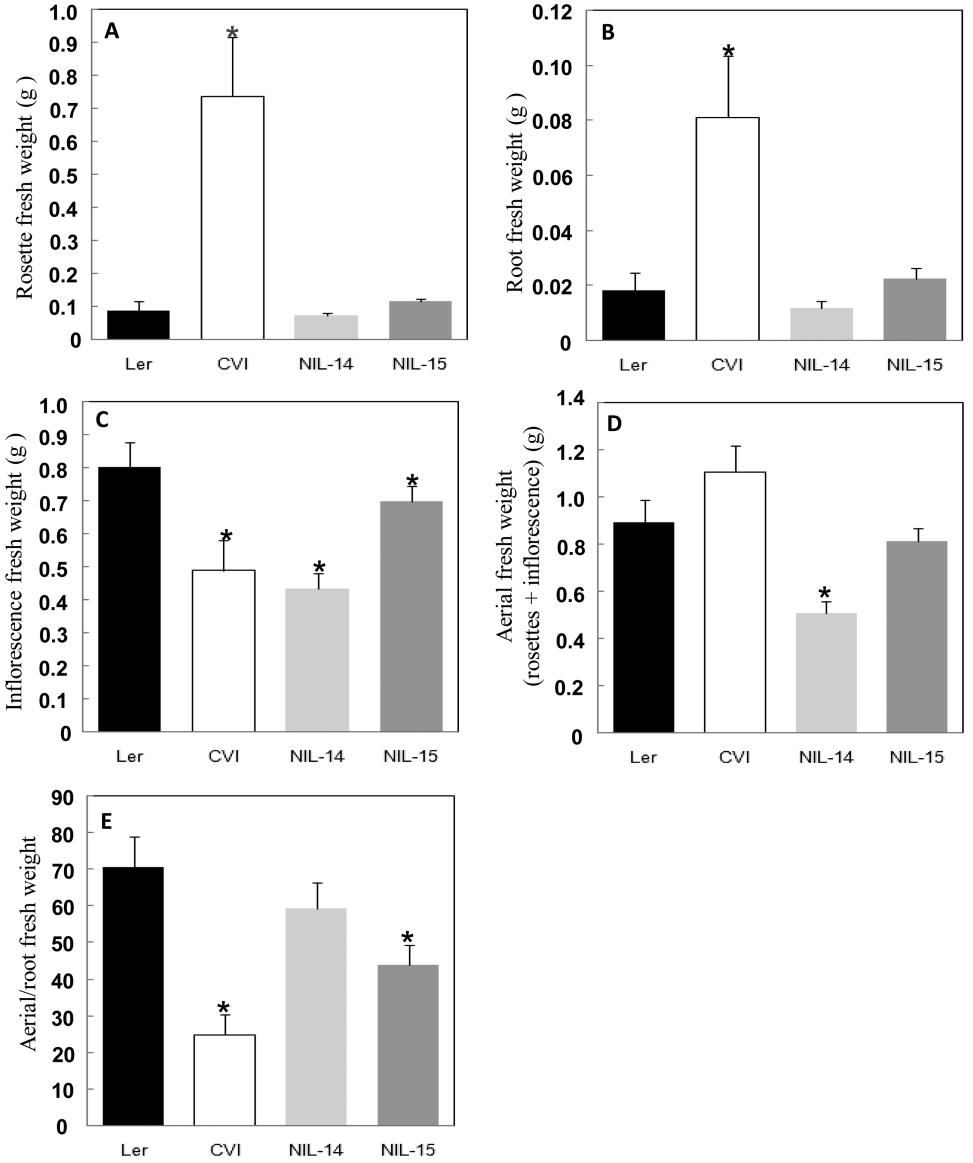

Fig. 3. Fresh matter accumulation in rosettes (A), roots (B), inflorescence (C), and aerial organs (D) of adult plants (30–40 d after germination). (E) Aerial to root ratios. Accessions: Ler, Landsberg *erecta*; CVI. Cape Verde Islands; NIL-14 and NIL-15 correspond to near isogenic lines harboring 1.8 Mb and 1.1 Mb CVI introgressions into the Ler genetic background. Bars indicate averages ±SE from two independent experiments with 10 individuals. An asterisk denotes a statistically significant difference in comparison with the Ler accession (*t*-test $P<0.01$).

vac-Inv antibody described in Rojo *et al.* (2003) (and kindly provided by the authors) and showed identical results (data not shown).

To summarize, the results described above indicate that the differences in the activity levels shown by the different genotypes are not directly linked to the amount of AT1G12240 protein in the analyzed tissues.

Kinetic parameters of recombinant purified vac-Inv enzyme

Both Ler and CVI cDNA alleles were cloned in the pPICZαB vector (Fig. 5A). Expression of the recombinant vac-Inv (Ler and CVI) in *P. pastoris* resulted in soluble and active proteins. Maximum protein yields were obtained after 72 h induction. When examined by SDS–PAGE, they both showed the presence of a band of the expected size that was later confirmed by an immunoblot assay using an antibody against the c-*myc* epitope (Fig. 5B). After purification, protein extracts were used to determine the apparent kinetic constants at 30 °C. The initial rate of glucose production was determined and the results were analyzed by means of Lineweaver–Burk plots to obtain substrate affinity (K_m) and maximal velocity (V_{max}) values. The K_m values of both proteins were of the same order of magnitude as those found by Tang *et al.* (1996), being 5.65 ± 0.7 mM and 5.46 ± 0.96 mM for Ler and CVI, respectively. Maximal velocities, however, differed between the proteins. V_{max} was higher from the Ler recombinant protein ($3.37e^{-07}$±$7.45e^{-09}$ mmol glu µg protein^{-1} s^{-1}) than the CVI recombinant protein ($1.08e^{-07}$±$4.35e^{-09}$ mmol glu µg protein^{-1} s–^{1}) (Fig. 5C), in stark contrast to the results obtained in tissues where the CVI allele resulted in higher enzyme activities with no clear changes in total amount of the protein itself (Fig. 4).

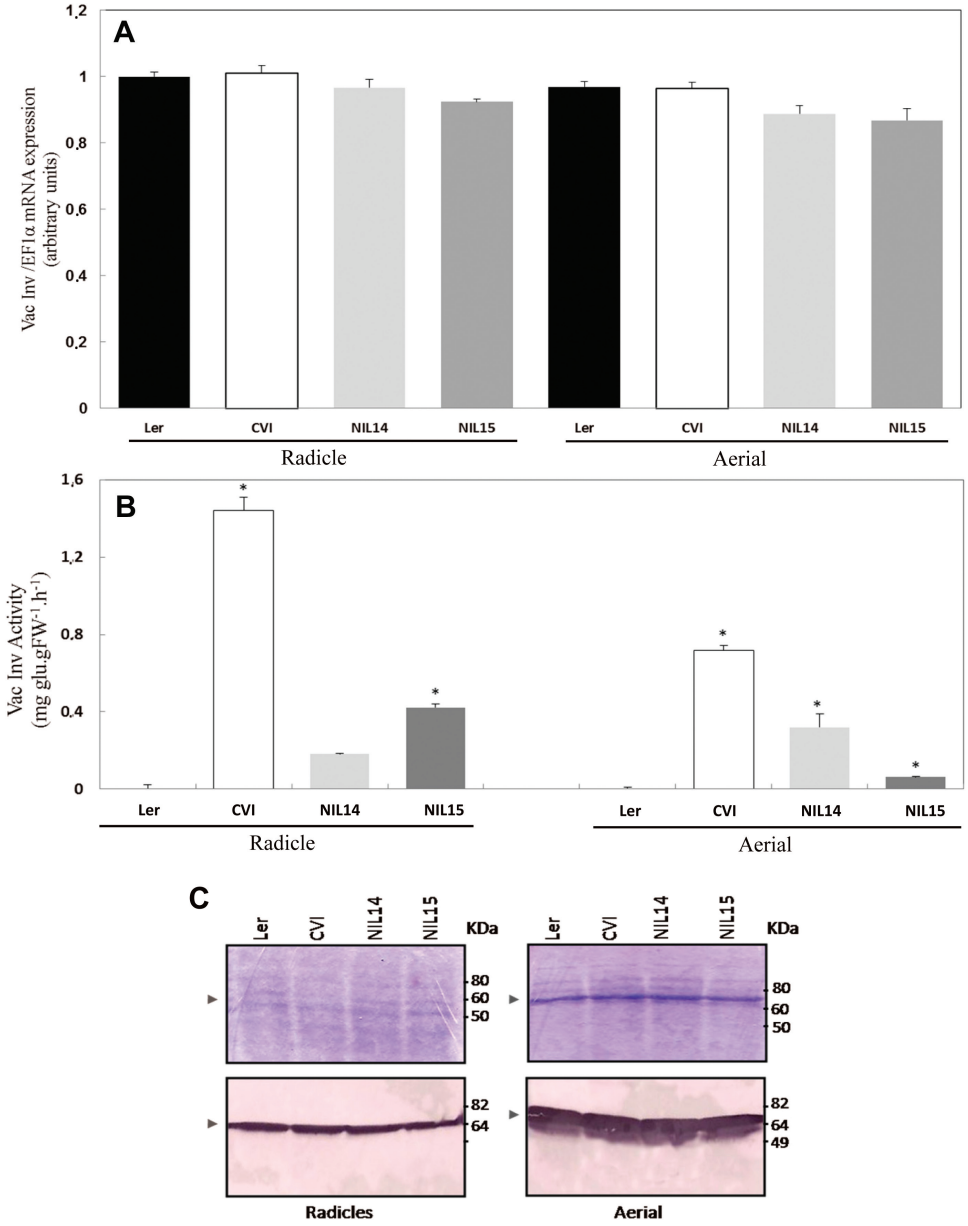

Fig. 4. Invertase expression and enzymatic activity analyses in seedlings 7 d after germination. (A) Vac-Inv mRNA accumulation measured by qRT-PCR. (B) Soluble acid invertase activity in radicles and aerial organs, and (C) SDS–PAGE (upper panel) and western blot analyses (lower panel) of the enzyme hybridized with anti-vac-Inv antibody raised against 20-mer and 15-mer peptides of the N-terminal portion of the protein. Results of mRNA expression analyses are normalized to values of the Ler accession measured in samples from radicle tissues. Accessions: Ler, Landsberg *erecta*; CVI, Cape Verde Islands. NIL-14 and NIL-15 correspond to near isogenic lines harboring 1.8 Mb and 1.1 Mb CVI introgressions into the Ler genetic background. Bars indicate averages ±SE from two independent experiments with 10 individuals. An asterisk denotes a statistically significant difference in comparison with the Ler accession (*t*-test $P<0.01$). Entire vac-Inv peptides are indicated by arrowheads.

Interaction of vac-Inv with its inhibitor

The above results indicate that the Ler and CVI alleles have differing effects on Inv activity, depending upon whether the activity is measured in crude plant extracts or using recombinant purified protein. It is very well known that Inv enzymes are regulated by small peptides in a pH-dependent way (Weil *et al.*, 1994; Brummell *et al.*, 2011; McKenzie *et al.*, 2012; Su *et al.*, 2015). In order to explore the implications of these inhibitors on vac-Inv differential activity between the accessions, assays involving the interaction of the proteins with their inhibitor(s) were performed. Treated [where the activity of the inhibitor(s) was minimized by foaming, exactly as described by Pressey (1966)] and non-treated extracts from 7-day-old seedlings of the four genotypes were assayed for vac-Inv activity. The results showed again that genotypes harboring the CVI allele have higher vac-Inv activity. Moreover, in seedlings from the Ler genotype, the activity of treated extracts was significantly higher than that of non-treated extracts (Fig. 6A), indicating that the level of inhibition is ~80% in this genotype (Fig. 6A inset graph). In contrast, activity was hardly changed between treated and untreated extracts in CVI, NIL-14, and NIL-15. These results demonstrate that Ler and CVI vac-Inv proteins interact differentially with their inhibitor(s) and suggest that this is a plausible explanation for the QTL action reported by Sergeeva *et al.* (2006).

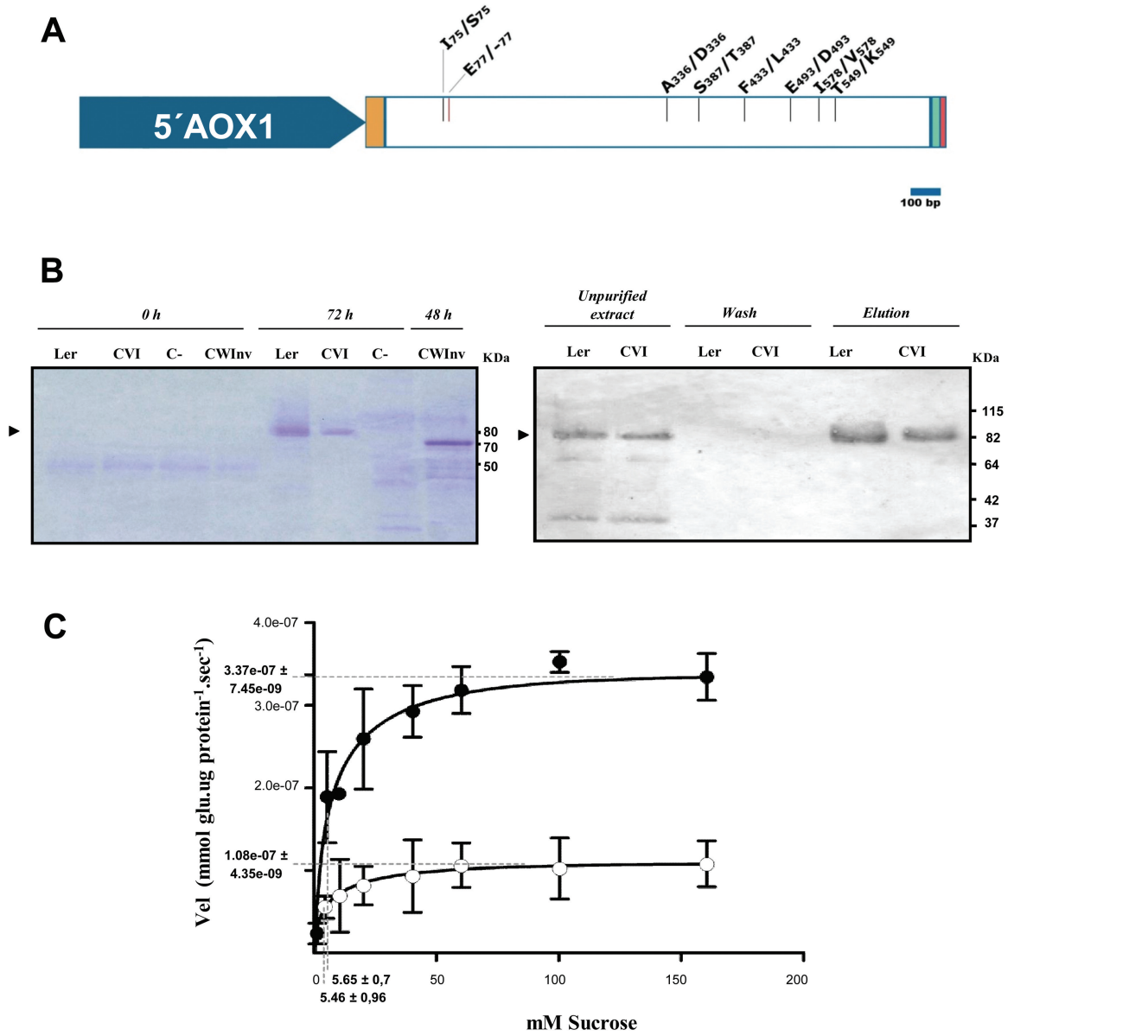

Fig. 5. Vac-Inv heterologous expression in *P. pastoris*. (A) Schematic representation of the allelic variants recombined into the *P. pastoris* (X-33 strain) genome. Amino acid polymorphisms between the two variants are shown. Yeast secretion signal peptide is depicted as a light gray box at the N-terminal, and the c-*myc* epitope and His-tag are depicted as light and dark gray boxes, respectively, at the C-terminal. (B) Coomassie blue staining of an SDS–PAGE gel (left) of purified protein extracts from the supernatant of recombinant yeasts expressing the two vac-Inv variants (CVI and Ler) after 72 h of induction in methanol-containing medium and western blot analysis (right), hybridized with an anti-Myc antibody before and after the purification steps. (C) Michaelis–Menten kinetics of the vac-Inv enzyme measured in the purified extracts shown in (B). Filled circles correspond to Ler and open circles to CVI. Ler, Landsberg *erecta*; CVI, Cape Verde Islands; C–, negative control; CW-inv: cell wall invertase. Measurement data were analyzed with the Graph Path Prism software. (This figure is available in colour at *JXB* online.)

In order to understand the cause of the different effect on vac-Inv activity between Ler and CVI accessions, we tried to express and purify two vac-Inv inhibitors (Inv-inh1 and Inv-inh2) from the parental lines in different systems including bacteria (*E. coli*), yeast (*P. pastoris*), and plants (*Nicotiana benthamiana*) with the aim of performing *in vitro* assays; however, we were not successful (data not shown). Tauzin *et al.* (2014) reported that heterologous expression of *Solanum lycopersicum* vacuolar inhibitor (SolyVIF) in *P. pastoris* was not successful and, when expressed in *Rosetta gami*, peptide was found in inclusion bodies and recovery was very low. Next, we explored the hypothesis relating transcriptional regulation of the inhibitor to the control of acid Inv activity posited by Brummell *et al.* (2011) and supported by experiments recently reported by Su *et al.* (2015). qPCR experiments on radicles and aerial organs from 7- day-old seedlings of the four genotypes showed that mRNA levels of the two vac-Inv inhibitors were significantly lower in the CVI genotype than in the Ler genotype (Fig. 6B). With the exception of inh2 in radicles, mRNA levels in the NILs were more similar to those found in the CVI genotype. These results support the idea that post-translational regulation of vac-Inv is related to the amount of Inv inhibitor mRNAs present in

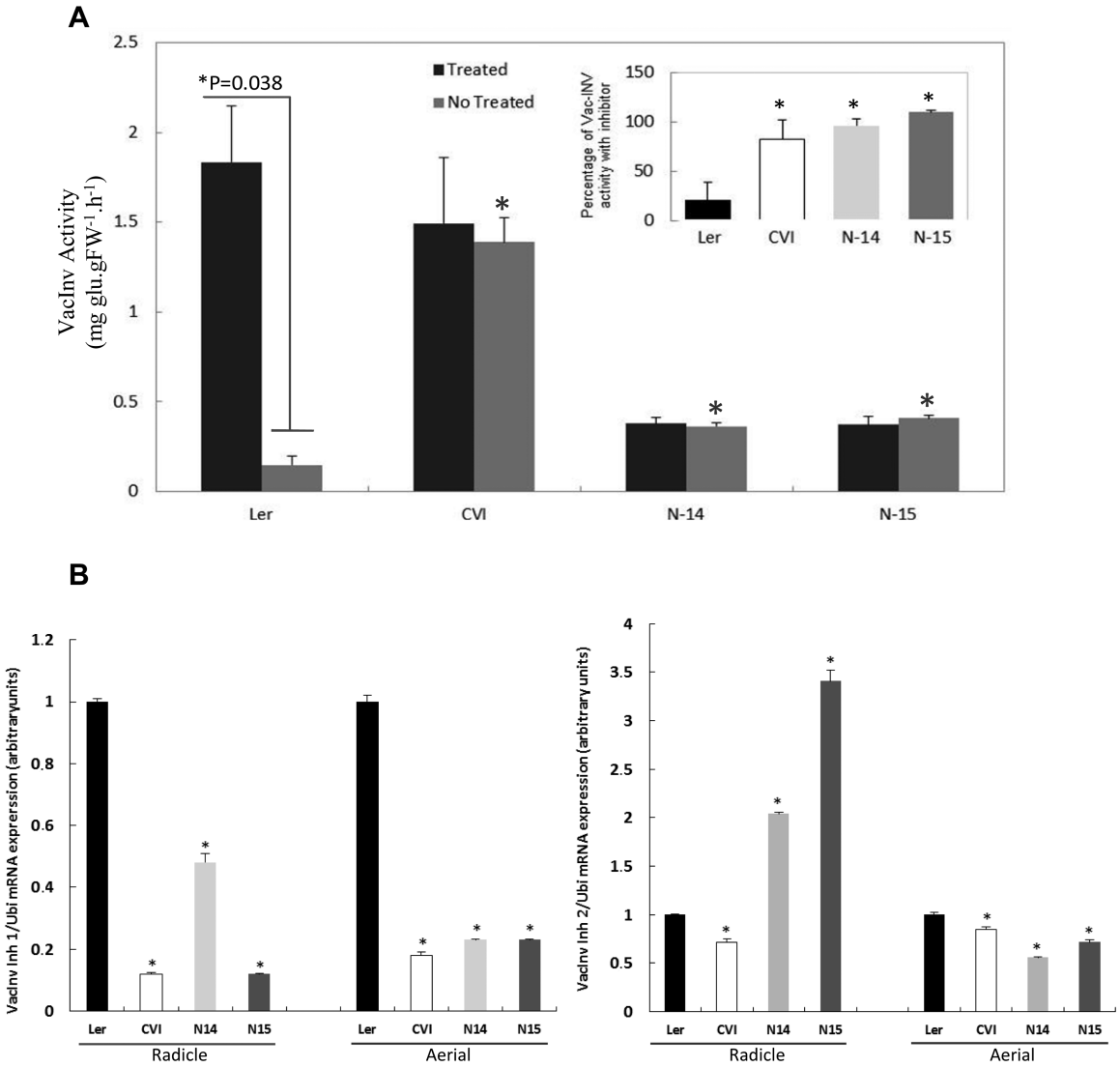

Fig. 6. (A) Vac-Inv enzyme activity measured in treated (where the activity of the inhibitor was minimized by foaming as described by Pressey, 1966) and not treated, 7-day-old extracts of Arabidopsis seedlings. An asterisk denotes a statistically significant difference by comparing every genotype against the Ler accession (*t*-test $P<0.05$). Vac-Inv enzyme activity calculated as a percentage of the total activity (without inhibitor) relative to the activity measured in crude extracts is shown in the inset graph. (B) Vac-Inv inhibitors (inhibitor 1, *At1g47960*; and inhibitor 2, *At5g64620*) mRNA abundance in radicles and aerial organs of 7-day-old seedlings from NILs and parental lines measured by qRT-PCR. Accessions: Ler, Landsberg *erecta*; CVI, Cape Verde Islands; NIL-14 and NIL-15 correspond to near isogenic lines harboring 1.8 Mb and 1.1 Mb CVI introgressions into the Ler genetic background. Bars indicate averages ±SE from two independent experiments. An asterisk denotes a statistically significant difference in comparison with the Ler accession (*t*-test $P<0.05$).

each Arabidopsis accession seedling and suggest a pivotal role for this later in the control of biomass accumulation. This is not surprising as it is well known that activities of many enzymes responsible for primary metabolism are regulated at the post-translational level (Friso and van Wijk, 2015) as shown for cw-Inv in tomato (Jin *et al.*, 2009; Ruan *et al.*, 2009). Along these same lines, Husain *et al.* (2001) reported that the different Inv activity found between red and green tomato fruits is not related to changes in Inv protein as large amounts of it were found in both tissues. They postulate that the increase in activity during fruit ripening is therefore due to post-translational regulation of Inv activity probably by alterations in the amount of the Inv inhibitor protein as described for tomato and other species (Goetz and Roitsch, 1997; Greiner *et al.*, 1998; Jin *et al.*, 2009; Ruan *et al.*, 2009).

Discussion

The main aim of this study was to understand the molecular mechanism(s) behind one of the major QTLs for root biomass accumulation reported in Arabidopsis (Sergeeva *et al.*, 2006). Analyses of a NIL population derived from a cross between the Ler and CVI Arabidopsis ecotypes revealed that this QTL co-localized with acid Inv activity. Our analyses focused on the two parental genotypes and two of the NILs of the population (NIL-14 and NIL-15) harboring single CVI genomic segments in the Ler genetic background spanning the *At1g12240* locus, which is one of the two known paralogs encoding different isoforms of the vac-Inv enzyme (Haouazine-Takvorian *et al.*, 1997).

Seven-day-old seedlings from genotypes harboring the *At1g12240*-CVI allele have longer roots than those observed

in the Ler genotype. Further, this difference is more probably related to post-germinative growth events (mostly radical growth rates) than to differences in the dormancy exit of the seeds. While the onset of germination is under temperature and humidity control, the root growth parameters evaluated here are controlled by multiple environmental conditions, including water, light, or temperature, and, at the same time, it is well known that soluble sugars can act as signaling molecules during early post-germinative events (Mitsuhashi *et al.*, 2004).

Sequence analyses of the *At1g12240* genomic clone revealed a number of SNPs and InDels between Ler and CVI alleles and confirmed that the two NILs harbor the CVI allele. Most of these polymorphisms were found in the intronic regions, but a number of amino acid changes were also detected. However, they were neither inside conserved regions nor in the reported catalytic sites of this enzyme (Fridman *et al.*, 2004; Le Roy *et al.*, 2007). Although this is somewhat expected in cases where enzymes have high functional requirements like Invs, we cannot rule out the possibility that the detected SAS can affect the enzymatic activity as described in earlier studies of variation at Inv loci in other species (Fridman *et al.*, 2004; Le Roy *et al.*, 2007). In contrast to cw-Inv, whose activity can be regulated at both the transcriptional and post-transcriptional levels (Ruan *et al.*, 2009; Su *et al.*, 2015), our results showed that for the vacuolar isoform (at least the two allelic variants assayed in this work), the observed differences in enzymatic activity are more related to post-translational regulation and that this is linked to a regulation of its inhibitor(s) at the mRNA levels. This is supported by the fact that no major differences between Ler and CVI alleles were found in the transcript level for *At1g12240*, the vac-Inv protein level, or the apparent K_m values of the recombinant protein *in vitro*. There were differences between the maximum activity of CVI and Ler forms, but the allele effect differed from assays conducted in crude plant extracts, where the CVI form showed higher activity than the Ler form, and assays of purified recombinant proteins, where the CVI form showed lower activity than the Ler form. The use of a foaming treatment, that releases inhibitor protein(s) from Inv, indicated that the two forms differ in the way they may be inhibited by Inv inhibitor protein. This treatment had almost no effect on the CVI form, both in CVI and in the NIL-14 and NIL-15 introgressions, but led to a very large stimulation of activity of the Ler form.

Two vac-Inv inhibitors are encoded in the Arabidopsis genome; Inv-Inh1 and Inv-Inh2 (*At1g47960* and *At5g64620,* respectively). They show 29% similarity (Link *et al.*, 2004) and possess apoplastic and vacuolar localization, respectively, which implies that each one may play a specific role in the plant. Lack of differences in the amino acid sequences of both inhibitors between Ler and CVI genotypes (Supplementary Fig. S2) suggests that any possible change in the interactions would be by the concentration of the inhibitors and/or by the enzyme protein structure. This is also supported by the observation that activity was not increased by the foaming treatment in NIL-14 and NIL-15, in which the CVI form is introgressed into a Ler background.

Mapping of the observed SAS to the crystal structure of the Arabidopsis cell wall INV1 in complex with a protein inhibitor from tobacco (Hothorn *et al.*, 2010) showed that five (out of eight) of these SAS are conservative and positioned on the surface of the protein opposite to the substrate- and inhibitor-binding sites and thus are unlikely to modify the enzyme activity parameters, unless this happens by long-distance conformational changes. Two other SAS (I/S^{75} and E/–^{77}) mapped near the INV1 N-terminal extension, whose function is to anchor the protein to the membrane before it solubilizes in the vacuole. Although more work is needed to understand the role played by these two changes, the facts that different maximum rates (V_{max}) were observed and the differential effect of the protein inhibitor, without a significant change in apparent K_m *in vitro*, suggest that the N-terminal region of the mature protein might acts as an allosteric modulator of vac-Inv activity, that behaves like a classical non-competitive inhibitor, and also modulates Inv protein–inhibitor interaction.

Brummell *et al.* (2011) found that during cold storage in potato, two spliced isoforms of Inv-Inh2, INH2α and the hybrid INH2β mRNA, accumulate to higher abundance in cultivars resistant to cold-induced sweetening than in susceptible cultivars, and suggested that this may contribute to the suppression of acid Inv activity and prevent cleavage of sucrose as a way of aiding adaptation. Parental genotypes used here are quite divergent and originally from very different ecological regions (Ler-0 is frequently listed as originating from the La-0 accession of Landsberg, Germany and CVI from Cape Verde Island, insular Africa). Thus, Brummell *et al.*'s hypothesis of vac-Inv inhibitors as effectors of adaptative traits is attractive and could be extended to Arabidopsis supported by evidence presented here.

Another line of evidence that SAS I/S^{75} and deletion E/–^{77} are functionally relevant in determining the levels of enzyme activity is the significant association found between the haplotypes defined by these two substitutions and the acid-soluble Inv activity measured in adult rosettes from 79 Arabidopsis accessions (Supplementary Fig. S1B). In this regard, Sulpice *et al.* (2010) reported that 57% of the variation in acid Inv activity in rosettes from adult plants in a collection of 129 different Arabidopsis accessions is explained by the genetic variation, representing the highest genetic contribution in the 39 enzymes measured in this study (Sulpice *et al.*, 2010). A more recent report by Le Roy *et al.* (2013) has shown that two amino acid residues are sufficient to convert a tobacco cw-Inv into an inactive form (Nin88), highlighting the importance of few SAS in determining the enzyme specificity.

In order to study whether the phenotypic differences observed in seedlings are maintained throughout development, we made a comparative analysis of the biomass accumulated in adult plants. As far as radical biomass is concerned, the introgressed lines did not show significant differences from that recorded in the Ler genotype. However, the presence of the *At1g12240*-CVI allele correlated negatively with the biomass accumulation in inflorescences and with the aerial to root ratio, but neither mRNA accumulation nor enzymatic activity levels showed patterns associated with the presence

of the CVI allele in rosettes and roots assayed at different phenological stages of adult plants (data not shown). These results, together with the lack of correlation between the vac-Inv enzyme activity and the *At1g12240* transcript levels found in seedlings of the whole NIL population (Keurentjes *et al*., 2008), allow to postulate that whilst AT1G12240 vac-Inv plays an important role during early stages of development, in adult plants its role is more related to carbon partitioning (or signaling) than to providing respiratory substrate to support growth. Earlier experiments with vac-Inv iRNA carrot plants sustain this hypothesis (Tang *et al.*, 1999), and several other studies also proposed a link between vac-Inv activity and cell wall signaling mediated by a wall-associated receptor-like kinase (Kohorn *et al.*, 2006). Moreover, Arabidopsis normal root growth has been related to interactions between an alkaline/neutral Inv and phosphatidylinositol monophosphate 5-kinase (PIP5K), a component of phosphatidylinositol signaling pathways (Lou *et al.*, 2007).

In summary, data presented here show that the link between the vac-Inv activity and biomass accumulation in Arabidopsis seedlings suggested by the QTL analysis reported by Sergeeva *et al.* (2006) cannot be explained either at the transcriptional or at the translational regulation levels of the *At1g12240* gene, along with the fact that no differences were found in the kinetic values of the recombinant proteins. Instead, our results suggest that differential interactions between the Inv alleles related to differential expression levels of the inhibitor-encoding genes cause the observed phenotypes. Although more experimentation is needed to understand the precise role of these interactions completely, data presented here aid in the understanding of carbon metabolism of two of the most used genotypes of the plant model species *A. thaliana* and indicate the care that must be taken when analyzing the genetic basis of a given trait by QTL mapping with biparental populations.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Alignment of Ler and CVI vac-Inv protein.

Figure S2. Alignment between Ler and CVI vac-Inv inhibitors *At1g47960* (Inh1) and *At5g64620* (Inh2).

Table S1. Comparison between the no synonymous substitutions, the synonymous substitutions, and the amino acid identity of the coding regions of Ler and CVI ecotypes from *Arabidopsis thaliana.*

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