Vacuolar invertase regulates elongation of *Arabidopsis thaliana* roots as revealed by QTL and mutant analysis

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The possible role of the sucrose-splitting enzymes sucrose synthase and invertase in elongating roots and hypocotyls of *Arabidopsis* was tested by using a combination of histochemical methods and quantitative trait locus (QTL) analysis. Lengths of roots and hypocotyls correlated better with invertase activities than with sucrose synthase activities. The highest correlations were observed with activities in the elongating zones of roots. The genetic basis of these correlations was studied by using QTL analysis. Several loci, affecting invertase activity, colocated with loci that had an effect on root or hypocotyl length. Further fine mapping of a major locus for root length, but not for hypocotyl length (top chromosome 1), consistently showed colocation with the locus for invertase activity containing a gene coding for a vacuolar invertase. The analysis of a functional knockout line confirmed the role of this invertase in root elongation, whereas other invertase genes might play a role in hypocotyl elongation. Thus, we show the power of QTL analysis, combined for morphological and biochemical traits, followed by fine-mapping and mutant analysis, in unraveling the function of genes and their role in growth and development.

Arabidopsis natural variation | sucrose synthase | hypocotyls

Total plant yield depends on the acquisition of raw material, i.e., photosynthesis and mineral (plus water) uptake, and on the ability of the plant to cope with stress. However, the economic yield of a crop is to a large extent also determined by the partitioning of dry matter over the harvestable and nonharvestable parts of the plant. The molecular and physiological basis of the regulation of assimilate partitioning in plants is still poorly understood. In terms of biomass, the most important components in assimilate partitioning and in total yield are carbohydrates. There is increasing evidence that a limited number of key enzymes, involved in primary (carbohydrate) metabolism, might be pivotal in this process (1).

Functionally, a plant can be divided into sources (the sites of assimilate production) and sinks (the sites of use and/or storage). Sinks can either be rapidly growing, expanding organs, such as elongating stems and roots, or storage sinks accumulating reserves, such as fruits, seeds, or tubers (2).

In most plant species, carbon is transported from source to sink in the form of the disaccharide sucrose. Upon arrival in the sink, sucrose has to be hydrolyzed. In plants, two pathways are available for sucrose cleaving: via invertase (Inv), yielding glucose and fructose, and via sucrose synthase (Susy), yielding fructose and UDP-glucose. In several cases, it has been suggested that sink strength might depend on the activities of these sucrose-splitting enzymes. There is increasing evidence that in storage sinks, the predominant pathway is via Susy, whereas in growing sinks, the Inv route is most important. In potatoes, the elongating rhizomes (stolons) exhibit high Inv activity, whereas a switch toward Susy-catalyzed sucrose breakdown occurs upon tuber formation, which is accompanied by starch accumulation (3–6). In lentil pods, Inv activity was associated with pod elongation, whereas the pattern of Susy activity highly paralleled the phase of rapid seed filling (7). However, in some studies these correlations were not clear. A maize mutant, which lacks Inv activity in the primary root and has normal root growth, has been described, casting doubt on the requirement of Inv for root elongation (8). The relative usage of Inv or Susy pathways might also be due to energetic reason, because Susy requires less ATP to breakdown sucrose as compared to Inv, in combination with hexokinase (9).

When trying to establish a possible role for these enzymes in the regulation of sink activities, several problems may arise, including: (*i*) all plant species tested so far have multiple genes for both Susy and Inv (10–12), and it is unlikely that they are all involved in regulation of sink strength in different organs, and (*ii*) expression of these genes may have profound organ-specific patterns and may vary during plant and/or organ development (5, 10, 12, 13).

To overcome these problems, we have chosen a quantitative trait locus (QTL) approach to unravel loci/genes involved in organ and tissue-specific activities of key enzymes, supposedly involved in source–sink interactions. QTL analysis uses naturally occurring within-species allelic variation and is especially suited to study polygenic traits (14). It has been used to find loci involved in regulation of enzyme activities: Inv, phosphoglucomutase, and other enzymes in *Arabidopsis* and maize (15–17).

QTLs for Inv have been studied in tomato (18, 19) and potato (20), where genetic colocation or heterologous complementation indicate that Inv polymorphisms are responsible for sugar content/composition.

As a model system, *Arabidopsis* seedlings were chosen. In seedlings, hypocotyl, root, and shoot apex are the main sinks, assimilates being supplied by the cotyledons. Using the available Landsberg *erecta* (L*er*)/Cape Verde Islands (Cvi) recombinant inbred line (RIL) population, Borevitz *et al.* (21) detected QTLs for hypocotyl length (HL), suggesting natural variation for this trait to be present in this population. We decided to combine QTL analysis of HL and root length (RL) with a study of tissue-specific activities of Inv and Susy by using a histochemical technique described in refs. 17 and 22. Because hypocotyls and roots of seedlings are growing rather than storing organs, it was expected to find colocation of one or more QTLs for Inv with QTLs for elongation. Some papers suggest a negative correlation between Susy and Inv activities (3–5), and, therefore, colocation between QTLs for Susy and for elongation might also be observed but with opposite allelic effects.

Conflict of interest statement: No conflicts declared.

Abbreviations: CW-Inv, cell-wall bound invertase; HL, hypocotyl length; Inv, invertase; QTL, quantitative trait locus; RIL, recombinant inbred line; RL, root length; Susy, sucrose synthase; Vac-Inv, vacuolar invertase.

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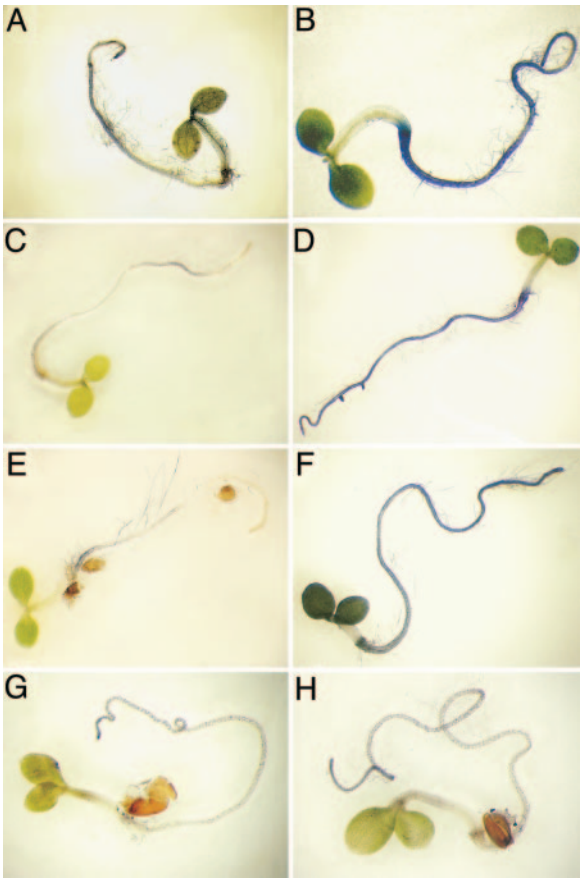

Fig. 1. Histochemical staining for Inv (*A–F*) and Susy (*G* and *H*) enzyme activities in 7-day-old *Arabidopsis* seedlings. Blue color indicates activity. (*A*) Inv, L*er*. (*B*) Inv, Cvi. (*C*) Inv, NIL147-4. (*D*) Inv, NIL15-3. (*E*) Inv, KO (SALK_100813). (F) Inv, Col. (*G*) Susy, L*er*. (*H*) Susy, Cvi.

The observed colocation of a major QTL for RL with a QTL for vacuolar invertase (Vac-Inv) activity and further fine mapping and analysis of an Inv knockout line confirmed the role of this enzyme in determining sink strength in roots. This approach highlights the potential of QTL analysis to unravel relations between multiple quantitative traits and, thereby, the function of genes.

Results

Visualization and Localization of Invertase and Sucrose Synthase Activities. Fig. 1 shows the results of histochemical staining for Inv and Susy activities in intact seedlings of two *Arabidopsis* accessions, namely, L*er* and Cvi. For Inv, the two accessions showed a different staining pattern, Cvi having more intense staining in the roots. For Susy, the staining patterns were largely similar; the highest intensities for both accessions were observed in the root tip. Control incubations, lacking the substrate sucrose, did not stain either for Inv or for Susy.

Enzyme Activities and Organ Lengths in Seedlings of RIL Population. The L*er*/CviRIL population was used to determine quantitative data for a series of traits related to sucrose metabolism and RL and HL for subsequent QTL analysis. Activities of acid Inv, both cell wall-bound and Vac-Inv (CW-Inv) forms, were determined in whole-seedling extracts. Susy activity was not or hardly detectable in extracts, probably due to low activities in the seedlings. Frequency distributions of Inv activities in the RIL population are given in Fig. 2 *A* and *B*. For both Vac-Inv and CW-Inv, a wide range of activities was observed in the RIL population, with transgression beyond the parental values. Fig. 2 *C* and *D* shows the distribution of RL and HL of 7-day-old seedlings of the lines of the RIL population. Also for these traits, a large variation and transgression were observed.

Plotting HL against RL showed a slight, but significant ($P < 0.05$), negative correlation (Fig. 3), indicating a certain degree of competition for a limited pool of assimilates and/or other nutrients between roots and hypocotyls as sinks.

If the hypothesis that activities of sucrose metabolizing enzymes are related to sink strength is correct, a significant correlation would be expected between Inv or Susy and RL or HL. Across the RIL population, HL showed a significant positive correlation with the activity of Vac-Inv but not with CW-Inv (Table 1).

To test whether activities in specific organs might be related to elongation, the activities of Inv and Susy, as revealed by histochemical staining, were semiquantified as described by Sergeeva *et al.* (17). It should be noted that this method does not discriminate between Vac-Inv and CW-Inv. Significant positive correlations were observed between HL and Inv activities in the cotyledons and in the upper part of the hypocotyl. However, Inv activity in the lower part of the hypocotyl did not correlate with HL and neither did the Inv activities measured in any part of the root.

RL showed positive correlations with Inv staining in roots, especially in the lower parts of the roots, but not with staining in hypocotyls (Table 1).

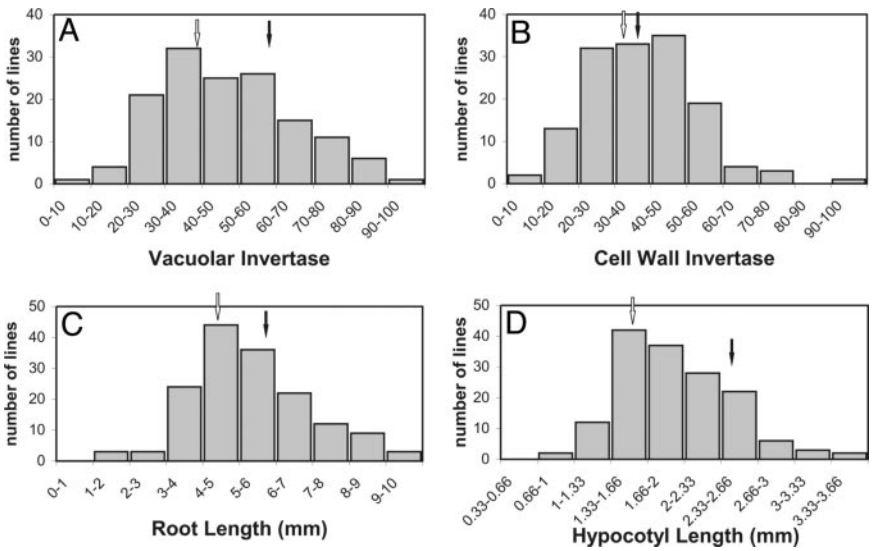

Fig. 2. Frequency distributions of vacuolar and cell wall Inv (*A* and *B*) and lengths of root and hypocotyls (*C* and *D*) in seedlings of the L*er*/Cvi RIL population. Inv activities were determined in extracts and are expressed as milligrams of glucose formed per gram of dry weight per hour. Arrows, parental values; open arrows, L*er*; filled arrows, Cvi.

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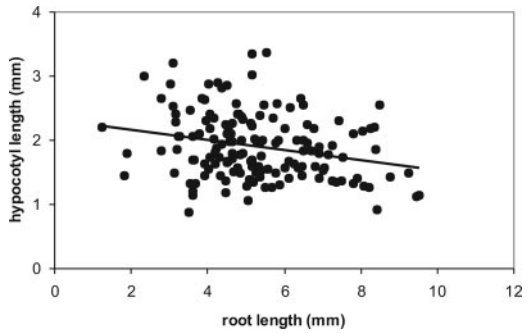

Fig. 3. Correlation of lengths of roots and hypocotyls of 7-day-old seedlings of the Ler/CviRIL population. Each dot represents one line of the RIL population.

Similarly, we tested possible correlations between Susy activities in various parts of seedlings and RL and HL across the RIL population. As shown in Table 1, only one significant, but negative, correlation was observed, namely between Susy activity in the root tip and the HL.

Cell division and cell elongation occur predominantly in the lower part of the root and in the upper part of the hypocotyl. Thus, these findings at least suggest a positive relationship between Inv activity and cell division or elongation. For Susy, such a relationship is much less obvious. As a control, we also checked possible correlations between organ lengths and the activity of phosphoglucomutase (PGM), an enzyme that is not supposed to be involved in elongation. No significant correlations were found (Table 1).

QTL Mapping of Enzyme Activities and Organ Lengths. From the correlation studies, it appears that Inv is more likely to be involved in regulating elongation than Susy. However, it is also clear that Inv, even when analyzed at the organ level, explains only part of the variation observed for elongation. Besides technical and biological variation, the reason might be the presence of isoforms of the enzyme, of which only one or a few might be involved in elongation. Therefore, we performed QTL analysis for tissue-specific Inv and Susy activities and for RL and HL, expecting that some, but not all, QTLs for Inv activity would colocate with QTLs for length and that the Susy QTLs would not colocate.

QTLs detected for RL and HL are listed in Table 2, which is published as supporting information on the PNAS web site, and summarized in Fig. 4. For HL, seven significant loci were found. In total, these loci explained 64.3% of the observed variation. L*er* mainly contributed negative alleles, except for the QTL on chromosome 3, explaining the overall shorter hypocotyls of L*er* seedlings. For RL, four significant loci with a total explained variance of 50.1% were identified. Also for roots, L*er* conferred mostly negative alleles, consistent with the shorter roots of L*er* seedlings. QTLs for RL and HL were found at different positions with one exception, namely at the bottom of chromosome 5.

Using the same RIL population, QTLs were detected for Inv activities, both in whole-seedling extracts and by histochemical staining of seedlings and subsequent quantification in different organs (details in Tables 3 and 4, which are published as supporting information on the PNAS web site).

As summarized in Fig. 4, many significant QTLs could be detected for organ-specific Inv activity, e.g., at the bottom of chromosome 2, QTLs were detected for activities in the shoot and root neck but not in the rest of the root. For all traits, more than one QTL was detected, and many small-effect QTLs were present. At the top of chromosome 1, ≈15 cM, QTLs were found for RL, Vac-Inv activity, and Inv activity in several organs, all with the same allelic effect. At this position, no QTLs were found for HL and neither for CW-Inv activity. At the lower end of chromosome 1, ≈90–100 cM, QTLs were found for HL, but not for RL, and for Inv activities in all organs and for Vac-Inv activity in extracts. All these QTLs showed the same allelic affect. At this position, no QTL for CW-Inv was detected. The two regions on chromosome 1 containing QTLs for various traits colocate with two known Vac-Inv genes, namely, At1g12240 and At1g62660.

Around the *ERECTA* locus (chromosome 2, 48 cM), QTLs were found for HL, but not for RL, and for Inv staining in hypocotyl and various parts of the roots. At the bottom of chromosome 5, QTLs were detected for RL and HL, with opposite effects, and also for Vac-Inv activity in extracts. This region might be more complicated, because the staining data indicate two closely linked loci affecting Inv activity in the roots, with opposite allelic effects. At the other chromosomes, various QTLs were observed but without a clear colocation for the various traits.

We also detected QTLs for organ-specific Susy activities. From >10 loci detected affecting Susy activity, only one colocated with a QTL for HL and none with RL (data not shown).

Fine Mapping and Candidate Gene Approach By Using Near Isogenic Lines (NILs) and a Knockout (KO) Mutant. At the top of chromosome 1, a major QTL for RL was detected, explaining 20.2% of the variance. The region also contained QTLs for Inv activities (Fig. 4) and a gene encoding a Vac-Inv (At1g12240) (23) is located between 4,154 and 4,158 kb in this area. Two partly overlapping NILs, each containing only one small, but different, Cvi-introgression in L*er* background, were used to confirm and fine map this QTL. NIL147-4 does not contain the Cvi allele of this Inv gene, whereas the NIL15-3 does (details in Fig. 5*A*). Because this Inv gene was an obvious candidate for the observed QTL for Inv activities, we also included a KO line for this gene (SALK_100813) and its corresponding wild type (Col) in the analysis.

Roots of seedlings of the NIL containing the Cvi allele of the Vac-Inv gene (NIL15-3) were significantly longer than those of L*er* and NIL147-4, confirming the observed QTL and showing that the underlying gene is located between 3,528 and 4,174 kb on chromosome 1. This region indeed contains the Inv gene (At1g12240). Furthermore, the line in which this gene was functionally knocked out had significantly shorter roots than the wild type (Fig. 5*C*). For HL, no differences were found between L*er* and the NILs, whereas Cvi was significantly longer (Fig. 5*B*), consistent with the absence

Table 1. Correlations between lengths of roots or hypocotyls and invertase or sucrose synthase activities

Staining	Cotyledon	Hypocotyl upper	Hypocotyl lower	Root neck	Root upper	Root middle	Root lower	Root tip	Extracts of whole seedlings		
									Vac-Inv	CW-Inv	PGM
Invertase											
Hypocotyl length	0.182*	0.197*	0.097	0.099	0.04	−0.011	−0.022	−0.086	0.314**	0.143	−0.132
Root length	−0.055	−0.019	0.013	0.038	0.068	0.190*	0.24**	0.253**	0.074	−0.080	−0.056
Sucrose synthase											
Hypocotyl length	0.138	0.044	0.085	−0.163	−0.130	−0.136	−0.175	−0.304**			
Root length	−0.179	−0.129	−0.173	−0.073	−0.062	−0.128	−0.130	0.078			

Numbers denote Spearman correlations. *, significant at $P < 0.05$. **, significant at $P < 0.01$.

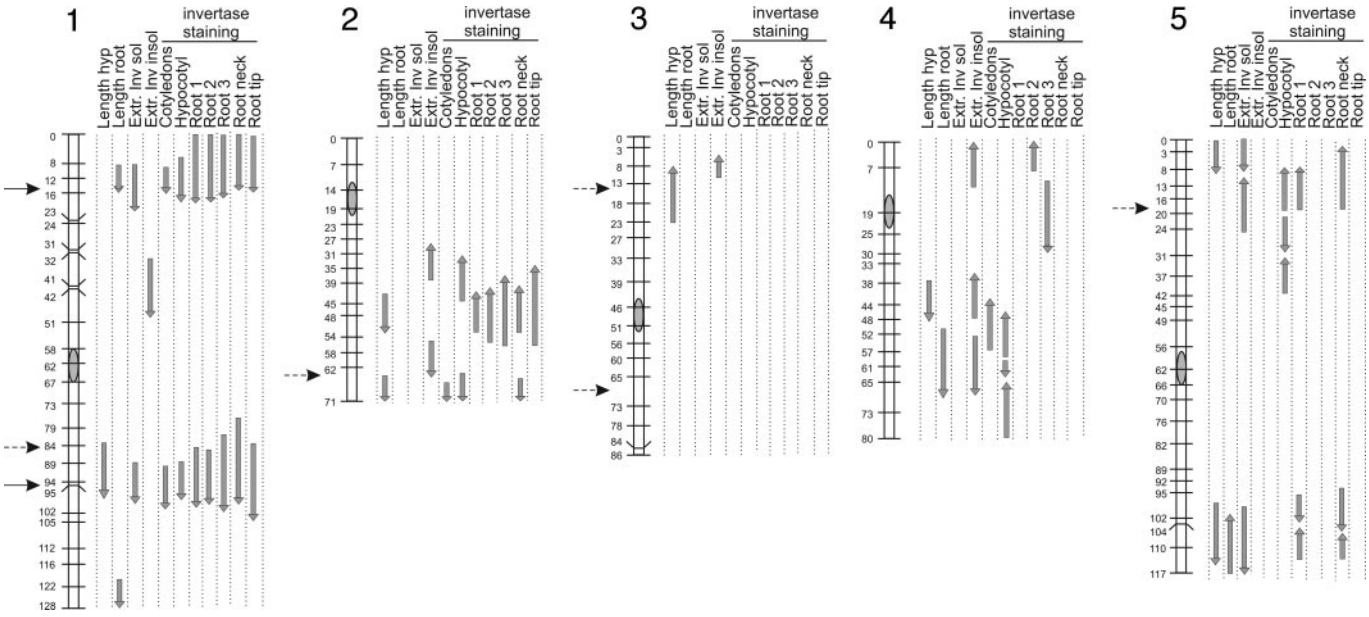

Fig. 4. Combined genetic map (chromosomes 1–5) showing QTLs for lengths of roots and hypocotyls, Inv activities in extracts, and Inv activities as calculated from histochemical assays for various organs of the seedlings. Lengths of the arrows indicate the two-logarithm of odds intervals, and directions of the arrows indicate the allelic effects: upward, L*er* alleles increase; downward, Cvi alleles increase trait values. Horizontal arrows: approximate positions of annotated Inv genes; solid arrows, vacuolar Inv genes; dashed arrows, cell-wall Inv genes.

of a QTL for HL at this position. HL of the KO line did not significantly differ from the wild type (Fig. 5*B*).

Fig. 5 *D* and *E* shows Inv activities in extracts of seedlings of the NILs, parents, and KO. For Vac-Inv, Cvi exhibited higher activities than L*er*. The activity in NIL147-4 was similar to that of L*er*, and significantly lower than in NIL15-3 and Cvi. Comparing the KO with its parental line revealed a 50% reduction in Vac-Inv activity. For CW-Inv, no significant differences were

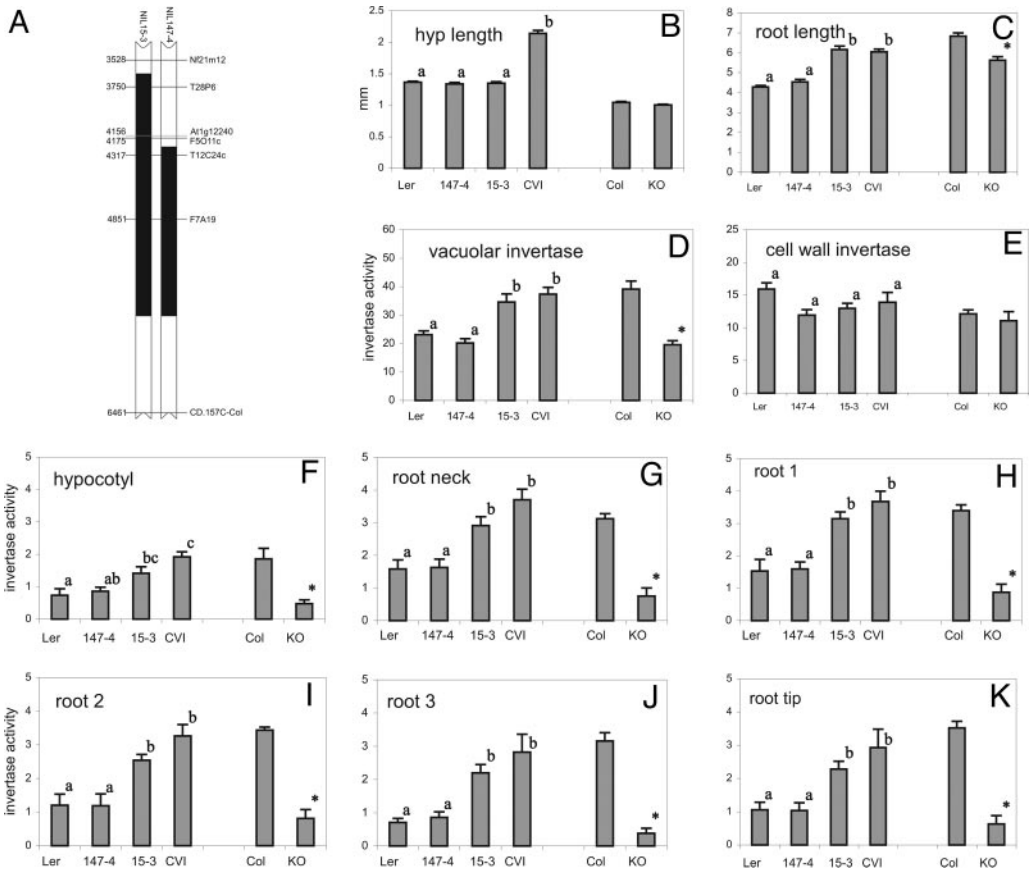

Fig. 5. Genotype of NILs (*A*), lengths of roots and hypocotyls (*B* and *C*), Inv activities in extracts (*D* and *E*), and activities of Inv in various organs as quantified from histochemical staining (*F–K*). Bars indicate average values ± SE. For NILs and parents (four left bars), bars labeled with the same letter are not significantly different. For KO, significant differences with Col are indicated with an asterisk. Inv activities are expressed as mg of glucose formed per g of dry weight per hour (*B* and *C*) or on a relative scale (*F–K*).

observed, neither comparing the NILs and parents nor between Col and the KO.

We also determined Inv activities in various organs of these lines. The general tendency was that activities in L*er* and NIL147-4 were lower than those in NIL15-3 and Cvi (Fig. 5 *F–K*), except for the activity in the hypocotyls, for which the values did not significantly differ between the two NILs (Fig. 5*F*). The relative differences between NIL147-4 and NIL15-3 increased from 1.6 to ≈2.5 from the hypocotyl toward the lower parts of the roots, suggesting that allelic differences of the Inv gene mainly influenced Inv activities in the lower parts of the roots. In all organs, including the hypocotyl, the activity in the KO was significantly lower than in Col.

Discussion

Invertase vs. Sucrose Synthase. Inv and Susy, the two types of enzymes capable of splitting sucrose in higher plants, have both been suggested to be involved in sink strength. However, they might play a role in different types of sinks being either consuming or accumulating sinks (1).

Hypocotyls and roots of *Arabidopsis* seedlings, as analyzed in the present study, represent growing, consuming sinks. In these seedlings, relatively high activities of Inv could be detected, whereas Susy activity was barely detectable. Moreover, we did not observe significant correlations between organ-specific activities of Susy and length of these organs, whereas such correlations were observed for Inv, both in hypocotyls and in roots (Table 1). These data support a role for Inv in consuming sinks. By contrast, Susy activities in *Arabidopsis* have been linked to phloem loading (*AtSUS1*) (24) and to the accumulation of triglycerides and proteins in seeds, i.e., in storage sinks (*AtSUS2*) (10). We observed an unexpected negative correlation between HL and Susy activity in the root tip. The physiological relevance, if any, of this correlation is not clear.

A Role for Vacuolar or Cell Wall Invertase in Controlling Elongation? Three different forms of invertases have been described in plants: acid soluble, located in the vacuole; acid insoluble or cell wall-bound, present in the extracellular space; and neutral soluble, presumed to be present in the cytoplasm (25). The role of the latter one is largely unknown, but the two acid isoforms have both been implicated in the control of sink strength. CW-Inv has been described as important for phloem unloading, maintaining a sucrose gradient between sieve element and apoplast. Vac-Inv is important for osmotic purposes and to supply energy. In *Daucus carota*, antisense suppression of CW-Inv or Vac-Inv resulted in altered source-sink ratios and carbohydrate levels, although the effects were more pronounced in antisense CW-Inv plants (11). In tomato fruits, a QTL for total soluble solids turned out to be a CW-Inv (18). In potato, a cold-sweetening QTL was due to an Inv gene, that, based on homology, was classified as cell wall-bound (20).

Our data suggest a role for Vac-Inv in organ elongation: A positive correlation was observed between Vac-Inv activity in extracts and HL (Table 1), a QTL for HL colocated with QTLs for Vac-Inv activity in extracts (lower arm chromosome 1, top chromosome 5), and the major QTL for RL on chromosome 1 coincided with an annotated Vac-Inv gene. The phenotype of the KO for this gene confirms the role of Vac-Inv in RL. A role for Vac-Inv has also been proposed for other elongating plant organs (2, 26).

Much less evidence was obtained for a role of CW-Inv in controlling RL: only at the lower part of chromosome 4, colocation between QTLs for RL and CW-Inv in extracts, with similar allelic effect, was observed. However, CW-Inv might play a role in HL: of six annotated CW-Inv genes, four colocated with QTLs for HL. A fifth CW-Inv has been described, located at chromosome 3, 68 cM. At this position, we observed a QTL for HL that was just below significance (logarithm of odds = 2.44, data not shown in Fig. 4). Around the sixth annotated CW-Inv gene (top chromosome 5) two QTLs were observed for Inv staining in the hypocotyl with opposite effects (Fig. 4). However, without further fine mapping and confirmation, these genes are only interesting candidates for the observed trait.

A role of CW-Inv in organ growth was suggested by the finding that the miniature-1 mutant in maize is deficient in CW-Inv (27).

Comparison with QTLs from Other Experiments. Using the same L*er*/CviRIL population, Borevitz *et al.* (21) mapped QTLs for HL in *Arabidopsis* seedlings, grown at various light conditions. In white light, they found four significant loci, of which only one on chromosome 4, ≈45 cM, colocates with a locus we identified. Some of the QTLs we found coincide with QTLs detected by Borevitz *et al.* (21) under other light conditions with the same allelic effects. The reason for this apparent discrepancy might be differences in growth conditions, either light quality or the supply of nutrients. For seedlings of the L*er* × Col population, Kobayashi and Koyama (28) reported three QTLs for RL, of which the QTL on the top of chromosome 1 might well be similar to the one we described in detail. These authors also reported a QTL around the *ERECTA* gene (chromosome 2), which we also detected.

The RL QTL identified as *BRX* (*Brevis radix*, At1g31880) in the Uk-1 × Sav population (29) and located at 11.4 Mb on chromosome 1 does not colocate with RL QTLs found in our study.

Does Invertase Play a Role in Controlling Root Elongation? Several findings in the present paper indicate that Inv activity might have a controlling role in RL in seedlings: (*i*) RL correlated positively with Inv activity, especially in the lower parts of the roots (Table 1). (*ii*) Some of the QTLs for RL colocate with QTLs for Inv activity, which colocation for the major QTL near the top of chromosome 1 could be confirmed by fine mapping to a region 789 Kb (Fig. 5). This region, although small, still contains 211 genes, among which a gene described as a Vac-Inv (23), as based on the Col sequence and annotation. (*iii*) The role of this Inv was verified by comparing Inv activity and RL in a KO line for this gene. In this KO line, Inv activity was lower and the roots were shorter (Fig. 5). Taken together, these data show that variation in the Vac-Inv (At1g12240) gene most likely also controls the variation for RL at this position.

At the lower part of chromosome 1, At1g62660 encodes for another Vac-Inv, which is highly homologous to At1g12240. Interestingly, at this position, no RL QTL was observed, but QTLs for other traits were found (Fig. 4). This finding suggests that if these genes are duplicated versions (30), they have different functions.

From the comparison of QTLs for Inv activity and RL (Fig. 4), it is evident that they do not always colocate. Thus, not all loci affecting Inv activity also affect RL, e.g., around the *ERECTA* locus significant QTLs are found for Inv staining in roots but not for RL.

Competition Between Hypocotyl and Root? In the RIL population, a significant, although small, correlation was observed between RL and HL, suggesting some level of competition for available resources. However, the correlation is relatively low, indicating that increasing the sink strength of the hypocotyl is not fully compensated for by a similar decrease in sink strength of the root and vice versa. This observation is consistent with the limited number of QTLs colocating for RL and HL and having opposite allelic effects. Only one such colocation was found on chromosome 5. The noncolocating QTLs show that elongation of the two organs is at least partly independently regulated, as confirmed by the phenotype of the KO of At1g12240 in which the Inv activity is reduced both in hypocotyls and in roots, but only elongation in roots is affected (Fig. 5).

Physiological Role of Vacuolar Invertase in Root Elongation. The highest correlation between Inv activity and RL was observed for the lower parts of the roots (Table 1), i.e., the zones in which cell division and cell elongation occur. However, Inv activity was not

confined to the very tip of the roots, so there is no evidence that it was exclusively in the cell division zone. Rather, the largest difference between the two NILs, only one of which contained the more active Cvi allele of the Inv gene, was observed in root zone 3, i.e., just above the root tip. A possible role for vac-Inv might be the hydrolysis of sucrose, yielding fructose and glucose in the vacuole, thus leading to osmotic water uptake and a subsequent increase in turgor as a driving force for elongation. Alternatively, but not excluding the former option, Inv could play a role in phloem-associated processes. When acting in parenchyma cells, vac-Inv might stimulate phloem unloading and, thus, sink strength by maintaining a steady gradient of sucrose from phloem to parenchyma cells, thus driving unloading (31).

In conclusion, the present data show that QTL analysis is a useful tool to unravel the role of genes in physiological processes; quantitative biochemical data can be linked with morphological traits, resulting in relative rapid identification of the role of a vacuolar Inv in determining elongation of roots.

Materials and Methods

Plant Material. The RIL population derived from crosses between the laboratory strain L*er* and the accession Cvi was used. These RILs have previously been genotyped with AFLP and CAPS markers (32).

Two NILs (NIL15-3 and NIL147-4), derived from a cross between L*er* and NIL45 [described as *EDI*-Cvi in Alonso-Blanco *et al.* (32) and as NIL45 in Swarup *et al.* (33)], were used. NIL15-3 and NIL147-4 contain a Cvi introgression of ≈3 and 2.5 mbp, respectively, in a L*er* background determined by selection against molecular markers (Fig. 5*A*). A line homozygous for a T-DNA insert in At1g12240 (SALK_100813) was obtained from the Salk Institute Genome Analysis Laboratory (34) and was compared with its isogenic Col0 wild type.

Growth and Test Conditions. Seedlings were grown in light (Philips TLD 50W/830 fluorescent tubes, 13 W/m^2 for 7 days as described in ref. 17). Samples were taken 4 to 6 h after the start of the light period and immediately frozen in liquid nitrogen (for enzyme extraction) or fixed (for histochemical staining and for length measurements).

Samples for the three different assays were taken from independent experiments. Because of low germination rates or poor seedling vigor, several RILs were discarded from the analyses, resulting in 142 RILs for Inv assays in extracts and 162 RILs for the histochemical staining and 138 RILs for length measurements.

For length measurements, seedlings were transferred to Petri dishes, straightened, and measured by using a stereomicroscope.

Enzyme Activities in Extracts. *Arabidopsis* seedlings were extracted according to Sergeeva *et al.* (17). Vac-Inv and CW-Inv were determined according to Appeldoorn *et al.* (4) with some modifications. Sucrose synthase activity was determined as described by Xu *et al.* (35) with some modifications. Blanks were carried out with substrate solution without sucrose. Details are listed in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Localization of Enzyme Activities. Seedlings (20–40 per line) were fixed according to Sergeeva *et al.* (17). Staining for Inv and sucrose synthase activity was as described in refs. 36 and 37, respectively, with some modifications. In control reactions, sucrose was omitted.

The seedlings were studied for staining with a binocular (Leica, Wetzlar, Germany); the intensities of staining patterns were (semi-) quantified on an arbitrary scale, ranging from 0 to 5, indicating no and very intense staining, respectively (17). Details are listed in *Supporting Materials and Methods*.

Statistical Analysis. For the RIL population, Inv assays in extracts were done once, each sample containing >50 seedlings; lengths of roots and hypocotyls, Susy, and Inv staining were determined in two independent experiments, with 15–25 seedlings per replicate for lengths and 40–50 seedlings per replicate for stainings.

Assays on NILs, knockout line, and parental lines were repeated five times for Inv activities in extracts, three times for Inv staining, with two independent replicates, and three times for measurements of lengths, also with two replicates. Statistical analysis was performed by using a Tukey test in SPSS, version 11.5.0 (SPSS, Chicago).

QTL analyses were done as described in ref. 17 and as listed in detail in *Supporting Materials and Methods*.

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