Towards understanding sucrose-mediated changes in meristem activity in *Arabidopsis thaliana*

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Carbon nutrient availability is of critical importance for plant growth and development, which in its turn is dependent on activity of the shoot apical meristem (SAM). Little is known, however, about the molecular mechanisms involved in integrating carbon nutrient availability with changes in SAM activity. Here, an optimized seedling-based assay is presented for studying these changes in the presence of sucrose and in the absence of light. Using these growth conditions, several associated genomic loci were identified though genome-wide association (GWA) mapping on natural accessions of *Arabidopsis.* In addition, Cyclin D3 (CYCD3;1), Cyclin B1 (CYCB1;1) and *Arabidopsis thaliana* homeobox gene 1 (ATH1) were characterized as genetic markers for early shoot meristem activation events. Thus, these findings provide a foundation for studying the molecular mechanisms behind sucrose-mediated changes in meristem activity in *Arabidopsis*.

Laymen's Summary (Dutch)

De beschikbaarheid van suikers, waaronder sucrose, is cruciaal voor de groei en ontwikkeling van planten, wat afhankelijk is van de activiteit van het scheut apicaal meristeem (SAM). Echter, er is momenteel weinig bekend over de mechanismen die de beschikbaarheid van sucrose detecteren en vervolgens signalen afgeven die ervoor zorgen dat het SAM geactiveerd wordt. Omdat planten van zichzelf suiker aanmaken door middel van fotosynthese, is het moeilijk om de suiker-gereguleerde groei van planten te bestuderen in het licht. In het verleden zijn er daarom verschillende groeicondities gepresenteerd die het mogelijk maken om zaailingen in het donker te laten groeien, maar hier zitten nadelen aan die het moeilijk maken om goed onderzoek te doen naar de eerdergenoemde mechanismen. In dit onderzoek is daarom een geoptimaliseerd groeisysteem geïntroduceerd om deze mechanismen effectief te bestuderen in de aanwezigheid van sucrose en afwezigheid van licht. Vervolgens zijn er met deze verbeterde groeicondities een aantal regio's in het genoom geïdentificeerd die geassocieerd zijn met veranderingen in activiteit van het SAM. Om deze regio's verder te kunnen bestuderen, is het van belang dat veranderingen in activiteit van het SAM in een zo vroeg mogelijk stadium kunnen worden waargenomen. Deze veranderingen vinden op een moleculair niveau plaats, veel eerder voordat de eerste zichtbare verandering plaats vindt. Tenslotte zijn er daarom drie zogenaamde "marker" genen geïdentificeerd om deze veranderingen te kunnen detecteren.

Introduction

Plant growth and development critically depend on carbon nutrient status. Processes that especially require significant energy input are developmental phase transitions and the initiation and outgrowth of new shoot organs. These processes are of vital importance to plant productivity and are dependent upon changes in activity and/or size of shoot meristems. Reproductive output is therefore directly linked to activity of shoot meristems since it is dependent on developmental phase transitions that lead to flowering and the development of new shoots. Because of this, activity of shoot meristems has a direct impact on yield in many crops (reviewed in Rolland *et al.,* 2006; Bartrina *et al.*, 2011; Lastdrager *et al.,* 2014).

Previous research points to the importance of carbon nutrients controlling meristem activity for plant growth, development and yield, but no systematic analyses of the mechanisms involved have been performed (Smith & Stitt, 2007; Baena-González & Sheen, 2008; Smeekens *et al.,* 2010). One of the challenges that arise in studying nutrient-sensing signaling mechanisms is that plants are photoautotrophs and are therefore able to produce sugars through photosynthesis. Feasible analysis of these mechanisms

would require controlled conditions without having the plant producing its own sugars. Although activity of the shoot apical meristem (SAM) and the consequent development of the plant normally occurs only in light grown plants, previous research has shown that dark grown seedlings were also able to grow and develop in the presence of sucrose (Araki & Komeda, 1993; Roldán *et al.,* 1999). In darkness, there is an absolute requirement for metabolic sugar availability (sucrose, glucose or fructose) at the SAM to induce its activity and maintain development. Since this dark development phenotype is responsive to low, physiologically relevant sugar levels and occurs in the absence of photosynthate (Róldan *et al.,* 1999), it provides an excellent model system for identifying and understanding the links between metabolite provision and meristem activity. Despite the advantages of such a model system, however, it is still limited for studying the mechanisms that link carbon nutrient status with meristem activity.

Among other techniques, a genome-wide association (GWA) study has been used for a multitude of phenotypes in *Arabidopsis* and other species and has proven to be an effective method for identifying the underlying genetics of traits (Atwell *et al.,* 2010; Huang *et al.*, 2011; Filiault & Maloof, 2012). A GWA study on sugar-activated SAMs therefore provides a fitting way to uncover novel signaling components that ultimately link carbon nutrient availability to changes in meristem activity (Korte & Farlow, 2013). To confirm whether potential signaling components identified through GWA mapping are actually involved in sugar-mediated changes in SAM activity, morphological and/or molecular markers are required that signal early meristem activation events resulting from activity of these components. Several genes have shown to play vital roles in various aspects of meristem function, and could therefore be potential marker genes for sucrose-induced early meristem activation events. These include meristem identity genes (WOX family, CLV family, KNOX family) (Kim *et al.,* 2007), genes involved in control of cell proliferation (cyclin-dependent kinases, kip-related proteins) (Ormenese *et al.*, 2004; Verkest *et al.*, 2005), in lateral organ initiation (ANT, ARP family) (Golz & Hudson, 2002; Zhou *et al.*, 2014; Byrne *et al.,* 2000) and axillary meristem initiation and outgrowth genes (LAS, TCP family) (Greb *et al*., 2003; Koyama *et al.*, 2007). In particular, the cyclins CYCB1;1 and CYCD3;1 are rapidly expressed in the shoot apex when seedlings are exposed to light after a 3-day dark period. Under normal conditions, light induces a developmental switch from an inactive SAM at the completion of embryogenesis and seed maturation to an active SAM, and expression of these two cyclins shortly after light exposure makes them therefore interesting candidate marker genes for meristem activation events (López-Juez *et al.,* 2008). Finally, *Arabidopsis thaliana* homeobox gene 1 (ATH1) was reported to be expressed in the shoot apex of 7-day old seedlings in the presence of sucrose, but not in the presence of sorbitol (Gómez-Mena & Sablowski, 2008). This makes ATH1 another candidate gene for marking developmental changes in the SAM.

To identify and study new signaling components that integrate carbon nutrient availability with meristem activity, an optimized model system is presented here with enhanced and more controlled growth conditions than previously reported (Araki & Komeda, 1993; Roldán *et al.,* 1999). Furthermore, a comprehensive score system is presented to effectively detect small changes in SAM development. These optimized growth conditions and this detailed score system were then used to perform a GWA study, which led to the identification of distinct genomic regions that are associated with sucrose-mediated SAM development in dark-grown seedlings. Finally, it is shown that CYCD3;1, CYCB1;1 and ATH1 are molecular markers that signal early activation events in the shoot apex. These findings contribute towards better understanding sucrose-mediated changes in meristem activity in *Arabidopsis*.

Materials and Methods

Plant material and growth conditions. Columbia-8 seeds (N60000) were used for optimizing the seedling-based meristem activity assay. 254 different accessions of *Arabidopsis thaliana* were used to conduct GWA mapping and are from the 1001 genomes project (Supplementary Table I) (Weigel & Mott, 2009). The GUS-lines studied were CYCD3;1:GUS, CYCB1;1:DB-GUS (López-Juez et al., 2008) and pATH1:GUS (Proveniers *et al.,* 2007) and are all in the Col-8 background. Seeds were chlorine gas sterilized in a desiccator for 4 h using a mixture of 4 ml 37% HCl and 100 ml commercial bleach (Chlorix). 10-20 seedlings were grown in 100 ml bottles closed with cotton wool plugs and containing 20 ml liquid medium half-strength Murashige and Skoog (MS) medium with vitamins and MES buffer (pH 5.8). Shortly before adding the seeds to the medium they were suspended in 0.1% (w/v) agarose to ease the process of adding seeds to the medium. To trigger germination, the seeds were exposed to 4 h of fluorescent light

prior to stratification after which the bottles were wrapped in aluminum foil to avoid any light exposure during germination and growth. Filter-sterilized 50% sucrose or sorbitol was added to the medium to a 1% final concentration either before sowing seeds, or, using a 1 ml syringe with a needle attached, after 0-13 d of growth by penetrating the needle through the cotton wool plugs. Sown seeds were stratified at 4 °C for 4 d in GWAS experiments, and 3 d in all other experiments. After stratification, bottles containing sown seeds were placed in a shaker and grown at 22 °C.

GUS staining. Seedlings were transferred from liquid medium to a 24-well tissue culture plate containing 1-2 ml X-GLUC staining solution (0.2% triton, 50 mM NaPO4 (pH 7.0), 10 mM ferrocyanide, ferricyanide, 2mM X-GLUC). The plates were wrapped in aluminum foil and incubated overnight at 37 °C. The next day, the X-GLUC solution was removed followed by immersion of the seedlings with 70% ethanol until the seedlings turned completely white. Subsequently, the seedlings were transferred to another 24-well tissue culture plate containing 2 ml FAA fixation solution (50% EtOH, 5% formaldehyde, 10% acetic acid).

Morphological analysis of meristem development. For determining visible meristem development, seedlings were transferred from liquid medium to plates containing half-strength MS medium with 1% plant agar. For analysis, scans of the plates were made using a regular flatbed computer scanner and SAM development was examined using a score system consisting of 5 scores (Supplementary Fig. 1). An improved score system was devised for GWA mapping for use with a binocular (Fig. 2). GUS expression was analyzed with a Zeiss Stemi SV 11 binocular, using 1x and 2.5x magnifications. Images were captured in 1280x1024 pixels resolution using a Nikon DXM1200 camera along with Nikon ACT-1 image capture software.

Results

Liquid medium setup allows for controlled sugar exposure times to study SAM development. To investigate the optimal conditions for the SAM to develop, as well as for detailed analysis and timing of subsequent stages in sugar-mediated SAM development, two different liquid cultures were set up. As previously reported, *Arabidopsis* plants are able to grow and promote morphogenesis in the dark, in both liquid and solid medium cultures (Araki & Komeda, 1993; Roldán *et al.,* 1999). However, a liquid medium culture provides some distinct advantages over solid medium cultures: (1) sugars and other compounds can be added at variable time points allowing for controlled metabolite exposure times; (2) disparate metabolite availability to particular seedlings resulting from unequal germination times is prevented by adding carbon nutrients after all seedlings in an experiment have germinated and (3) seedlings are completely submerged, ensuring more equal exposure to nutrients among seedlings. *Arabidopsis thaliana* seedlings of the Columbia ecotype were grown in bottles containing half-strength MS medium supplemented with either 1% sucrose or 1% sorbitol (control). In the first setup, which will be referred to as setup A, SAM development was scored after 4-14 d of growth in the presence of sorbitol or sucrose (Fig. 1a,c), whereas in the second setup, which will be referred to as setup B, SAM development was scored after 14 d of growth and sucrose or sorbitol was added after 0-13 d (Fig. 1b,d). For observation, seedlings were given one of the following five scores based on the developmental stage of the SAM: (1) no response; (2) opening of the cotyledons; (3) visible true leaf primordia; (4) first pair of true leaves have formed; (5) several pairs of true leaves have formed (Supplementary Fig. 1). The first signs of meristem activity (score 2) were observed in some seedlings after 8 d of continuous sucrose exposure in setup A, but only 3 d in setup B. The second stage (score 3) was also reached after 8 d of continuous sucrose exposure in setup A, but 10 d were required for consistent development in multiple seedlings and to achieve an average score of 3. In setup B, only 4 d of sucrose exposure were required for seedlings to reach score 3, but 5 d to reach an average score of 3 of the top 5 seedlings. Score 4 was achieved after 11 d in setup A and 7 d of sucrose exposure in setup B, and averages were achieved after 13 d and 8 d, respectively. The fourth stage (score 5) was observed after 11 d in setup A, and the average after 13 d. In setup B, score 5 was reached after 11 d of sucrose exposure in all seedlings (Fig. 1). These results indicate a much more efficient nutrient uptake in setup B conditions as sucrose exposure times are much shorter than in setup A. Furthermore, significant variation between individual seedlings was observed at multiple time points in setup A (Fig. 1c). This was reduced in setup B, but still present (Fig. 1d). Thus,

these findings demonstrate the extent of SAM development at different time points and show that sucrose exposure time can be minimized by altering the timing of sucrose supplementation.

GWA study identified genomic loci associated with SAM activity. To identify genomic loci that are associated with sucrose-mediated shoot meristem activity, a GWA study was conducted using 254 natural accessions of *Arabidopsis* (Supplementary Table I). 10-20 seedlings were grown in the dark for 14 d using the liquid medium setup described above. Sucrose was added after 8 d, because seedlings exhibited an average score of 3 at at 14d in this set-up (Fig. 1b,d). In this way, seedlings can be given both a lower and a higher score depending on the extent of SAM development. This allows for the identification of both hypo- and hypersensitive accessions after 14 d of growth. Furthermore, this time point provided a uniformity of scores (Fig. 1d), limiting the possibility of detecting differences in SAM development between accessions that are not related to genetic differences.

The score system that previously was used for analysis of subsequent stages in SAM activity was based on interpretation of scanned pictures by the naked eye (Supplementary Fig. 1). However, smaller advances in SAM development cannot be detected with this system because it would require a larger magnification. In addition, newly formed leaves can block the detection of small primordia, resulting in false scores for particular seedlings. For proper GWA analysis, therefore, a more detailed score system using a stereo microscope is presented here to overcome these shortcomings. This new score system also discriminates between five stages of SAM development: (1) closed cotyledons; (2) opened cotyledons, non-elongated leaf pair primordia; (3) opened cotyledons, elongated leaf pair primordia; (4) opened leaves, non-elongated second leaf pair primordia; (5) opened leaves, elongated second leaf pair primordia (Fig. 2). For effective application of this new score system, normal performing accessions should provide an average score of 3, similarly to the previously used score system (Supplementary Fig. 1), after 14 d of growth when sucrose is supplemented at day 8. This will allow for the identification of both lower and higher scoring accessions.

Each individual seedling of every accession was given a score using the newly devised score system. In cases where the stage of SAM development was unclear, a decimal score of 0.5 was given between the two most likely scores. An average score was calculated for each accession based on the rounded top 75% of seedlings, with a minimum of 6 germinated seedlings to filter out seedlings that demonstrated poor development for unclear reasons and deviated from the majority of seedlings. Accessions with only 5 germinated seedlings were additionally included if the minimum score of each of these 5 seedlings was 2, and was limited to only one seedling. Finally, accessions with 4 germinated seedlings were included if all seedlings were given an equal score. The majority of accessions had an average score of around 3 (Supplementary Table I), confirming the effectiveness of the new score system because over- or underperforming accessions could be given a higher score (3-5) or lower score (1-3). Using the averages of each accession, GWAS was conducted with the online program GWAPP (https://gwas.gmi.oeaw.ac.at; Seren et al., 2012). In a Manhattan plot, the -log(p-value) of associated SNPs were plotted against their positions on the *A. thaliana* genome, providing peaks on certain positions of the genome that were found to be associated with the phenotype. Higher peaks correspond to higher p-values, and hence have a higher probability to be associated with the phenotype. Two peaks, on chromosome 1 and 3, were identified which exceeded the 95% certainty Benjamini-Hochberg-Yekutieli threshold. In addition, the peak on chromosome 1 also exceeded the multiple-Bonferroni criterion. Furthermore, a peak was identified on chromosome 4 that reached the threshold, but did not exceed it. This was also found on chromosome 2, although this peak consisted of only few SNPs (Fig. 3). However, if the criteria for the selection of accessions to be analyzed with GWAPP were changed to only include the rounded top 75% of seedlings with 6 or more germinated seedlings, the peak on chromosome 4 was raised to above the threshold. On the other hand, this caused other peaks to be lowered down (Supplementary Fig. 2). From the most significant peaks on each of chromosomes 1-4, genes were identified surrounding the highest SNP of each peak within a region of 5 kb upstream and 5 kb downstream. This resulted in a selection of 16 genes (table I). Thus, GWA mapping resulted in the identification of various genomic loci that are associated with sucrose-mediated activity of the SAM.

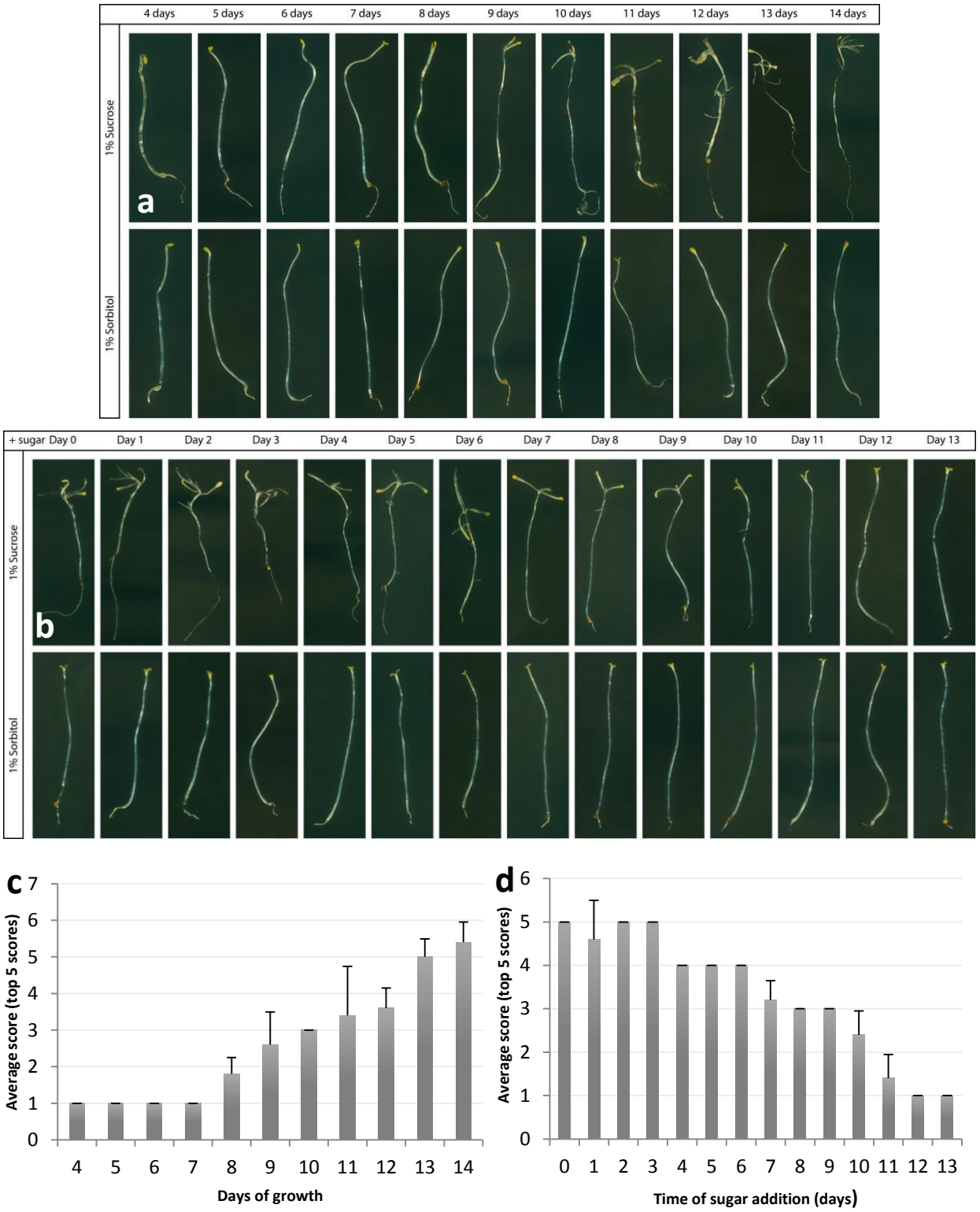

Figure 1. Impact of different sucrose exposure times on SAM development in liquid medium setup. (a,b) SAM development of seedlings grown in liquid medium supplemented with either 1% sucrose or 1% sorbitol. **(a)** SAM development was scored after a variable number of days of continuous sucrose or sorbitol exposure (setup A) or **(b)** after 14 d with variable moments of sucrose or sorbitol supplementation (setup B). **(c,d)** The extent of SAM development was observed using a score system consisting of 5 scores of seedlings grown **(c)** for a variable number of days under continuous sucrose-treatment conditions (setup A) or **(d)** for 14 d with variable moments of sucrose supplementation (setup B). Average scores were calculated from the top 5 scores of individual seedlings for each time point.

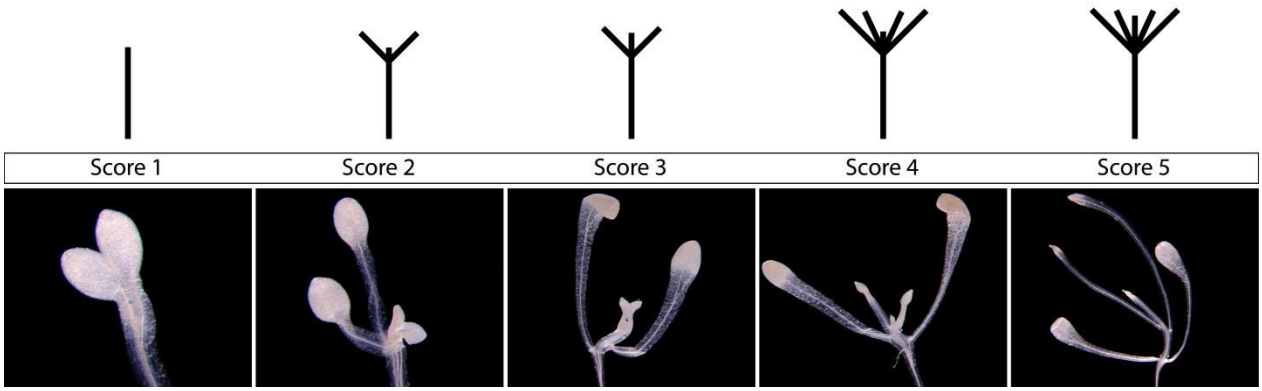

Figure 2. Detailed score system for SAM development. The extent of SAM development was scored using 5 different scores: (1) closed cotyledons; (2) opened cotyledons, non-elongated leaf pair primordia; (3) opened cotyledons, elongated leaf pair primordia; (4) opened leafs, non-elongated second leaf pair primordia; (5) opened leafs, elongated second leaf pair primordia. All seedlings depicted were washed in 70% EtOH prior to taking photographs.

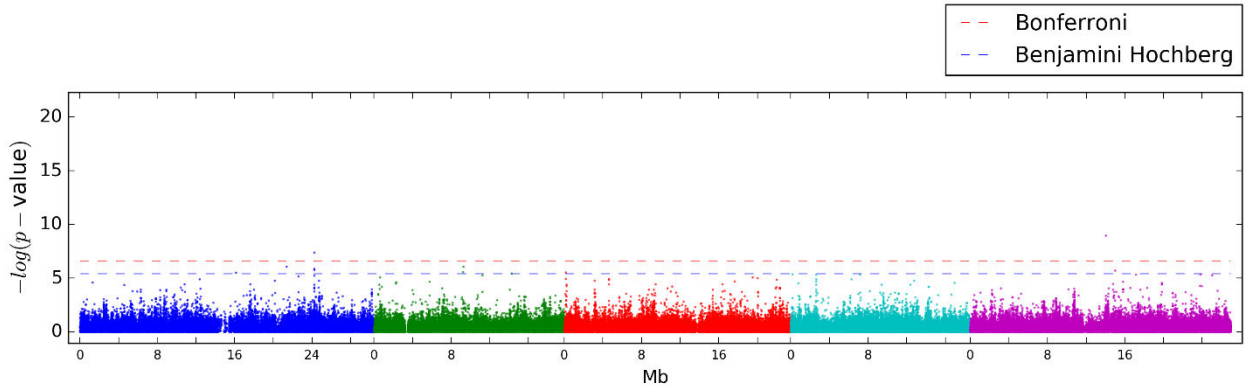

Figure 3. Identification of associated genomic loci with Manhattan plot. The -log(p-value) of SNPs within the genomes of each analyzed HapMap accession was plotted against their positions on the genome. The blue dotted line represents the Benjamini-Hochberg-Yekutieli multiple testing procedure, which implies a 5% false discovery rate; the red dotted line represents the multiple-Bonferroni criterion. Peaks of multiple SNPs indicate associated regions, with variable probability depending on their height. The five different colors represent the five chromosomes of A. thaliana. The plot was created by the GWAPP program.

Sucrose availability increases CYCD3;1, CYCB1;1 and ATH1 expression in shoot apex. To further study the potential role of the newly identified genes in sugar-mediated changes in SAM activity, morphological and/or molecular markers are required that signal early meristem activation events correlated to activity of these new genes. To discover these markers, several candidate marker genes were selected based on their biological function. Among these genes are two cyclins, CYCD3;1 and CYCB1;1. CYCD3;1 is involved in the regulation of the G1/S transition and is active in proliferating tissues in the shoot apex (Dewitte *et al.,* 2003; López-Juez *et al.,* 2008). CYCB1;1 is a mitotic cyclin of which its activity is restricted to the G2/M transition (Colón-Carmona *et al.,* 1999; López-Juez *et al.,* 2008). Both cyclins demonstrated clear expression in the SAM upon light exposure of several hours to a few days following a 3 d dark period (López-Juez *et al.,* 2008), indicating that they are correlated to developmental events in the SAM. To investigate this correlation, GUS constructs of CYCB1;1 and CYCD3;1 were analyzed in seedlings exposed to either 1% sucrose or 1% sorbitol (control). CYCD3;1 exhibited expression in the hypocotyl in both sucrose and sorbitol conditions, but expression was extended to the shoot apex after 4 and 5 d under sucrose conditions (Fig. 4a). CYCB1;1 was clearly expressed after 4, 5 and 6 d in the presence of sucrose, whereas only very slight (4 and 5 d) or no expression (6 d) was observed in the presence of sorbitol (Fig. 4b). To allow for seeds to fully germinate prior to sugar addition, sucrose or sorbitol was supplemented after seedlings containing the GUS constructs of CYCB1;1 were

Table I. Selection of candidate genes. Identified genes from the 4 most significant peaks on the Manhattan plot shown in Fig. 3. Genes were selected within a region of c. 5 kb upstream and 5 kb downstream from the highest SNP of each peak. Genes in bold contain the SNP with the largest -log(p-value) of a peak.

Gene model	Gene name/description
AT1G65260	PLASTID TRANSCRIPTIONALLY ACTIVE 4 (PTAC4)
AT1G65270	**Unknown function**
AT1G65280	DNAJ heat shock N-terminal domain-containing protein
AT2G26300	G PROTEIN ALPHA SUBUNIT 1 (GPA1)
AT2G26310	FATTY-ACID-BINDING PROTEIN 2 (FAP2)
AT2G26320	**AGAMOUS-LIKE 33 (AGL33)**
AT2G26330	ERECTA (ER)
AT3G01420	ALPHA-DIOXYGENASE 1 (DOX1)
AT3G01430	Unknown function
AT3G01435	Involved in regulation of transcription from RNA polymerase II promoter
AT3G01440	**PHOTOSYNTHETIC NDH SUBCOMPLEX L 3 (PnsL3)**
AT3G01450	ARM repeat superfamily protein
AT3G01460	METHYL-CPG-BINDING DOMAIN 9 (MBD9)
AT4G05095	Unknown function
AT4G05100	**MYB DOMAIN PROTEIN 74 (MYB74)**
AT4G05110	EQUILIBRATIVE NUCLEOSIDE TRANSPORTER 6 (ENT6)

grown in the dark for 3 d. GUS expression was then observed after 1-6 d following sugar addition. Seedlings exhibited only marginal expression after 1 d. However, after 2-4 d, expression was highly induced under sucrose conditions, whereas expression was minimal to none in the presence of sorbitol. Expression started to reduce after 5 d, where levels were identical between sucrose and sorbitol treated seedlings. After 6 d, expression was completely gone (Fig. 4c). Interestingly, expression of both CYCD3;1 and CYCB1;1 started to occur in the shoot apex after 4 d of continuous sucrose exposure, while it took 8 d to see any morphological changes (Fig. 1a,c). Thus, these findings show that expression of both CYCD3;1 and CYCB1;1 is induced in the shoot apex upon exposure to sucrose and occurs much earlier than the first observable morphological changes.

ATH1 was shown to be expressed in the shoot apex after 1 week in the presence of sucrose, but not in the presence of sorbitol (Gómez-Mena & Sablowski, 2008). This indicates that ATH1 may be involved in sugar-mediated SAM activation, and is therefore another potential candidate gene. To test this hypothesis, early expression of ATH1 was studied here in the presence and absence of sucrose or sorbitol. Seedlings containing pATH1:GUS constructs were grown in the dark for 3 d after which 1% sucrose or 1% sorbitol was supplemented, and GUS expression was observed 4-7 d later. Because Gómez-Mena & Sablowski compared sugar-treated seedlings to seedlings grown in the absence of sucrose, additional seedlings were grown here that were deprived of any sugars to mimic experimental conditions and to rule out any possible effect of sorbitol on ATH1 expression. Seedlings exhibited GUS expression in both sucrose- and sorbitol-treatment conditions, as well as in the absence of both sugars, indicating that sugars are not required to induce ATH1 expression. However, GUS expression was slightly increased under sorbitol-treatment conditions as compared to no sugar conditions, pointing out that sorbitol might have some effect on ATH1 activity. Moreover, GUS expression was highly increased in the SAM region under sucrose conditions after 4 and 5 d following sugar supplementation, and was gradually decreased after 6 and 7 d (Fig. 5). This is similar to expression of CYCB1;1, which was also increased after 4 d following sucrose supplementation, and decreased after 5 and 6 d (Fig. 4c). Thus, these results demonstrate increased ATH1 activity stimulated by sucrose and clear overlap of expression patterns with CYCB1;1.

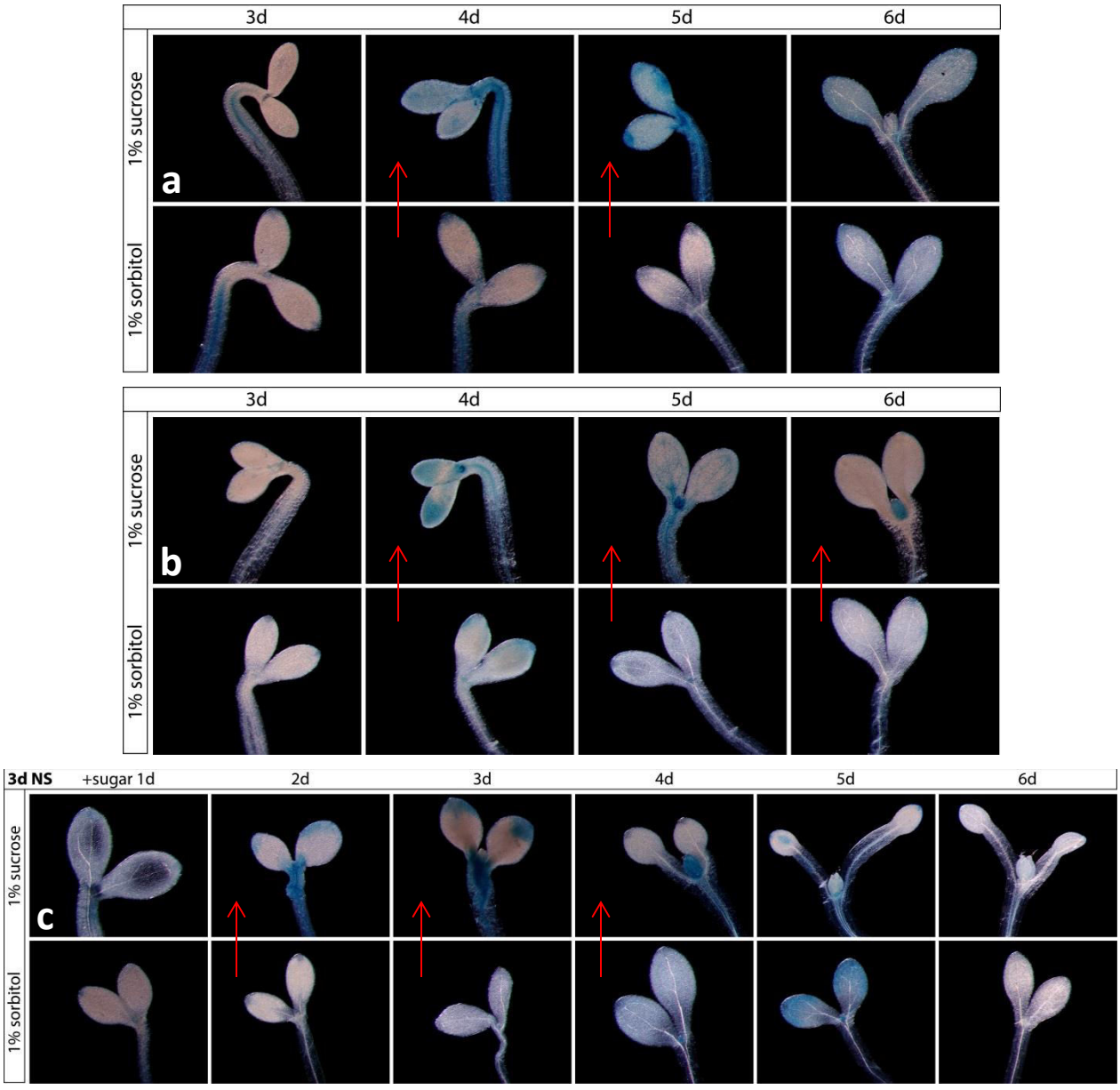

Figure 4. Sucrose availability induces CYCD3;1 and CYCB1;1 expression in the shoot apex. (a,b) Seedlings were grown under continuous sucrose or sorbitol (control) exposure for 3-6 d and were observed for **(a)** CYCD3;1:GUS expression and **(b)** CYCB1;1:DB-GUS expression. **(c)** Sucrose or sorbitol was supplemented after seedlings were grown in the dark for 3 d, and CYCB1;1:GUS expression was observed the subsequent 1-6 d. Arrows indicate an increase in GUS expression.

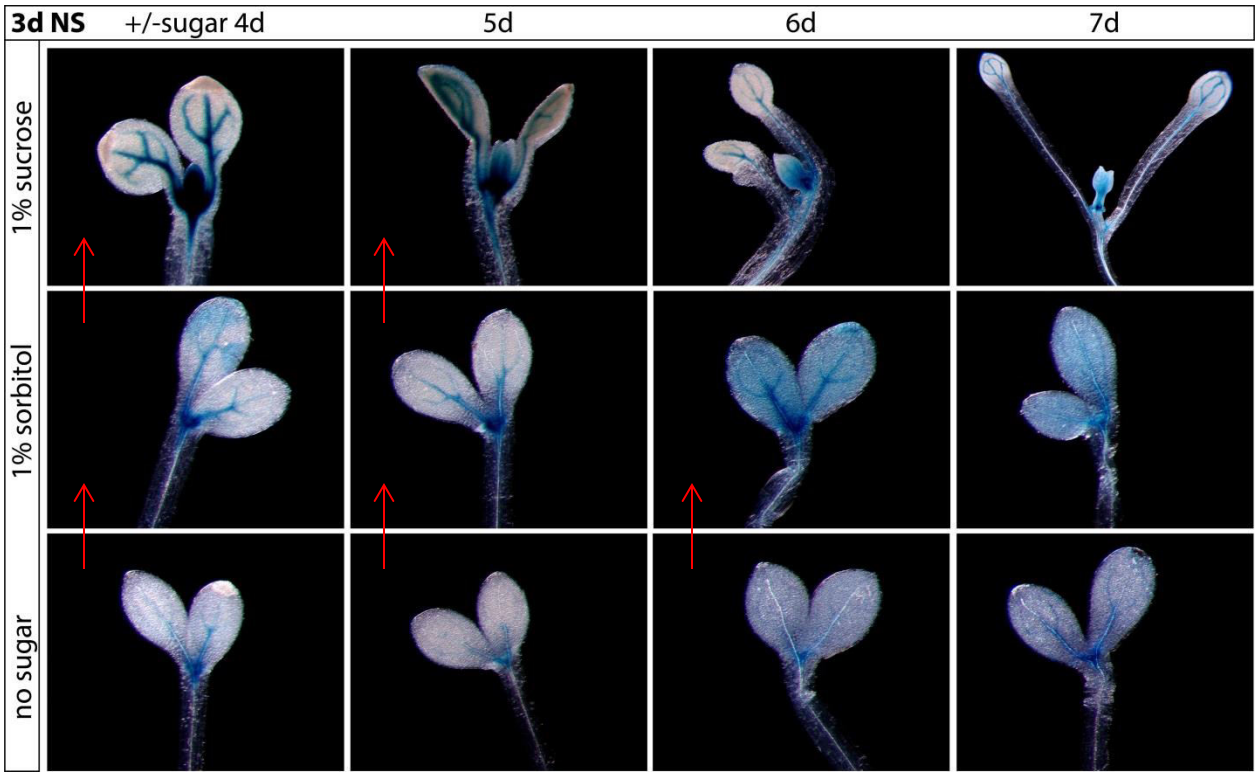

Figure 5. Sucrose availability increases pATH1 expression in the shoot apex. Seedlings were grown in liquid medium in the dark for 3 d, after which they were supplemented with sucrose or sorbitol to a 1% concentration. Control seedlings were additionally included to which no sugar was supplemented. pATH1:GUS expression was observed the subsequent 4-7 d. Arrows indicate an increase in GUS expression.

Discussion

The availability of carbon nutrients and the ability to sense this availability is of major importance for the development and survival of plants. Several growth controlling regulatory networks have been discovered that respond to the nutrient status of plants. Although some of these networks have been extensively studied, knowledge is still limited on how plants sense the availability of carbon sources and how this is translated into growth and development (Rolland et al., 2006; Smith & Stitt, 2007; Baena-González & Sheen, 2008; Smeekens *et al.,* 2010; Lastdrager *et al.,* 2014). In this study, an optimized seedling-based assay is presented to study the effects of sucrose availability on SAM development in dark-grown seedlings, and several genomic loci were identified to be associated with these effects through a GWA study. In addition, CYCD3;1, CYCB1;1 and ATH1 were found to be molecular markers that signal early activation events of the SAM, and were expressed much earlier than the first morphological signs of SAM activation. The findings presented here allow for the detection and scoring of small changes in SAM activity with much more consistency than in previously reported experimental settings. Furthermore, the candidate genes that were identified through GWA mapping can be studied for a potential function in sucrose-mediated SAM activity using the new experimental setup and the identified markers can be used to signal potentially early meristem activation events resulting from activity of these candidate genes. Thus, this study provides a groundwork towards better understanding the sugar-sensing mechanisms involved in changes in meristem development.

Optimal growth conditions were determined here in the absence of light, to ensure complete dependence on the availability of sugar. Dark morphogenesis was initially studied using liquid cultures, but a solid medium setup was presented to overcome some of the problems associated with liquid cultures, including the expression of light-regulated genes and the reported limited effectiveness of a liquid culture to Columbia (Araki & Komeda, 1993; Roldán *et al.,* 1999). A solid medium setup, however, has major limitations that impact reproductive success of experiments. Seedlings require a carbon source at the

aerial part of the plant, which was accomplished by growing seedlings vertically on solid agar plates containing half-strength MS medium supplemented with sucrose. However, this requirement is not always met as seedlings frequently grow in other directions, thereby avoiding contact of their aerial part with the medium. Initially, this study performed a GWA study using the solid medium setup as proposed by Roldán *et al.*, which showed that the aerial part of seedlings separated from the medium in 40% of analyzed accessions, even as plates were constantly in a vertical position (Supplementary Table II). Moreover, there is no guarantee that seedlings which appear to be in contact with the medium at the time of observation have not separated its aerial part earlier. Furthermore, it has been reported that germination and seed dormancy are very complex traits influenced by many factors, and can be very different between natural accessions of *Arabidopsis* (Bentsink & Koornneef, 2008). Unequal germination times will result in longer sucrose exposure time for particular seedlings, which may result in enhanced development of the shoot apex compared to seedlings with relatively late germination. This would cause unwanted variation between seedlings. In addition, the solid medium setup does not allow for the supplementation of sugars, as well as other chemicals such as plant hormones, at any time point other than from the start. This prevents the testing of involvement of certain chemicals in the process of meristem activation. To overcome these problems, the liquid culture as reported by Rédei *et al.* and repeated by Araki & Komeda was improved to allow for the addition of carbon sources at later time points. Depending on the time point of sucrose addition, and the time it takes for seeds to germinate, this system allows for the addition of sucrose after all seeds have germinated, thus ensuring equal exposure time to sucrose for all seedlings. A time series was presented in which sucrose was supplemented after 0-13 d, and SAM development was observed after 14 d to observe the stage of SAM development as well as variation between individual seedlings at each time point. One of the problems that was reported to occur in a liquid shaken culture, is the stress-induced expression of light-regulated genes (Araki & Komeda, 1993). However, no changes in SAM activity were detected under sorbitol conditions in the setup used in this study, which would be expected if stress had any influence on development.

Observation of shoot meristems was initially carried out with a specially designed score system (Supplementary Fig. 1). However, since no magnification has been taken into account with this score system, small advances in SAM development could not be detected. At least part of the variation observed in the two liquid medium setups presented here (Fig. 1c,d) could therefore be attributed to a failure to detect particular developmental stages in the SAM in some seedlings. For this reason, a new score system was designed to achieve better accuracy and consistency between seedlings in scoring SAM development (Fig. 2). This is of critical importance for GWA mapping, as variant phenotypes resulting from inefficient scoring could lead to false associations of particular SNPs with the phenotype. Using this improved score system, GWA mapping on 254 natural accessions of *Arabidopsis* let to the identification of several genomic regions that were found associated with sucrose-mediated SAM activity (Fig. 3). Further examination of these regions revealed that the most significant SNP of the highest peak on the Manhattan plot was found in AT1G65270, a gene with unknown function (Fig. 3; Table I). It has been reported, however, that this gene produces mobile mRNAs, in root to shoot direction (Thieme *et al.,* 2015). Furthermore, the Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter *et al.,* 2007) shows high expression for this gene in most tissues, especially in the shoot apex. This indicates that this gene may have an important function in meristem function. Another interesting observation is the finding of the MADS-box type I gene AGAMOUS-LIKE 33 (AGL33), because a second MADS-box type 1 gene, AGAMOUS-LIKE 38 (AGL38, also known as PHERES2 (PHE2)), is located adjacent to the 10 kb region around the highest SNP of the most significant peak on chromosome 2 (De Bodt *et al.,* 2003; Li *et al.,* 2005). Likewise, the R2R3-type MYB transcription factor MYB DOMAIN PROTEIN 74 (MYB74) was identified from the most significant peak on chromosome 4, but a second MYB transcription factor of the same group, MYB DOMAIN PROTEIN 57 (MYB57), was found surrounding the most significant peak on chromosome 3 (Song *et al.,* 2011; Xu *et al.,* 2015). This peak consists of two separate peaks when zoomed in, and MYB57 is located near the highest SNP of the lower second of these sub-peaks. These discoveries are interesting because they were found under distinct peaks on multiple chromosomes. Studies have shown that MADS-box type I genes are largely involved in female gametophyte regulation and seed development, but their possible involvement in vegetative meristem development has not been explored (Masiero *et al.,* 2011; Smaczniak *et al.,* 2012). However, members of the MADS-box family are thought to be broadly involved in almost all plant developmental processes (Smackzniak *et al.,* 2012). Hence, further research on the two type I MADS-box genes identified here would shed light on their

possible roles in vegetative SAM development. MYB57 and MYB74 were reported to regulate normal stamen development by gibberellin through jasmonate and to be involved in regulating transcriptional responses to salt stress, respectively (Cheng *et al.*, 2009; Xu *et al.,* 2015), but no studies have been performed on their potential influence on meristem development. In addition, the eFP browser shows no increased expression in the shoot apex. Finally, ERECTA (ER) was found under a peak on chromosome 2. This gene has a major role in controlling SAM size by regulating WUSCHEL (WUS) expression (Mandel *et al.,* 2014). WUS has an important function in determining the fate of stem cells by maintaining their active state, and it was reported to be expressed in dark-grown seedlings grown on medium supplemented with sucrose (Pfeiffer *et al.,* 2016). ER is therefore a meaningful finding considering that sucrose-dependent SAM development is the phenotype under study. Thus, various candidate genes were identified that may have critical roles in sucrose-mediated SAM activity.

In order to ultimately unravel the sugar-sensing genetic mechanisms involved in changes in SAM activity, it is essential to include genetic markers that signal the earliest signs of meristem activation on a molecular level. These markers would signal any possible influence of new candidate genes, such as the ones listed in table I, on meristem activity by detecting small developmental changes in the SAM. It has been reported that the cyclins CYCD3;1 and CYCB1;1 were expressed in the shoot apex shortly after dark-grown seedlings were transferred to light (López-Juez *et al.,* 2008). Furthermore, sucrose induced the expression of CYCD3;1 in cell suspension cultures (Riou-Khamlichi *et al.,* 2000). This led to the hypothesis that CYCD3;1 and CYCB1;1 are markers for SAM reactivation and would therefore also be expressed in dark-grown seedlings in the presence of sucrose. The findings presented here show that CYCD3;1 was clearly expressed under sucrose-treatment conditions after 3-5 d of growth in the dark. Although expression under these conditions was limited to the hypocotyl after 3 d, similarly to sorbitol-treatment conditions, it was clearly located in the shoot apex after 4 and 5 d. No detectable expression in the shoot apex was visible in the presence of sorbitol (Fig. 3a). Likewise, CYCB1;1 expression was clearly increased under sucrose-treatment conditions after 4-6 d, with almost undetectable expression levels in sorbitol-treated seedlings (Fig. 3b). Interestingly, expression of both cyclins started after 4 d (Fig. 3a,b), while it took 8 d to see visible SAM development (Fig. 1a,c). More detailed analysis of seedlings after 4 d, using special microscopy techniques, would uncover any possible morphological changes that occur in the shoot apex at this stage. Furthermore, if sucrose or sorbitol were supplemented after 3 d of growth in the dark, GUS expression was enhanced under sucrose-treatment conditions after only 2 d of exposure to sugar and expression continued to remain high after 3 and 4 d of sucrose exposure. However, expression started to reduce after 5 d and almost disappeared after 6 d of sucrose exposure. These observations make CYCD3;1 and CYCB1;1 suitable genetic markers for detecting early shoot meristem activation events. Moreover, the sucrose-induced expression of CYCD3;1 and CYCB1;1 correlates with the light-induced expression of both cyclins (López-Juez *et al.,* 2008), and previous research pointed to interactions between the light- and sugar-regulated pathways (Thum *et al.,* 2003). To test whether there is any overlap in light- and sugar-signaling towards meristem activation, signaling intermediates in light signaling should also be studied in darkness.

In addition to CYCD3;1 and CYCB1;1, ATH1 has been reported to be expressed in the presence 1% sucrose in the shoot apex of 7 d old dark-grown seedlings, whereas no expression was detected in the presence of 1% sorbitol (Gómez-Mena & Sablowski, 2008). This led to the hypothesis that ATH1 may be a suitable marker to detect developmental changes in the shoot apex, and possibly much earlier than 7 d. The results presented here show ATH1 expression under both sucrose- and sorbitol-treatment conditions, as well as in the absence of sugars. However, a pATH1:GUS construct was used here instead of a ATH1:ATH1-GUS construct that was used by Gómez-Mena & Sablowski, which might result in different expression patterns. Furthermore, seedling were grown in liquid medium here rather than solid medium, allowing for a more consistent uptake of sugars. Nevertheless, expression was highly increased in the presence of sucrose after 4 and 5 d of exposure to sucrose, and reduced to levels similar to sorbitol-treatment conditions after 6 and 7 d of sucrose exposure (Fig. 4). Thus, these findings demonstrate that ATH1 is a suitable early marker for detecting changes in shoot meristem activity.

Interestingly, ATH1 exhibited similar expression patterns to CYCB1;1. Expression of both genes was increased after 4 d following sucrose supplementation, and decreased after 5 and 6 d (Fig. 4c; Fig. 5). However, expression of CYCB1;1 already started to increase after only 2 d following sucrose supplementation (Fig. 4c). Additional time points are needed for ATH1 to see whether this early

expression overlaps with CYCB1;1 expression as well. There may also be overlap with CYCD3;1, since expression of both CYCD3;1 and CYCB1;1 is induced in the presence of sucrose after 4 d if sucrose is added to the medium from the start (Fig. 4a,b). Further experimentation is required to analyze whether CYCD3;1 shows the same expression pattern as ATH1 if sucrose is supplemented after 3 d of growth. Equal expression patterns among all three markers would be very powerful to confirm early meristem activation events in future studies. To this end, TCP5 was initially considered as an additional candidate marker gene for meristem reactivation, as it has been reported that this gene is downregulated instead of upregulated by light (López-Juez *et al.,* 2008). Confirming its role as a marker gene and using it in combination with CYCD3;1, CYCB1;1 and ATH1 would provide additional evidence for meristem activation since both up- and downregulation would be measured.

In conclusion, this study provides a framework for studying sucrose-mediated changes in SAM development in the dark. Growth conditions that have previously been used were revised and improved here, and were utilized to conduct a GWA study. Phenotypes of natural accessions of the HapMap population were analyzed with a newly formulated score system that allows for accurate observation of small developmental progressions of the SAM. This led to the identification of 4 genomic loci that are associated with these phenotypes. Finally, the two cyclins CYCD3;1 and CYCB1;1, as well as ATH1 were shown to be early expressed upon exposure to sucrose, which makes them excellent genetic markers for early activation events in the SAM.

Future work could include the evaluation of additional growth conditions for the liquid medium setup to reduce the number of days required to achieve the desired extent of SAM development. It is further recommended to perform an in-depth analysis on several of the candidate genes identified from GWA mapping. Such an analysis would consist of mutant studies of these genes to observe the effects of lost gene function on SAM development, as well as studying expression patterns and interaction with other known factors involved in meristem function. These genes could potentially prove to be essential in better understanding the sugar-sensing regulatory networks that influence SAM development and therefore growth that ultimately impacts yield. Finally, it is worthwhile to analyze expression of CYCD3;1, CYCB1;1 and ATH1 at time points corresponding to each visible subsequent stage of SAM development in the liquid medium setup. It would be interesting to observe how early visible changes in SAM development translate to changes on a molecular level.

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Supplemental data

Supplementary Table I. List of accessions that were used for GWA mapping, with country of origin and given score.

Accession	Country	Score	Accession	Country	Score	Accession	Country	Score
RRS-10	USA	3.00	Hn-0	GER	3.50	Sei-0	ITA	4.00
Aa-0	GER	2.36	Je-0	GER	3.00	Sg-1	GER	3.42
Amel-1	NED	2.00	Jl-3	CZE	3.05	Sh-0	GER	3.00
An-2	BEL	3.60	Jm-1	CZE	3.19	Si-0	GER	3.00
Ang-0	BEL	3.10	Kl-5	GER	3.23	Tha-1	NED	2.06
Ann-1	FRA	2.00	Kn-0	LTU	3.65	Ting-1	SWE	2.90
Arby-1	SWE	3.15	Knox-11	USA	3.73	Tiv-1	ITA	2.17
Benk-1	NED	3.21	Krot-2	GER	3.10	Tscha-1	AUT	3.00
Blh-2	CZE	3.38	Li-3	GER	4.00	Tsu-0	JPN	2.83
Boot-1	UK	3.30	Li-5:2	GER	1.86	Ty-0	UK	3.00
Bs-2	SUI	3.00	Li-7	GER	3.17	Uk-1	GER	3.27
Bu-8	GER	2.30	Mnz-0	GER	2.69	Uk-2	GER	3.27
Ca-0	GER	3.05	N4	RUS	3.00	Utrecht	NED	3.00
Cha-0	SUI	3.43	N7	RUS	3.00	Ven-1	NED	3.00
Chat-1	FRA	4.50	Nc-1	FRA	3.29	Wa-1	POL	3.00
CIBC2	UK	3.00	NFC20	UK	2.94	Wag-3	NED	3.38
CIBC4	UK	3.38	No-0	GER	3.00	Wag-4	NED	3.00
CIBC5	UK	3.00	Nok-1	NED	3.00	Wag-5	NED	3.13
Cit-0	FRA	3.00	Nw-2	GER	3.17	Wl-0	GER	3.00
Cnt-1	UK	3.25	Nz1	NZL	3.11	Wt-3	GER	3.20
Co-2	POR	3.69	Ob-1	GER	2.88	Ors-1	ROU	3.00
Co-4	POR	3.90	Pa-2	ITA	3.06	11ME1.32	USA	2.95
CSHL-5	USA	2.94	PHW-13	UK	3.73	11PNA4.101	USA	3.45
Com-1	FRA	3.00	PHW-20	UK	3.22	627ME-4Y1	USA	3.40
Da(1)-12	CZE	3.30	PHW-22	UK	3.00	Ag-0	FRA	2.95
Db-0	GER	3.14	PHW-26	UK	1.40	Alc-0	ESP	3.00
Di-1	FRA	4.00	PHW-28	UK	3.10	ALL1-2	FRA	3.20
Do-0	GER	2.75	PHW-35	FRA	3.00	ALL1-3	FRA	2.36
Dra-2	CZE	3.11	PHW-36	FRA	3.20	An-1	BEL	3.30
Ep-0	GER	3.00	PHW-37	FRA	3.38	App1-16	SWE	3.00
Es-0	FIN	3.14	Pla-0	ESP	3.20	Ba1-2	SWE	3.00
Est-0	RUS	2.20	Pn-0	FRA	3.59	Belmonte-4-94	ITA	3.55
Fi-1	GER	3.50	Pog-0	CAN	3.15	Bg2	USA	3.10
Ge-1	SUI	2.67	Pr-0	GER	2.80	Bla-1	ESP	2.95
G”-0	GER	3.80	PUZ24	CZE	3.93	Blh-1	CZE	2.89
Gr-5	AUT	3.00	Rhen-1	NED	3.00	Bor-1	CZE	3.00
Gu-1	GER	3.29	Rou-0	FRA	3.50	Bor-4	CZE	3.00
Ha-0	GER	3.29	S96	UNK	3.11	Br-0	CZE	1.50
Hau-0	DEN	3.85	Sav-0	CZE	2.59	Bro1-6	SWE	3.40

Supplementary Table I (continuing). List of accessions that were used for GWA mapping, with country of origin and given score.

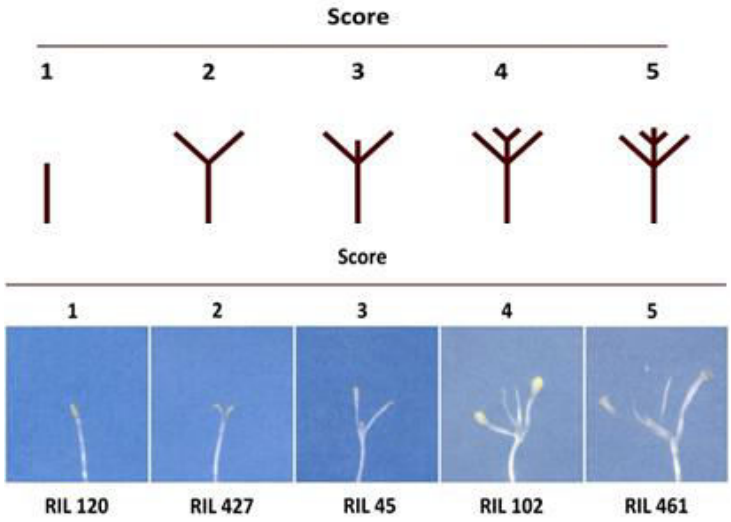
Accession	Country	Score	Accession	Country	Score	Accession	Country	Score
Bu-0	GER	2.89	LDV-14	FRA	3.20	Sap-0	CZE	3.10
BUI	FRA	3.00	LDV-34	FRA	3.00	Sav-1	CZE	3.00
Bur-0	IRL	3.68	LDV-58	FRA	3.38	Se-0	ESP	3.00
C24	POR	3.17	Ler-1	GER	2.41	Sha	TJK	4.81
CAM-16	FRA	3.83	LI-OF-095	USA	2.43	SLSP-30	USA	3.90
CAM-61	FRA	3.77	Liarum	SWE	3.09	Ste-3	USA	3.60
CIBC17	UK	3.82	Lip-0	POL	4.18	T1040	SWE	3.14
Col-0	USA	2.90	PHW-33	NED	3.33	T1110	SWE	4.00
Ct-1	ITA	3.10	LL-0	ESP	3.44	T510	SWE	3.55
CUR-3	FRA	4.00	Lm-2	FRA	3.17	T540	SWE	2.82
Cvi-0	CPV	3.29	Lp2-2	CZE	3.38	T620	SWE	3.20
DraIV1-14	CZE	3.45	Lp2-6	CZE	3.00	Ta-0	CZE	3.00
DraIV1-5	CZE	3.53	Map-42	USA	3.82	Tad01	SWE	3.00
DraIV1-7	CZE	3.00	MIB-15	FRA	3.00	TDr-18	SWE	3.32
DraIV6-16	CZE	3.61	MIB-22	FRA	2.42	Tottarp-2	SWE	3.00
DraIV6-35	CZE	3.10	MIB-28	FRA	3.55	TOU-A1-115	FRA	3.05
Duk	CZE	3.44	MIB-84	FRA	3.00	TOU-A1-12	FRA	3.25
Eden-2	SWE	3.08	MNF-Pot-48	USA	3.73	TOU-A1-62	FRA	3.00
Edi-0	UK	3.17	MNF-Pot-68	USA	3.64	TOU-A1-67	FRA	3.00
Est-1	RUS	3.00	Mr-0	ITA	3.10	TOU-A1-96	FRA	2.18
Fei-0	POR	3.00	Mrk-0	GER	3.45	TOU-C-3	FRA	2.13
Fja1-5	SWE	3.00	Mt-0	LIB	3.82	TOU-E-11	FRA	2.91
Ga-0	GER	3.50	Mz-0	GER	3.45	TOU-H-13	FRA	3.00
Gd-1	GER	3.00	N13	RUS	3.05	TOU-I-2	FRA	2.70
Ge-0	SUI	2.75	NC-6	USA	3.09	TOU-J-3	FRA	3.00
Gul1-2	SWE	3.86	Nd-1	SUI	3.31	TOU-K-3	FRA	3.17
Gy-0	FRA	3.68	NFA-10	UK	2.57	Ts-1	ESP	3.42
Hi-0	NED	3.42	NFA-8	UK	3.23	Udul1-34	CZE	3.09
Hod	CZE	3.00	PAR-4	FRA	3.00	UKID37	UK	3.33
HR5	UK	3.40	PAR-5	FRA	3.06	UKID48	UK	2.46
Hs-0	GER	3.35	Paw-3	USA	3.72	UKNW06-386	UK	3.00
HSm	CZE	3.20	Pent-1	USA	3.09	UKNW06-436	UK	3.23
In-0	AUT	2.40	Per-1	RUS	3.00	UKNW06-460	UK	2.14
JEA	FRA	3.00	Petergof	RUS	3.14	UKSE06-062	UK	3.32
Ka-0	AUT	3.15	Pro-0	ESP	3.00	UKSE06-192	UK	2.65
Kas-2	IND	2.00	Pu2-23	CZE	3.75	UKSE06-272	UK	2.81
KBS-Mac-8	USA	3.00	Rak-2	CZE	3.00	UKSE06-349	UK	2.20
Kin-0	USA	2.67	Rennes-1	FRA	3.00	UKSE06-429	UK	2.83
PHW-3	GER	3.00	Rmx-A180	USA	3.00	UKSE06-466	UK	3.14
LAC-3	FRA	2.80	ROM-1	FRA	3.00	UKSE06-482	UK	3.60
LAC-5	FRA	3.00	Rsch-4	RUS	3.75	UKSE06-520	UK	3.00

Supplementary Table I (continuing). List of accessions that were used for GWA mapping, with country of origin and given score.

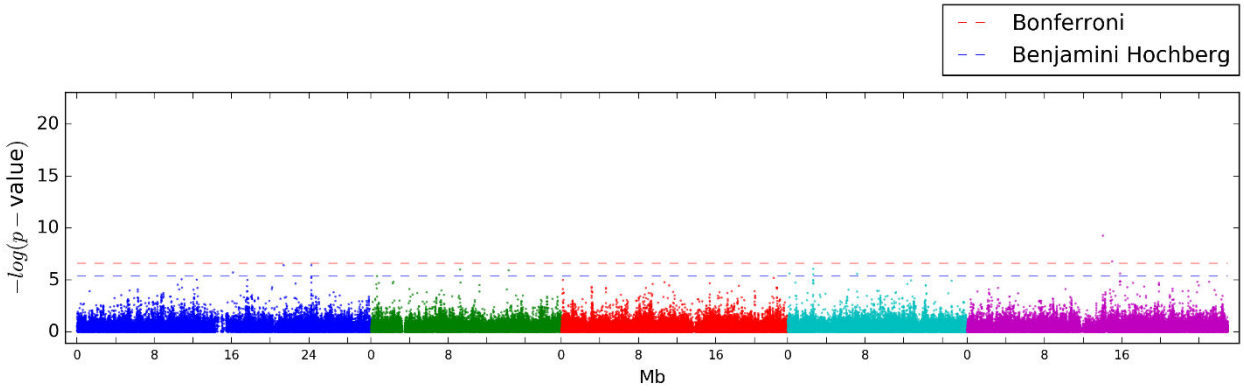
Accession	Country	Score	Accession	Country	Score	Accession	Country	Score
UKSE06-628	UK	3.75	VOU-1	FRA	2.69	Wt-5	GER	2.00
Ull2-3	SWE	3.00	VOU-2	FRA	3.00	Zdr-6	CZE	3.17
Ull3-4	SWE	3.22	Wei-0	SUI	3.17	ZdrI2-24	CZE	3.32
Uod-7	AUT	3.00	Wil-1	LTU	3.80	ZdrI2-25	CZE	2.86
Van-0	CAN	3.00	Ws-0	RUS	3.00			

Supplementary Table II. Number of accessions containing seedlings with separated aerial parts. Accessions were counted from an unpublished GWA study that used a solid medium setup.

% of total number	# accessions	% of total
0-50%	112	35.9
50-100%	13	4.2
Total	**312**	**100**



Supplementary Figure 1. Score system for phenotypic analysis. To analyze visible SAM development, a score system was used to give seedlings one of five scores according to their extent of SAM development: (1) No response; (2) Opening of the cotyledons; (3) Visible true leaf primordia; (4) First pair of true leaves have formed; (5) Several pairs of true leaves have formed.



Supplementary Figure 2. Manhattan plot with changed criteria. An average score was calculated for each accession based on the rounded top 75% of seedlings with 6 or more germinated seedlings The plot was created by the GWAPP program and is described n Figure 3.