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Peer Review Report for 10.1105/tpc.16.00465

UUAT1 Is a Golgi-Localized UDP-Uronic Acid Transporter that Modulates the Polysaccharide Composition of Arabidopsis Seed Mucilage.

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Review timeline:

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	1st Decision:	July 19, 2016 *revision requested*
TPC2016.00465-RAR1	1st Revision received:	Sept 21, 2016
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REPORT: (Note: The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2016.00465-RA 1st Editorial decision – *revision requested* July 19, 2016

Reviewer 1 has asked for additional experiments to more completely characterize the second substrate used by the UUAT antiporter transport mechanism. In your resubmission you should address this question.

Both reviewers 1 and 2 would like to see a more complete description and presentation of the results of the genetic screen used to identify the putative UUAT transporter sequence. Also, the authors should consider and discuss the functions of additional, closely related sequences in the complete Arabidopsis genome sequence.

Reviewer 3 also has suggested slightly altering the title of the article, and it might be worth considering this.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2016.00465-RAR1 1st Revision received Sept 21, 2016

Reviewer comments and author responses:

Reviewer #1 (Comments for author):

Point 1. The authors of this manuscript claim to have identified a UDP-glucuronate (UDP-GlcA)- specific nucleotide sugar transporter (NST) from Arabidopsis that belongs to clade V of the NST-TPT superfamily of transporters in this model organism. The starting point of their inquiry was the analysis of changes in the monosaccharide composition or abundance of seed coat mucilage in insertion mutants with gene disruptions in those family members for which a function had not already been described. Somewhat curiously, the authors do not comment on potential changes in

mucilage composition in the 11 targeted candidate genes but jump ahead to three insertion mutants in a single gene (At5g04160).

RESPONSE: We have taken into account the comments of the reviewer and made a more detailed description of the screening process that led us to the identification of UUAT1 (See below). We also included the measurement of soluble mucilage (based on the content of uronic acids) done during the screening of NSTs mutants. The following text is included at the beginning of the results section:

From the 11 target genes expressed throughout seed development and without a known function (Supplemental Figure 1), At5g17630 was discarded of the analysis since it belongs to the clade of the triose phosphate translocators. Out of the 10 remaining genes, we were able to obtain homozygous mutants for 8 of them. Soluble mucilage content was assessed by measuring the amount of uronic acid released upon seed imbibition for 10 minutes in water. The results showed that a mutant line on At5g04160 exhibited the lowest level of uronic acid in mucilage; thus we named *uuat1* (Supplemental Figure 2). To confirm that this gene is expressed at the stages when mucilage is produced (6 to 8 DAP) we measured the expression level of *UUAT1* during seed development. Supplemental Figure 3 shows a peak of expression of this gene at 8 DAP, a pattern that is similar to the expression of genes involved in the synthesis of mucilage (Macquet et al., 2007; Saez-Aguayo et al., 2013; Rautengarten et al., 2014). *UUAT1* encodes a polytopic transmembrane protein with 10 putative membrane spanning domains (Supplemental Figure 4). It belongs to a subclade composed by 5 paralogues, with identities ranging from 81 to 49% (Supplemental Table 1); however, their expression levels are much lower than UUAT1 (Supplemental Figure 3); provided these results, we decided to focus our study on *UUAT1*, analyzing its role in the biosynthesis of seed coat mucilage."

Point 2. One of the three alleles (dubbed *uuat1-2* by the authors) resulted in a loss of function and was chosen for detailed analysis. A study of the mutant plants by methods of carbohydrate chemistry indicated a marginal (but statistically significant) reduction in rhamnose and galacturonate content in seed coat mucilage that is consistent with (but not necessarily indicative of) a UDP-GlcA transport defect. An analysis of other cell wall or mucilage components revealed a highly significant increase in galacturonate methylation and a decrease in the abundance of an arabinan epitope that in at least some instances correlated with a reduction in the abundance of total arabinose. One noteworthy aspect of the insufficiently documented mutant screen was the application of a filtering procedure: Only genes with significant expression during seed development were considered, and the source data were taken from a publicly available database apparently without inclusion of genes that were not present on the ATH1 Genome Array that is generally used as the source of expression data for GENEVESTIGATOR and related applications. Although the vast majority of the NST-TPT genes are represented on this array, At3g10290 (the closest homolog to At5g04160) is not. This creates a situation where the authors may have inadvertently disregarded an important candidate gene, and a mutation in At3g10290 (or a At5g04160, At3g10290 double mutation) may lead to much more significant phenotypes than those described in this manuscript.

RESPONSE: *At5g04160*/ *UUAT1* co-expresses with *MUM4*, a gene involved in the synthesis of UDP-rhamnose and it is critical for mucilage biosynthesis in Arabidopsis. Furthermore, it also co-expresses with *URGT2*, a UDP-rhamnose/UDP-galactose transporter, but they belong to different clades. Since UDP-rhamnose and UDP-uronic acid incorporation to the Golgi apparatus is critical for mucilage biosynthesis; and it is already known that URGT2 is involved in the transport of UDP-rhamnose, it is likely that At5g04160/UUAT1 may play a role transporting UDP-glucuronic acid, the other substrate needed for the synthesis of rhamnogalacturonan I, the most abundant polymer in mucilage. On the other hand, *At3g10290* is the closest paralogue (Supplemental Figure 1) but in contrast to *UUAT1*, it exhibits low expression during seed development (Supplemental Figure 3); therefore, we did not consider it as an important player in the biosynthesis of seed coat mucilage. Furthermore, we measured the transcript levels of At3g10290 in other organs (leaves, roots) and it was also expressed at very low levels (data not shown). This information, in addition to the fact that we did not find mutants in At3g10290; to analyze the phenotype associated to the lack of this putative UDP-uronic acid transporter; led us to focus on *UUAT1.*

Point 3. The authors used a biochemical assay system to bolster their claim that they identified a bona fide UDP-GlcA NST but did not take into account that the second partner of these antiport systems cannot be predicted with certainty. To their credit, the authors used all four nucleoside monophosphates (AMP, GMP, CMP, and UMP), and observed seemingly significant antiport activity with UMP as one partner, and UDP-GlcA, UDP-GalA, and UDP-Arap as the second partner. Somewhat curiously, the UDP-Arap / UMP antiport activity is dismissed because of a lower transport

rate compared to UDP-GlcA and UDP-GalA but this does not strike me as a valid argument against a role of At5g04160 in UDP-Arap transport.

RESPONSE: As the reviewer pointed out there was a low transport activity when UDP-Ara*p* was used as substrate and we dismissed because, in addition we had also observed this low activity in other nucleotide sugar transporters such as the UDP-rhamnose/UDP-galactose transporters (URGTs) as well as the UDP-xylose transporter (UXT). Therefore, we believe this is not a specific function of UUAT1. We comment this in the Discussion section on the new version of the manuscript.

Point 4. The most serious flaw in the experimental design is a failure to use nucleoside diphosphates in addition to nucleoside monophosphates as potential substrates. While it is true that NMPs are the usual substrates for NSTs in their electroneutral transport mechanisms, UDP-GlcA and several other nucleotide sugars carry three negative charges under physiological conditions, and NMPs carry only two in their most highly ionized state. With some modification of their experimental design, the authors may very well find that At5g04160 acts as UDP-GlcA / UDP antiporter with the possibility of transporting other nucleotide sugars. The choice of an incorrect antiport partner may explain the unexpectedly high Km value reported in this manuscript. In summary, there is a considerable likelihood that At5g04160 functions as an NST with UDP-GlcA as one of the antiported substrates; however, the genetic evidence is by far too circumstantial to substantiate this claim, and the biochemical studies are inconclusive. Substantial further experimentation is needed to deduce the function of At5g04160 with the required degree of confidence.

RESPONSE: As the reviewer pointed out, the ionic state of UDP-glucuronic acid has an extra negative charge compared to other nucleotide sugars due to the carboxyl group present in the sugar. Then, it is reasonable to think that due to charge balance, UMP, the usual substrate for the antiporter mechanism of transport, may not be the most suitable substrate in this process and he suggested that UDP might be the likely antiporter substrate. Even though no measurements of the UDP content in the Golgi have been performed, the fact that an UDPase/Apyrase is located in the Golgi lumen (Orellana et al 1997; Chiu et al 2015) indicate that UDP is likely quickly metabolized to UMP and inorganic phosphate. Support for this idea came from the analysis of the metabolism of a nucleotide sugar (UDP-glucose) in the plant Golgi, suggesting that nucleotide sugars are rapidly metabolized and transformed into UMP and inorganic phosphate as final products (Neckelman and Orellana, 1998). Therefore, UDP may be present at very low levels. In any case, following the suggestion of the reviewer, we performed the assay using UDP instead of UMP. As it can be seen in Supplemental Figure 6 using UDP as antiporter substrate had no effect, and no transport of UDP-GlcA was observed under these conditions (This is also described in the results section). This result poses a question about the antiporter mechanism, a topic that needs to be addressed in future work.

Reviewer #2 (Comments for the Author):

Over 30 % of the world biomass (cell walls) requires that the nucleotide-sugar UDP-GlcA and its derivatives be made, transported to Golgi, and further metabolized to other activated-sugars that, in turn, are incorporated into different glycan types that are then integrated into cell wall polysaccharides.

The manuscript by Saez-Aguayo et al., is the first to describe and identify a Golgi transporter with such an important function in biomass production. The study of transporters is hard and not always appreciated. Here the group led by Ariel Orellana provides convincing and elegant biochemical, genetic and cell biology data to identify an important transporter - UDP-uronic acid. This transporter is not only significant to our understanding of how the wall is made, but it also provides information on how the flux of NDP-sugars between the cytosol and Golgi is perhaps regulated. Thus, I believe this manuscript is of general importance to the entire field of plant sciences. Beyond the finding of the transporter, I found it very intriguing that the knockout of this gene led to an altered glycan, in particular, to an increase in methyl esterified-homogalcturonan. Overall I think the manuscript is well written. I do have minor editorial comments to make and some specific but minor scientific questions that should be easily handled by the authors.

Point 1. Introduction: line 93. The Ahn 2006 paper does not describe the formation of UDP-apiose. You can refer, for example, to Guyett P et al. (2009).

RESPONSE: We have changed the reference following the suggestion of the reviewer.

Point 2. line 94. The authors should mention that UDP-arabinose pyranose is also made by a cytosolic enzyme (I forget the enzymatic/gene reference, but it is one of the predicted UDP-glc 4-epimerases that has dual activities). This cytosolic activity also explains that the cytosolic UDP-Arap is a precursor to UDP-Araf .

RESPONSE: We addressed this comment of the reviewer in the beggining of the Discussion section.

Point 3. line 94. The authors should also mention that a GalA kinase exists and this is used by a Sloppy PPase to give UDP-GalA in the cytosol (Yang et al 2009). This cytosolic metabolite may also be transported to Golgi, even though the GalAK and Sloppy enzymes belong to salvage system. It may work in pollen, during seed development or in stem organs. Such interpretation may fit in the Discussion section.

RESPONSE: We have addressed the comment of the reviewer in the discussion section.

Point 4. line 95. In the Introduction it would be important to add that the Golgi-localized NDP-sugar converting enzymes have their catalytic domain facing the Golgi (they are type-II). Thus, a transport mechanism from the cytosol to the compartment lumen is required.

RESPONSE: Following the comment of the reviewer, we have modified the text as suggested.

Point 5. line 103. The use of the wording "these scenario" is not clear. The authors should mention that the UDP-GlcA transporter was identified in animal systems (also give ref). Therefore, it is reasonable to find similar activity in plants. Further more, I think Dr. Orellana has an earlier work describing biochemically a transport assay of UDP-GlcA....so there is a compeling reaon for such a transporter in plant.

RESPONSE: We have replaced the phrase on line 103 (former version of the ms) by referring to the transport of UDP-GlcA observed in animal cells, which leads to abnormal development when is mutated.

Point 6. Results: line 159. I think Table 1S is a typo. Also, add a period after "Fig" (Fig.)

RESPONSE: Following the comment of the reviewer, we have modified the text as suggested.

Point 7. line 169. It is not clear how the authors determine what constitutes a promoter. Was it the generic 2,000 bp upstream the AUG? If so, please indicate.

RESPONSE: We cloned the intergenic region bewtween genes At5g04160 and At5g04170. This is now described in the Methods section.

Point 8. Fig. 1A. I am not sure what the transcription data (by someone else) provides. If the authors have their own transcript data, then they should show it.

RESPONSE: The transcription data from seed development was taken from public databases EFp Browser, using the seed section. The data obtained for this section, is the one published by Le et al., 2010 and used as a “proxy” that allowed us to identify NSTs being expressed during seed development; then, analyze mutants on those genes to explore changes in levels of uronic acids in mucilage. We have introduced some changes in the results section describing this process (See response #1 to reviewer 1). Once *UUAT1* was identified as a candidate, we quantified the transcript content of *UUAT1* and its closest paralogues located in the subclade, using qPCR (Supplemental Figure 3). This data is now shown as supplementary data. We have moved the *in silico* transcription data from Figure 1 to Supplemental Figure 1.

Point 9. "following the molecular analyses of UUAT1." Line 144-172. I found it odd that homologous proteins in Arabidopsis was not mentioned. I did a quick BLAST search and found 3 more genes with highly identical coding sequences to UUAT1. Please prepare a Table to indicate those. I assume the authors will discuss these later in the manuscript

RESPONSE: We have introduced in the results section a phrase that refers to the paralogues present in the subclade where UUAT1 is located. In addition, we made a comparison of the identity at the protein level among the members of the subclade, introducing a table (Table S1) showing this data. We have also measured the transcript levels during seed development of all five members of this subclade, and we focused on *UUAT1* since it the most expressed one (Supplemental Figure 3).

Point 10. Fig. 2: 2A is not clear. Several questions:

a) What is the control?

RESPONSE: The control assay uses proteoliposomes isolated and prepared from microsomes of yeasts transformed with the empty vector, in precisely the same manner as UUAT1. The control is now detailed in the Methods section.

b) What is proteo-liposome (is it a yeast microsome?)?

RESPONSE: A proteo-liposome is an artificial vesicle formed from phospholipids and membrane proteins extracted from a microsomal membrane fraction that may contain (or not, in the case of the control) UUAT1. This is now detailed in the Methods section.

c) Add an explanation why microsomes need to be pre-loaded with UMP (is it to deal with inside-out vesicles?).

RESPONSE: Nucleotide sugar transporters (NSTs) are antiporters and all NSTs use a nucleoside monophosphate as a counter substrate that moves in opposite direction to the nucleotide sugar, the other substrate of NSTs. Since the proteoliposomes containing UUAT1 are incubated with a nucleotide sugars that are outside the vesicle, the nucleoside monophosphate is needed inside the vesicle. This is achieved by pre-loading the proteoliposomes with UMP.

d) While I intuitively understand Fig. 2B, what does the Y axis mean? Is it the amount of activated-sugar detected inside the lumen of the yeast ER?

RESPONSE: The y-axis of Figure 2B is the amount of nucleotide sugars detected after the transport activity of UUAT1 containing proteoliposomes and the control. These proteo-liposomes were pre-loaded with UMP in order to act as the antiporter substrate needed for the incorporation of UDP-GlcA. We are unable to measure the endogenous luminal nucleotide sugars or nucleotides from yeast microsomes, any substrate is depleted during the proteoliposomes preparation and therefore must be pre-loaded prior to the transport assay.

e) Fig. 2C. If the authors have kinetic data to show transport of UDP-GalA, they should include it. It may suggest a preference for a particular UDP-uronic acid.

RESPONSE: We never conducted a kinetic analysis of UUAT1 with UDP-GalA, although we have shown that it can transport this substrate (Fig 2B). We did not undertake a detailed analysis, as it would be meaningless since the vast majority of UDP-GalA is biosynthesized within the lumen of the Golgi from UDP-GlcA. Only scavenged UDP-GalA (from GalA) would be transported by UUAT1 and we have no information as to the contribution or concentration of this substrate within the cytosol. Thus the main task for UUAT1 would be the transport of UDP-GlcA. Moreover, given their structural similarities, we would guess their Km's to be similar.

Point 11. line 201. What do the authors mean by "UUAT1 is saturable"? Based on Fig. 2C, it is obvious that saturation had not yet been achieved. The same is true of 'time dependable manner'. I would omit this sentence, unless the authors wish to convince me otherwise.

RESPONSE: We agree that there is not a great response with increased concentration, however there is a clear response over time (2 min). We will adjust the statement accordingly. Added to manuscript (Results section):

“Transport of UDP-GlcA by UUAT1 did not achieve saturation within the concentration range utilized in the assay (Fig. 2C), however transport was effected in a time dependent manner (Fig. 2D). Analysis of transport rates indicated that UUAT1 has an apparent Km for UDP-GlcA of 1.5 mM (Fig. 2C).”

Point 12. line 202. Kinetics. If the authors have Kcat /Km values, then it would provide catalytic efficiency values and the turnover number. While this is hard to achieve (I would imagine) because there is not a pure enzyme in the microsome prep, I suggest the authors consider running some of these calculations -- kinetics has immense biological value for a biochemist like me ☺.

RESPONSE: As we indicated in the Discussion, the apparent Km is higher than we would anticipate, especially when compared to the cellular concentration of UDP-GlcA. While it is clear that UUAT1 has preference for UDP-GlcA/GalA, the data indicate that there is a missing component in the assay e.g. cofactor, protein partner etc. This is currently under further investigation, but the potential permutations make this an arduous task. As a consequence, we decided it would be unhelpful to provide values, such as kcat, as it is likely to be biochemically meaningless in this instance.

Point 13. Fig. 3 and after line 217. I suggest the authors consider adding Kyte Doolittle's hydrophaty plot as a a pannel in figure 3 and perhaps indicate the theoretical membrane spanning domain UUAT1 has. Perhaps it can be useful (in the Discussion) to compare the number of membrane spanning transporters(?) with other transporters.

RESPONSE: We introduced a phrase in the results section referring to the putative features of the predicted protein and included a supplemental figure containing a Kyte-Doolittle hydropathy plot (Supplemental Figure 4)

Point 14. Fig. 3- what is AtWAK?

RESPONSE: Arabidopsis thaliana Wall associated kinase-2 (It is now incorporated in the figure legend)

Point 15. line 254. Are the authors referring to wall stiffness or tissue stiffness in this context?

RESPONSE: In this context we refer to wall stiffness.

Point 16. line-288. The GUS stain exp. I am not sure what the word ubiquitous is adding - remove it. Based on the leaf staining it seems the transcript is predominantly in the trichome region (so it is not ubiquitously expressed).

RESPONSE: The text was changed according to the recomendation of the referee.

Point 17. Discussion: lines 320-324 . This sentence needs re-wording. UDP-GlcA as you know is required and utilized in the cytosol and is being utilized in the Golgi lumen.

RESPONSE: Following the comment of the reviewer, we have modified the text as suggested.

Point 18. line 342. Kinetics. Unless the authors have Kcat/Km values the argument of affinity and metabolite cell concentration may not be relevant or indicative. I would simply omit lines 338-346 since no transport turnover rate values are provided.

RESPONSE: Following the comment of the reviewer, in the new version of the ms we deleted the section dealing with the kinetics.

Point 19. The authors should provide some data or discussion comparing the different 'potential homologs' of UUAT in the Arabidopsis genome. Expression? Membrane spanning domain? Compare to other transporters including the human UDP-GlcA transporter?

RESPONSE: Following the comment of the reviewer, we have modified the text as suggested. Furthermore, we have introduced a table in supplemental data containing a comparison of the paralogues and the degree of protein identity among them (Supplemental Table 1).

Point 20. Methods.

a) The manuscript lacks information regarding the cloning and over-expression of the plant genes (and or/controls) in yeast, and of the isolation and formation of liposomes.

RESPONSE: Added to the manuscript (Methods):

In Vitro Transport Analyses of UUAT1

Heterologous expression employed the uracil-auxotrophic *Saccharomyces cerevisiae* (strain INVSc1: MATa his3D1 leu2 trp1-289 ura3-52 MAT his3D1 leu2 trp1-289 ura3-52, Thermo Fisher Scientific). The coding sequence of UUAT1 was cloned into Gateway expression vector pYES-DEST52 (Thermo Fisher Scientific) and introduced into the yeast strain with the Sc EasyComp Transformation Kit (Thermo Fisher Scientific). The control was the yeast strain transformed with the vector pYES-DEST52. Microsomal fractions were obtained from 500 mL cultures grown at 30 °C. Yeast were pelleted and spheroplasts produced in 10 mL resuspension buffer (50 mM potassium phosphate, pH 7.1, 1.4 M sorbitol, 10 mM NaN3, and 40 mM 2-mercaptoethanol, 6,000 u Lyticase [Sigma-Aldrich]) for 1 h at 37 °C. Spheroplasts were harvested by centrifugation and washed with 0.8 M sorbitol, 10 mM triethanolamine / acetic acid pH 7.2, 1 mM EDTA. The spheroplasts were lysed with glass beads in 5 mL 0.8 M sorbitol, 10 mM triethanolamine / acetic acid pH 7.2, 1 mM EDTA, protease inhibitor cocktail and 1 mM PMSF. Microsomes were isolated by sequential centrifugation (8,000 g for 10 min (F1), and 100,000 g for 75 min (F2)). The F2 fraction was reconstituted in 10 mM Tricine-KOH pH 7.5, 50 mM potassium gluconate, 20% glycerol. Proteo-liposomes were generated with 600 mg of acetone washed soybean L-**α**-phosphatidylcholine (Avanti Polar Lipids) in reconstitution buffer (10 mM Tricine-KOH pH 7.5, 50 mM potassium gluconate and 20 % glycerol). Reconstitution of microsomal membranes obtained from the

yeast expressing UUAT1 or the control was undertaken using around 400 µg microsomal protein in reconstitution buffer, lipid at a ratio of 13 (lipid:protein), 10 mM exchange substrate and 50 mM octyl-β-glucoside. Unincorporated components were removed from reconstituted liposomes using Sephadex G50 (GE Healthcare). Aliquots of 200 µL were incubated with nucleotide sugar substrates at 25 °C for indicated times to assess transporter activities. Kinetic parameters were calculated by non-linear regression using the Prism 7 application (GraphPad Sofware, La Jolla, CA). Polyacrylamide gel electrophoreses was undertaken with 2.5 µg protein of proteo-liposomes on a 7-15 % SDS-PAGE gel. Immunoblotting was conducted with the anti-V5 antibody using a dilution of 1:10,000 (Thermo Fisher Scientific).

b) Analyses of nucleotide-sugars. This information must be included in the manuscript.

RESPONSE: Added to the Manuscript (Methods)

Analysis of Nucleotide Sugars by Tandem Mass Spectrometry

The transport assay reactions were purified using ENVI-Carb SPE columns (Sigma-Aldrich) and then lyophilized overnight as outlined previously (Ito et al., 2014), prior tandem mass spectrometry (LC-MS/MS). Nucleotide sugars were separated using a Hypercarb column (1**50 mm × 1 mm, 5 µm) at a flow rate of 50 µL min**-1 with an 1100 series HPLC system (Agilent Technologies, CA) and a 4000 QTRAP LC-MS/MS system (Sciex, CA) equipped with a TurboIonSpray ion source. Initial conditions were 95 % buffer A (LC-MS grade water with 0.3 % formic acid, pH 9.0 with ammonia) and 5 % buffer B (100 % acetonitrile) for 1 min followed by a gradient to 75 % (A) in 20 min, then 50 % (A) in 5 min before returning to 95 % (A) in 5 min. The QTRAP was operated in negative ion mode using the multiple reaction monitoring (MRM) scan type. A declustering potential (DP) of -40, entrance potential (EP) of -10, collision cell exit potential (CXP) was -15. The ion spray voltage was set at -4200 V, source temperature (TEM) at 425 °C, collision gas (CAD) was set to High and source gas 1 (GS1) and 2 (GS2) were both set to 20. A time of 100 ms was applied for each transition resulting in a duty cycle of 1.0501 s with both Q1 and Q3 resolution set to Unit. All data were acquired using Analyst 1.6 Build 3773 (Sciex, CA) operating in MRM mode. Nucleotide sugars were quantified using MultiQuant 2.1 (build 2.1.1296.02.1) software (Sciex, CA) by integrating the signal peak areas of samples against a range of nucleotide sugar standards (2.5 to 20 pmol).

c) The manuscript lacks information regarding the cloning of the UUAT to GFP.

RESPONSE: In the Methods section we described information about the cloning of UUAT1 to GFP: "Resultant PCR products were introduced in the pENTR 5-TOPO vector (Thermo Fisher Scientific) to generate the pENTR5-pUUAT1 entry clone. Both C-terminal GFP and HA fusions were obtained by recombining the entry clones pENTR-UUAT1 and pENTR5-pUUAT1 with destination vectors R4pGWB504 and R4pGWB513, respectively"

Reviewer #3 (Comments for author):

Point 1. I suggest modifying the title of the paper. The use of the phrase "defines the content" overemphasizes the role of UUAT1, which is more accurately identified as "one of the factors that that defines cell wall content". Furthermore, the Rha content of the mucilage is reduced to a greater extent than GalA or Ara and this is not reflected in the title.

RESPONSE: Following the suggestion of the reviewer we changed the title. The new title is now: "UUAT1, a Golgi-localized UDP-uronic acid transporter, plays a role defining the cell wall content of Arabidopsis seed mucilage."

Point 2. Some important information about the initial mutant screen is missing from the first section of the results. First, the introduction states that a mucilage release screen was performed and this was a good approach. However, the results are never reported in manuscript. We are only told at the end of the section that the mutant lines rescued by expression of the wild type gene have "wild type mucilage release". This is not meaningful without the context of the screening results. Second, lines 153-154 state that the authors tested "mucilage sugar composition in insertion lines obtained for the remaining 11 NST candidates". However, Table 1S shows only results for GalA and Rha for the three *uuat1* mutants and provides no additional information beyond what is reported in Fig. 1C. The manuscript would be strengthened by including the complete analysis with statistical significance in Table 1S. Finally, line 172 states that mucilage sugar contents in rescue lines were returned to wild type levels, but no statistical analysis comparing rescue lines to wild type and mutants is reported.

RESPONSE: We have addressed this comment above (See response to reviewer 1, first comment). It is now described at the beginning of the results section.

Point 3. In lines 261-263, the authors make the point that the *uuat1-2* mutant rescued with UUAT1 has reduced labeling of methylated HG compared to the mutant and this is well supported by additional results. Interestingly, the rescued mutants appear to have substantially more methylated HG than the wild type. This point deserves mention in the results and discussion.

RESPONSE: The reviewer is right, *uuat1-2* plants were rescued by the expression of the gene, although this was partial. This is now described in the results and discussion section.

There are different reasons why the rescue was only partial. Among them we can cite the following: 1) The upstream region to the *UUAT1* coding region did not contain all the elements required for the full expression of the protein. 2) The rescue was performed with a fusion protein between UUAT1 and GFP. It is possible that this protein, even though is active may not be fully functional. 3) The insertion locus of the transgenic plants may be in a context that is different to the normal *UUAT1* locus leading to differences in expression.

Point 4. The discussion is rather long and could be shorten substantially without sacrificing meaningful interpretation of the data. For example, in the paragraph that runs from line 408 to line 419, the authors begin an attempt to explain why only Ara, but not Xyl and GalA, are reduced in the root and pollen tube walls of uuat1 mutants. But, general statements like "the biosynthetic machinery in each organ responds differently to the lack of UUAT1" and "the altered influx of UDP-GlcA has a larger impact on this metabolic pathway" are not informative. The next paragraph speculates that the GalA in the cell walls of these tissues is derived from UDP-GlcA transported to the Golgi lumen by other NSTs. Here it would presumably be available for synthesis of Xyl as well as GalA. The paragraph after that suggests that UDP-Xyl is supplied to the Golgi lumen by UXT1. These are a few of many possible explanations for these results. In my opinion, the important points in these three paragraphs could be summarized in a single short paragraph. The rest of the discussion could also be condensed by careful revision and this would increase the impact of the paper.

RESPONSE: We have considered the comment of the reviewer and condensed the information previously located on lines 408 to 445. The Discussion section also has been shortened.

Point 5. The concluding statement at the end of the abstract, that "a UDP-GlcA transporter defines the sugar composition of the plant cell wall" is too broad and should be narrowed to encompass only the seed mucilage. My comment regarding the use of the phrase "defines the content" in the title applies here as well. The conclusion as now stated is not strongly supported by the data.

RESPONSE: Following the recommendation of the reviewer we have modified this section of the abstract as it follows: “These results suggest that a UDP-GlcA transporter plays a role defining the sugar composition of the seed mucilage and its absence produces pleiotropic effects in this extracellular matrix”

TPC2016.00465-RAR1 2nd Editorial decision – *accept with minor revision*	Oct. 23, 2016

Reviewer 1 suggests several minor, mostly grammatical, changes to the manuscript text. Additionally, Reviewer 3 notes that statistical analysis of the results presented in figures 1C and S5C was not included in the revised version of the manuscript. If possible, I would suggest including this analysis, or alternately provide a response explaining the rationale for these being left out.

TPC2016.00465-RAR2 2nd Revision received	Nov. 14, 2016

TPC2016.00465-RAR2 3rd Editorial decision – *acceptance pending*	Nov 15, 2016

We are pleased to inform you that your paper entitled "UUAT1, a Golgi-localized UDP-uronic acid transporter, plays a role defining the polysaccharide composition of Arabidopsis seed mucilage." has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership. The Plant Cell has appointed several Ph.D. Plant Scientists to serve as Science Editors in this capacity, and you will receive further information on this process very shortly.

Final acceptance from Science Editor	Dec. 31, 2016