**The Myo-inositol pathway does not contribute to ascorbic acid synthesis**

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**ABSTRACT**

- Ascorbic acid (AsA) biosynthesis in plants predominantly occurs <i>via</i> a pathway with D-mannose and L-galactose as intermediates. One alternative pathway for AsA synthesis, which is similar to the biosynthesis route in mammals, is controversially discussed for plants. Here, myo-inositol is cleaved to glucuronic acid and then converted <i>via</i> L-gulonate to AsA. In contrast to animals, plants have an effective recycling pathway for glucuronic acid, being a competitor for the metabolic rate. Recycling involves phosphorylation at C1 by the enzyme glucuronokinase.

- Two previously described T-DNA insertion lines in the gene coding for glucuronokinase (vtc1) show wild type-like expression levels of the mRNA in our experiments and do not accumulate glucuronic acid in labelling experiments disproving that these lines are true knockouts. As suitable T-DNA insertion lines were not available, we generated frameshift mutations in the major expressed isoform glucuronokinase1 (At3g01640) to potentially redirect metabolites to AsA.

- However, radiotracer experiments with <sup>3</sup>H-myoinositol revealed that the mutants in glucuronokinase1 accumulate only glucuronic acid and incorporate less metabolite into cell wall polymers. AsA was not labelled, suggesting that Arabidopsis cannot efficiently use glucuronic acid for AsA biosynthesis.

- All four mutants in glucuronokinase as well as the wild type have the same level of AsA in leaves.

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**INTRODUCTION**

Ascorbic acid (AsA) is an important antioxidant in plants and mainly involved in the removal of reactive oxygen species (ROS). Humans need to take up AsA with their food (vitamin C), which results in broad scientific interest in AsA biosynthesis and regulation in plants. The role of AsA for plants, the biosynthesis and attempts to increase AsA levels through transgenic approaches is covered by numerous comprehensive reviews and publications (e.g. Wheeler et al. 1998; Ishikawa et al. 2006). Early feeding experiments with radioactive precursors revealed that the biosynthesis of AsA follows different routes in plants and animals. The mammalian route involves UDP-GlcA (UDP-D-glucuronic acid) to attach a GlcA sugar to an unknown metabolite, from which, through the action of a glucuronidase, GlcA is cleaved off (Linster & Van Schaftingen 2007). GlcA is then reduced to L-gulonate, dehydrated to gulono-1,4-lactone and finally oxidised to AsA (Fig. 1). This route could not be confirmed for plants using tracer experiments (Loewus et al. 1956, 1962). Instead, it was found, that the label from myo-inositol goes into hemicellulosic and pectins of the plant cell wall via GlcA and UDP-GlcA (Loewus et al. 1962). New radiotracer feeding experiments revealed the AsA biosynthesis route in plants, in which D-mannose is activated to GDP-mannose, epimerised to GDP-L-galactose and hydroxylised to L-galactose (Fig. 1). This metabolite undergoes lactone formation and is finally oxidised to L-AsA (Wheeler et al. 1998). This route is backed up by mutants like vtc1, vtc2 and vtc3 which have reduced levels of AsA or the mutations are even lethal during embryogenesis (Conklin et al. 1997; John et al. 2007). Therefore the predominant route to AsA is the D-Man/L-Gal pathway, and the presence of alternative pathways has long been debated in plants (Smirnoff et al. 2001; Wheeler et al. 2015). The two favourite candidates are the D-galacturonate pathway and a mammalian-like pathway which use D-glucuronic acid. The mammalian-like pathway was suggested in two publications. In the first one, the enzyme myo-inositol oxygenase (MIOX) was overexpressed in Arabidopsis resulting in a two- to three-fold increase of AsA (Lorence et al. 2004). MIOX catalyses the oxygen-dependent ring cleavage of myo-inositol to GlcA. A follow-up paper identified alkaline phosphatase acting on phytic acid as a possibility to increase AsA. The complete dephosphorylation of phytate releases myo-inositol. When the phosphatase was overexpressed in Arabidopsis a two-fold increase in AsA was observed (Zhang et al. 2007). The steady level of phytate is reduced by ca. 7.5 nmol g FW<sup>-1</sup> in the overexpressor lines. The observed increase in AsA is ca. 3 μmol g FW<sup>-1</sup>. This would indicate that the flux through the phytate pathway must be 400 times higher than the flux of AsA turnover.

In independent experiments with the MIOX-overexpressor lines, the increase of AsA could not be confirmed, although the flux from myo-inositol into cell wall polymers was indeed increased (Endres & Tenhaken 2009). Furthermore, a...
quadruple knockout mutant in the whole MIOX gene family resulted in plants with the same AsA levels as measured in WT plants (Endres & Tenhaken 2011). The overexpression of MIOX genes in tomato (Cronje et al. 2012) or rice (Duan et al. 2012) also did not lead to increased AsA levels.

The mammalian-like pathway to AsA starting from d-GlcA requires three enzymes to catalyse the conversion of d-GlcA into AsA. First, d-GlcA is reduced to l-gulonate by glucuronoreductase. The second step involves the formation of a lactone catalysed by aldonolactonase. The product l-gulono-1,4-lactone is then finally oxidised to l-AsA. Using bioinformatics Ruggieri et al. (2016) found some candidate genes in tomato for the reductase (first step), but no candidate gene for the second step. Two candidate genes were predicted for the final oxidase step in Fig. 1). These data do not confirm enzyme activity or physiological relevance. Aboobucker et al. (2017) recently characterised two genes from Arabidopsis with l-gulono-1,4-lactone oxidase activity. The enzymes have a high K_m (33 mM) and a very low K_cat (0.005 s^{-1}). The terminal enzyme of the well-established Wheeler-Smirnoff pathway, l-galactono-1,4-lactone dehydrogenase, is far more active, with a K_m of 0.17 mM and a K_cat of 134 s^{-1}. This corresponds to a more than 25,000-fold higher catalytic activity of ④ compared to ⑤. Maruta et al. (2010) overexpressed candidate genes for l-gulono-1,4-lactone in tobacco BY2 cell cultures. They found no increase in AsA in overexpressing lines. Only after feeding high concentrations of l-gulono-1,4-lactone (10 mM) did the BY2 cells have elevated levels of AsA. Aboobucker et al. (2017) also overexpressed the l-gulono-1,4-lactone dehydrogenase genes in Arabidopsis without any increase in AsA levels.

Unfortunately, most of the papers describe the overexpression of genes as the starting point and the outcome in AsA levels at the end, without further measurements of metabolites which might support the data. Here we show that a frameshift mutant in the gene for glucuronokinase1 (GlcAK1) has a reduced flux of GlcA into cell wall polymers. Radiotracer experiments with myo-inositol show no flux of labelled GlcA into the AsA pool but instead accumulate GlcA. Thus, GlcA cannot be metabolised to AsA. The level of AsA in these mutants is the same as in WT plants.

**MATERIAL AND METHODS**

**Plant material and growth conditions**

In this work Arabidopsis thaliana (ecotype Columbia 0) was used as a WT plant. Previously described T-DNA insertion mutants glcak1-1 (SALK_076931) and glcak1-2 (SALK_127949c) were obtained from the Arabidopsis Biological Resource Center and another two mutant lines glcak1-3 (contains CAS9 nuclease) and glcak1-4 were created by CRISPR/Cas9 technology. Plants were grown in a growth chamber in pots on standard soil (type ED73) under long-day conditions with 16-h light at 150 μmol·m^{-2}·s^{-1}. Temperature during the light phase was 23 °C and 18 °C in the dark. For RT-PCR and labelling experiments seedlings were grown in liquid medium. Seeds were surface sterilised in ethanol and grown in 0.5 × MS (Basal Salt Mixture, Duchefa #M0245; Duchefa, Haarlem, the Netherlands), pH 5.7 (KOH), with 1 g l^{-1} sucrose for 10 days. Then the medium was changed to 0.5 × MS (or 0.5 × MS with 0.37 MBq myo-[2-^3H]-inositol; final concentration in medium is 0.625 μM) for the next 3 days. For sugar measurements sterile seeds were incubated on 0.5 × MS (without micronutrients; Duchefa #M0221), pH 5.7 (KOH), plates with 0.8% plant agar. Harvested samples were immediately used for experiments or frozen in liquid nitrogen and stored at −80 °C until used.

**Real-time PCR**

Total RNA was isolated from 13-day-old seedlings using Tri-Reagent method. The aqueous phase containing the RNA was further purified on a silica spin column and eluted in 40 μl DEPC-H₂O. Subsequently, RNA was converted to cDNA by RevertAid Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA) using an anchored oligo(dT) primer. Real-time PCR was performed with a Mx3000P qPCR system (Stratagene, San Diego, CA, USA) and PCR products were detected by SYBR green fluorescence. The obtained values were analysed using the 2^{−ΔΔC_T} method (Livak & Schmittgen 2001). RT-PCR primers are listed in Supplementary Material.
Resequencing of the position of the T-DNA insertion in GlcAK1 mutants

Position of the T-DNA insertion in glcak1-1 and glcak1-2 was determined by direct sequencing of PCR products obtained with primers binding to the left border of the T-DNA insertion and on exon1 of GlcAK1 (Table S1). Genomic DNA was isolated from a leaf of a 4-week-old plant by standard methods and 1 μl was used as a DNA template for PCR (reaction volume 30 μl). PCR product was purified with the GeneJET PCR Purification Kit (Thermo Scientific).

Construction of CRISPR/Cas9 knockouts

Mutants in GlcAK1 were generated using CRISPR/Cas9 technology (Fauser et al. 2014). A. thaliana (ecotype Columbia 0) were transformed with the CRISPR/Cas9 construct by Agrobacterium tumefaciens (strain GV3101) according to direct-dip protocol (Davis et al. 2009). Plants with a homozygous mutation in GlcAK1 were selected in T2 generation by sequencing of a PCR product spanning the targeted region of exon1 (Table S1). In this study, two lines, glcak1-1 and glcak1-2, were used.

Feeding of seedlings with 3H-myoinositol followed by metabolite separation on HPLC

Ten-day-old seedlings labelled with myo-[2-3H]–myo-inositol for 3 days were washed twice with 0.5 × MS medium containing 8 mM myo-inositol to exchange non-specifically bound 3H-myoinositol. The seedlings were carefully dried with a soft cosmetic tissue and snap frozen in N2 with two 3 mm stainless steel balls. Seedlings were homogenised in a liquid N2 cooled mortar and vortexed several times in between. A total of 350 μl H2O was added and samples were incubated in a cooled shaker for 10 min. After centrifugation, the upper phase was transferred to a new reaction tube and dried in a vacuum centrifuge. The dry pellet was re-dissolved in 10 μl H2O. 1 μl of this sample were mixed with 900 μl NaH2-acetate and applied to a Hilic column (125 × 4 mm Nucleodur 100-5; Machery-Nagel, Düren, Germany). HPLC analysis was performed with 100 mM Na-acetate (buffer A) and acetonitrile (buffer B) using isocratic conditions (80% B; flow rate 0.6 ml·min⁻¹). Samples were collected using a fraction collector, mixed with 2 ml scintillation cocktail (Rotiszint eco plus; Carl Roth, Karlsruhe, Germany) and counted. Standard compounds (AsA, myo-inositol and GlcA) were separated under the same conditions.

and an acetone extraction step. The pellet corresponding to crude cell walls was air-dried and counted with 2 ml scintillation cocktail (Rotiszint eco plus; Carl Roth, Karlsruhe, Germany). In some cases, the lower phase of the metabolite sample, containing the insoluble fraction of the cells, was further extracted with 70% ethanol as described above.

Measurements of GlcA levels in seedlings

Free GlcA was extracted from seedlings using methanol:chloroform (7:3) extraction as described above. The dried metabolites were dissolved in 200 μl H2O and separated on a CarboPac PA20 column (150 × 3 mm) on an ICS3000-HPLC system: Buffer A: 200 mM NaOH; buffer B: 15 mM NaOH; buffer C: 50 mM NaOH and 500 mM Na-acetate; Flow rate 0.45 ml·min⁻¹; t0: 100%; t1: 100% B; t12.1: 60% B and 40% C; t20 60% B and 40% C.

Ascorbic acid measurements

The AsA was measured from individual leaves of 4-week-old plants. The fresh weight was determined, and the leaf was then immediately homogenised in 1 ml 1 st HClO4 and sea sand in a small cooled mortar. The homogenate was transferred to a reaction tube, centrifuged for 2 min, and 500 μl of the clear supernatant was neutralised to pH 5 by addition of 42 μl 5 M K2CO3 and 200 μl HEPES-KOH buffer 0.1 M pH 7. The sample was briefly stored on ice and precipitated KClO4 removed by centrifugation for 2 min. 100 μl of this sample were mixed with 900 μl Na-Pi buffer pH 5.6 and the OD262 nm determined. One unit of ascorbate oxidase (Applichem, Darmstadt, Germany) was added to the assay and carefully mixed. After 3 min, the OD262 nm reached a stable value. The difference between the two values corresponds to reduced AsA. For total AsA, 200 μl of the neutralised extract were mixed with 200 μl Na-Pi buffer (100 mM pH 7.5) and 20 μl TCEP (25 mM) and incubated at room temperature for 30 min. The amount of total AsA was measured with 200 μl extract as described above.

RESULTS

The conflicting data on the role of a mammalian-like pathway in AsA synthesis in plants was investigated in knockout mutants of glucuronokinase1 (GlcAK1). It has previously been shown that GlcAK is part of a salvage pathway for GlcA leading via GlcA-1P to UDP-Gka, a precursor for roughly half of the biomass of primary cell walls in Arabidopsis (Pielinger et al. 2010). We therefore hypothesised that blocking the pathway to GlcA-1P should potentially redirect GlcA to AsA via a mammalian-like pathway (Fig. 1).

Verification of T-DNA insertional mutants glcak1-1 and glcak1-2

To study an influence of GlcAK1 knockout on the mammalian-like pathway, previously described T-DNA insertional mutants glcak1-1 and glcak1-2 (Xiao et al. 2017) were chosen (Fig. 2A). However, when relative GlcAK1 expression in mutant lines was compared to WT, no differences were found (Fig. 2B). To confirm the position of the T-DNA insertion, both lines were

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verified by sequencing. The T-DNA insertions was found in the promoter region, which differed in the two mutants from the positions previously described (Xiao et al., 2017), but corresponded to the locations found in the databases (http://signal.salk.edu/cgi-bin/tdnaexpress; Arabidopsis Information Resource (TAIR)) (Fig. 2A). The transcription level of GlcAK1 was not decreased, probably due to the position of the T-DNA insertion, which was detected in the promoter region 197 bp upstream of the ATG start codon (Fig. 2B).

Preparation of new GlcAK1 mutants with CRISPR technology

In order to obtain plants with a loss of GlcAK1 activity, CRISPR/Cas9 mutants were prepared. The gRNA was targeting a sequence in exon1. In the T2 generation, several plants with a homozygous mutation were identified. For this study, glcak1-3 and glcak1-4 containing a frameshift mutation resulting in a premature stop codon close by were used. Both lines showed a decreased transcript level of GlcAK1, around 28% of the WT values (Fig. 2B), possibly because the non-translated part of the mRNA makes it more sensitive to degradation.

Seedlings of CRISPR/Cas9 mutants glcak1-3 and glcak1-4 accumulate GlcA after 3H-Myo-inositol feeding

To investigate whether the mutation in GlcAK1 redirects GlcA from the MIOX pathway to AsA, 3H-labelled myo-inositol was fed to 10-day-old Arabidopsis seedlings. After 3 days of feeding, radioactivity in soluble and cell wall fractions was measured. As expected, in WT and the SALK mutants (glcak1-1; glcak1-2) the main portion of the label was incorporated into cell walls (73–79%; Fig. 3A). In contrast, the CRISPR/Cas9 frameshift-mutants (glcak1-3; glcak1-4) showed reduced values of label in cell walls (32–40% incorporated 3H), whereas labelled soluble metabolites are much more abundant in glcak1-3 and glcak1-4. The SALK mutants and WT had only 21–27% of the label, but glcak-3 and glcak-4 retained 68% and 60% label in the soluble fraction, respectively (Fig. 3B). As we have generated knockout mutants only in the major expressed isoform GlcAK1, certain amounts of label go into the cell wall fraction via the second isoform of glucuronokinase.

In order to identify the 3H-labelled metabolites derived from inositol feeding, soluble fractions of WT and glcak1-3 and glcak1-4 were further separated by HPLC on a HILIC column and eluates were collected in 0.33-ml fractions (Fig. 4). The HILIC column was chosen as it allowed the separation of the three metabolites of interest, myo-inositol, GlcA and AsA. All samples were measured by scintillation counting. Surprisingly, the largest portion of the label was found in the GlcA peak, where glcak1-3 and glcak1-4 mutants accumulated around 26 times more label than WT plants. We cannot fully exclude that some of label is also present in L-gulonate, the product of the enzyme glucurono reductase if this metabolite would co-elute with D-glucuronic acid. L-gulonate neither absorbs UV light nor has an aldehyde group, which we would need to detect this compound. Whether the enzyme glucurono reductase exist in plants cannot easily be answered by bioinformatics. This enzyme belongs to a larger gene family of conserved aldehyde reductases, which, however, act on various but diverse substrates. The gene from mouse was recently identified and the enzymatic function was confirmed in knockout mice (Takahashi et al., 2012). In fractions containing AsA, no increased radioactivity was detected in any plant line.
The data show that *myo*-inositol is almost quantitatively converted to GlcA, which strongly increases in concentration when the glucuronokinase is blocked but a redirection of GlcA into other pathways including the formation of AsA is partially blocked in *MIOX* enzymes but the use for cell wall biosynthesis thereafter is well established and functional in plants, as indicated by several radioative labelling experiments, in which the flux from *myo*-inositol into cell wall material was found (Loewus et al. 1962; Seitz et al. 2000; Kanter et al. 2005; Endres & Tenhaken 2011). Furthermore, *ugd2,3* mutants in the biosynthesis of UDP-GlcA, which have a defect in the formation of UDP-GlcA via UDP-Glc by the enzyme UDP-glucose dehydrogenase, show a severe root phenotype (Reboul et al. 2011). The short roots and the defects during development can be largely rescued by feeding *myo*-inositol or GlcA, which *via* the salvage pathway provides UDP-GlcA to the plant mutants. So, if plants would also use GlcA for AsA biosynthesis, there would be competition between the cell wall pathway *via* glucurononokinase and the hypothetical conversion to l-gulonate and further to AsA.

We have generated frameshift knockout mutants (*glcak1-3; glcak1-4*) in the GlcAK1 gene as several of the available T-DNA lines did not have changed relative gene expression compared to WT when tested. When low concentrations of *3H*-myo-inositol was fed to WT and the *glcak1-3* or *glcak1-4* mutants a clear difference was found in the labelled products. Whereas less *3H* label is present in the cell wall fraction of *glcak1-3/1-4*, a massive accumulation occurred in the soluble metabolites. A detailed HPLC analysis of the soluble products clearly shows that the label accumulates as GlcA in *Arabidopsis*. The inositol is quantitatively converted to GlcA by the MIOX enzymes but the use for cell wall biosynthesis thereafter is partially blocked in *glcak1-3* and *glcak1-4*. If plants use GlcA for AsA biosynthesis we would have expected to find the label in AsA rather than its accumulation in GlcA. The amount of fed *3H*-inositol is less than 1 μM excluding a perturbation of the metabolism by overloading the pathway. AsA is typically present in concentrations higher than 1000-fold (low mM range). Here we show that the flux from *myo*-inositol to AsA
does not occur in Arabidopsis, because accumulated GlcA cannot be converted to AsA. The Loewus group came to a similar outcome, but their data could also be explained by the predominance of the salvage pathway to cell wall precursors (Loewus et al. 1962). The possible explanation for the accumulation of label in GlcA might be due to absence of glucuronate reductase, which is responsible for the conversion to L-gulonate. To our knowledge, there are no clear data for the presence of this enzyme in plants.

The studies of Lorence et al. (2004) and Zhang et al. (2008) rely on a pathway which, according to the data presented in this paper, does not exist in Arabidopsis. Furthermore, AsA measurements in the same MIOX4-overexpressing plants as Lorence et al. showed no clear differences in the AsA concentration between WT and the transgenic lines (Endres & Tenhaken 2009). The differences in the results for the same MIOX4-overexpressing plants as in Lorence et al. remain difficult to explain. One aspect is the concentration of myo-inositol in the transgenic lines, which is different between WT and the MIOX4-overexpressors (Endres & Tenhaken 2009). Changes in the concentration of myo-inositol are associated with changes in galactinol, a dimer of galactose and myo-inositol. Galactinol was recently associated with stress gene expression in tobacco (Kim et al. 2008). The studies of Lorence et al. (2004) and Zhang et al. (2008) provide no direct link between higher transcript levels for MIOX4 and higher levels of AsA.
Inspired by the publication of Lorenc et al. (2004), other research groups have also overexpressed MIOX genes, for instance in tomato (Cronje et al. 2012) or rice (Duan et al. 2012), to increase the AsA level in these crops. None of the transgenic plants contained higher levels of AsA than the WT controls, which is in good agreement with our data. If one includes the labelling studies from Loewus et al. (1962), it can be concluded that a mammalian-like pathway to AsA is not functional in a diverse group of plants including a monocot.

Other groups have addressed the different pathways to AsA by searching for conserved biosynthesis genes (Wheeler et al. 2015; Ruggieri et al. 2016). These studies show that, for example, the enzymes gluconolactonase, as well as l-gulonolactone oxidase, are absent in the genomes of higher plants. Moreover, there were no expressed sequence tags for gluconolactonase in kiwifruit (Actinidia spp.), which is one of the most suitable candidates to study mammalian-like pathway as it is rich in AsA content and has much higher myo-inositol concentrations than other plants (Bieselski et al. 1997; Crowhurst et al. 2008).

Taken together, the information on the part of the mammalian-like pathway starting from GlcA is either ambiguous or the evidence for particular genes is missing. There is therefore a need to confirm the data biochemically to prove the function of the enzymes and their biological relevance.

Xiao et al. (2017) recently published experiments about a knockout in GlcAK1 and showed some changes in stress response, different expression of sugar-related and ABA-response genes. The changes were attributed to the knockout of the GlcAK1 gene. We have tested the same T-DNA lines (glcak1-1 and glcak1-2) in our feeding experiments, but neither T-DNA line show accumulation of GlcA as found in the frame-shift mutations glcak1-3/1-4 (compare Fig. 4). The paper of Xiao et al. (2017) suggests a T-DNA insertion position close to the ATG start codon, which we cannot confirm. Resequencing of the DNA insertion position by us revealed a position 197 bp upstream of the ATG start codon, which is identical to the position shown on the T-SIGNAL webpage (http://signal.salk.edu/cgi-bin/tdnaexpress). We also measured relative GlcAK1 expression with the glcak1-1 and glcak1-2 mutants, showing no difference between WT and mutant lines. This also explains why the flux of 3H-myo-inositol into cell walls is very similar in WT, glcak1-1 and glcak1-2 mutants.

Plants have established recycling pathways for many sugars, including GlcA. Mutations in the gene for GlcAK1 already lead to a significant increase in GlcA, although a second isoform exists in the Arabidopsis genome. The flux of 3H label from myo-inositol into the cell wall is also detected in glcak1-3 and glcak1-4 mutants, although at a lower level, which confirms the function of GlcAK2 (At5g14470) as a second isoform of GlcAK. The paper of Zhao et al. (2013), however, claims a biological function of this gene as a galactokinase rather than a glucuronokinase. This is highly unlikely as the only galactokinase in Arabidopsis is encoded by a different gene (At3g06580; Egert et al. 2012). A possible misinterpretation of the gene ontology terms might have caused the wrong annotation and questionable conclusions of the paper. In fact, we have confirmed the function of GlcAK2 as a true glucuronokinase in preliminary experiments with purified recombinant enzyme from transient expression in tobacco plants.

CONCLUSION

The mammalian-like pathway to AsA via myo-inositol and GlcA was proposed in previous publications. Here we show that a knockout in glucuronokinase1 reduces the flux of GlcA into cell wall polymers and leads to an accumulation of GlcA. We also found no evidence that GlcA is further used to synthesise AsA in Arabidopsis. Moreover, any direct evidence for a mammalian-like pathway to AsA in plants is lacking.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Primers used in this study.

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