Improving cadmium mobilization by phosphate-solubilizing bacteria via regulating organic acids metabolism with potassium

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HIGHLIGHTS

- Assessed organic acids metabolism and Cd mobilization of PSB affected by K⁺.
- K⁺ regulated TCA cycle via regulating enzyme activity and related genes expression.
- Cd mobilized by PSB increased with tartaric, fumaric and succinic acids secretion.
- Increasing bioavailable Cd concentrations up-regulated the biosynthesis of amino acids.

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ABSTRACT

Organic acids secreted by phosphorus-solubilizing bacteria (PSB) is one of the main biological metabolites with cadmium (Cd) mobilization capacity in the conversion of insoluble precipitate forms to bioavailable forms in contaminated soil. However, the fluctuating concentrations of nutrient elements caused by agricultural activities may result in the substantial variances of carbohydrate metabolism of microorganisms involved in Cd remediation, it is therefore essential to study how metabolic strategies, especially for organic acids, affected by the environmentally friendly fertilizers, such as potassium (K). In this study, adding K⁺ (KCl) concentrations from 0.0 to 100.0 mg/L in medium clearly accelerated Cd mobilization from 15.9 to 35.9 mg/L via inducing the secretion of tartaric acid, 3-hydroxybutyrate, fumaric and succinic acids, increased by 10.0-, 7.5-, 4.3- and 4.1-fold changes, respectively. Current data revealed that the significant differences of metabolic pathways and genes expressions with the varied K⁺ concentrations included: i) K⁺ induces a substantial up-regulation in metabolic pathway of pyruvic acid to oxaloacetate and tartaric acids; ii) the varied expression of genes involved in encoding enzymes of tricarboxylic acid cycle result in the up-regulated fumaric acid, succinic acid and 3-hydroxybutyrate; iii) the expression of genes related enzyme cysteine and glutamate metabolism processes promoted with the increasing bioavailable Cd concentrations. Besides, P-type ATPase activity increased with K⁺ levels, indicating that H⁺ efflux and medium acidification were strengthened. In general, an appropriate enhancement of K based fertilizer is an effective manner for soil Cd remediation via the regulation of organic acids metabolism and H⁺ secretion of PSB.

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manufacturing and disposition processes is a potentially serious threat to food safety (Tansel, 2017; Zhou et al., 2016). Considering Cd usually present in an insoluble precipitate form by the combination with carbonate, iron oxide and clay (Cui et al., 2019), the bioavailability and accumulation of soil Cd would be significantly inhibited (Hu et al., 2016). As an effective remediation technology of soil Cd, microbial-plant interaction model can participate in Cd mobilization from insoluble precipitate forms into bioavailable forms via secreting siderophore and organic acids (Guo et al., 2018), especially low molecular weight organic acids. However, the concentrations of soil nutrient elements, including nitrogen (N), phosphorus (P) and potassium (K), are not always feasible for intracellular pH, osmotic solute, and enzymes activity in cells, resulting in the different concentrations of organic acids secreted by microorganisms into soil (Epstein, 2003; Forieri et al., 2017; Ruan et al., 2015). For instance, in soil with K deficiency, the biosynthesis of jasmonic acid and genes related to nutrient transport and protein kinases were repressed in watermelon and rice root, respectively (Fan et al., 2014; Zhang et al., 2017). Consequently, the metabolism and secretion of organic acids by plants will be significantly inhibited, and the microbial-plant system for Cd mobilization and remediation achieved at a low level (Ashley et al., 2005; Jha and Subramanian, 2016; Shen et al., 2017).

Considering the outstanding problems of aquatic environment caused by N and P based commercial fertilizers (Galloway et al., 2008), the application rates of these two fertilizers gradually decreased in recent years. It is therefore essential to investigate the effects of K+ concentrations in soils on metabolism and secretion of organic acids by microorganisms, and their influences on heavy metal mobilization and remediation. Organic acids are mainly produced in glycolysis pathway, tricarboxylic acid (TCA) cycle and other routes, such as tartaric and fatty acids metabolism (Luo et al., 2017). Nevertheless, the activity of enzymes related to organic acids metabolism in glycolysis pathway and TCA cycle are significantly influenced by intracellular pH, substrate concentrations, and environmental conditions. In fact, the common soil nutrient element K+ can be used to regulate the intracellular organic acids strategies as the activators of intracellular pyruvate kinase, glutamine synthase, citrate synthase, and fructose-6-phosphokinase (Hess and Haeckel, 1967; Moat et al., 2003). Meanwhile, most studies on organic biosynthesis also indicated that this process can provide an energy support for K+ acquisition and organic acids secretion (Yang et al., 2013; Yu et al., 2016). However, the variance of enzymes activities in glycolysis pathway and TCA cycle associated with different bioavailable K+ concentrations whether or not result in the increased production of organic acids with a better Cd mobilization ability have remained scarce.

To reveal the effects of K+ on organic acids metabolism and Cd mobilization by microbes, Agrobacterium sp., one of phosphate-solubilizing bacteria (PSB), was selected as the model bacterium. It widely distributed in the vicinity of rhizosphere and provides nutrients such as bioavailability phosphorus for plants via secreting protons, organic and amino acids (Chen et al., 2006; Jha and Subramanian, 2016). Of course, insoluble Cd in soil also can be transformed into bioavailable forms accompanied by the above biological metabolites of PSB (Jeong et al., 2013; Khan et al., 2014). As an expansion of transcriptomic analyses technology, genetic basis underlying the physiological processes of cation binding and transport, cellular enzymes biosynthesis, carbon and nitrogen metabolism, and organic acids secretion have been identified (Rahman et al., 2014; Wen et al., 2015). Hence, to optimize microorganisms for Cd mobilization, more detailed biological information about the influences of K+ on the organic acids metabolism and secretion are needed to be studied with metabolicomic and transcriptomic analyses.

Therefore, the biological mechanisms of Cd mobilization by Agrobacterium sp. were investigated with distinct K+ concentrations ranged from −0.0 to 100.0 mg/L based on the bioavailable K concentrations in soils of south China (He et al., 2015; Simonsson et al., 2007). We aim to acquire that: i) changes of Cd mobilization performance affected by organic acids secreted by Agrobacterium sp.; ii) variance of organic acids strategies in glycolysis and TCA cycle, and amino acids pathways in cells; iii) molecular mechanisms of K+ in regulating expression of genes related to cation binding and transport, carbohydrate and amino acids metabolism, and organic acids secretion.

2. Materials and methods

2.1. Cd mobilization trial

2 ml. Agrobacterium sp. solution was inoculated into a modified Pikovskaya medium (100 mL). This medium was prepared with denitized water and solute listed as follows (in mg/L): Glucose (1000.0), (NH4)2SO4 (500.0), Ca3(PO4)2 (668.0), NaCl (300.0), FeS2O7H2O (30.0), MgSO4·7H2O (534.3), MnSO4·H2O (22.7). In addition, 6.0 mg CdCl2 and 30.0 mg potassium feldspar were affiliated into this medium as the insoluble resources of Cd and K elements, respectively. The experimental treatment was established with four K+ concentration levels of 0.0, 25.0, 50.0 and 100.0 mg/L (K0, K25, K50, and K100, respectively) with the addition of KCl. Medium without Agrobacterium sp. was set up as control treatments (CK0, CK25, CK50, and CK100, respectively). Each treatment was performed with six replicates. After a 7-d culture period (28°C, 180 r/min), the supernatant was obtained by centrifuging at 4000 rpm for 10 min and then filtered with a 0.22 μm microfiltration membrane.

2.2. Determination of Cd concentration and extracellular metabolites

OD600 value of the bacterial solution was determined with visible spectrophotometry (UV–mini-1240, Shimadzu, Kyoto, Japan) at 1, 3, 5, 7 d periods, respectively. The pH of supernatant was measured with a five easy plus pH meter (Mettler Toledo, Switzerland). K and Cd concentrations in culture solution were analyzed with an atomic absorption spectrometer (AA-7000, Shimadzu, Kyoto, Japan). Inorganic pyruvate (PA) concentration and pyruvate kinase (PK) activity of strain var were respectively determined with 2,4-dinitrophenylhydrazine colorimetric method and lactate dehydrogenase (LDH)-coupled assay, respectively (Kim et al., 2015). P-ATPase activity was analyzed in accordance with the enzyme-linked immunosorbent assays (ELISA) methods.

Extracellular metabolites in supernatant referred to the previous studies (Lisec et al., 2006; Yang et al., 2018a). The dried residue was derivatized by 20 μL methoxamine hydrochloride (20 mg/ml in pyridine at 37°C for 2 h) and 70 μL N-methyl-N-(trimethylsilyl) trifluoroacetamide (at 37°C for 30 min). Samples (1 μL) were injected into gas chromatography–mass spectrometry (GC–MS) in a splitless mode (GC–MS-QP2010, Shimadzu, Kyoto, Japan). GC–MS data were carried out with the automated MS deconvolution and identification software and then compared with data in the NIST database. Matching degree of >80% and retention index difference of <20 were screened as the qualitative results of metabolites (Stein, 1999; Yang et al., 2018b). Extracellular metabolites were quantified using an internal standard method (Kumari and Parida, 2018).
Whole genome of Agrobacterium sp. was acquired with DNA sequencing and deposited on the NCBI database (No. CP040640-CP040642). The bacterial solution of every three medium in each treatment was mixed to yield one mRNA sequencing sample. Total RNA extraction was carried out with the standard methods provided by TRIzol® manufacturer after bacterial solution was frozen (−20 °C) (Rio et al., 2010). A certain amount of total RNA samples was settled at an appropriate temperature reaction for a while to remove DNA contaminations. Clean mRNA is collected with the application of rRNA purified reagent and then fragmented into small pieces with the fragmentation reagent. Single-strand circle DNA (cDNA) was generated using random primers reverse transcription, followed by double-strand cDNA synthesis. This cDNA was subjected to end-repair and then was phosphorylated (5’) and adenylation (3’). Afterward, cDNA fragments were performed with polymerase chain reaction (PCR) amplification. PCR product is transformed into a single strand, and then we obtained the single strand cDNA. Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA) and ABI StepOne Plus Real-Time PCR system (TaqMan Probe) were used to analyze the quality and concentration of library, respectively. The qualified library transformed into a strand chain by addition of NaOH. It was diluted and then added into FlowCell and hybridized with the junction on FlowCell. Finally, the prepared FlowCell was sequenced by Illumina HiSeq 4000 platform in BGI company (Shenzhen, China). The raw sequencing database from RNAseq was uploaded to the NCBI database (No. PRJNA542961).

2.4. Raw reads analysis, functional annotation and differentially expressed genes identification

Raw data of reads were firstly performed with quality control (Q30 is more than 80% or Q20 is more than 90%), and then we removed reads containing joints or with unknown base (N) above 5%, and reads whose base quality value bellowed 10 or it accounted for more than 20% of total base numbers. Clean reads were compared with the reference whole genome with Bowtie software. Genetic expression was standardized by the fragments per kilobase of exon per million fragments mapped (FPKM) method. For genes with one more alternative transcript, the longest transcription was selected to calculate its FPKM value.

The biological function of each gene in Gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG), NCBI non-redundant protein database (NR) is obtained by BLASTX searches with an e-value of 10−5. To identify differentially expressed genes (DEGs) in transcriptome of Agrobacterium sp. between different experimental treatments, NOISeq method was used to analyze genes expression with a reference level of FPKM value (log2(Ratio) >1) and probability level (>0.8). According to GO or KEGG annotation results, we classified DEGs into different functional categories. Additionally, phyper function in R software was used to analyze GO and KEGG enrichment.

2.5. Statistical analysis

The difference in metabolites evaluated principal component analysis (PCA) with Simca-P 12.0 (Bundy et al., 2010; Guo et al., 2018). Metabolites in each treatment were deposited to metaboanalyst 3.0 platform (http://www.metaboanalyst.ca/) to annotate their metabolic pathway (Guo et al., 2018). One-way analysis of variance (ANOVA) and student’s t-tests were used to obtain the significant differences of pH, OD600 value and Cd mobilization between different treatments (SPSS Ver. 22.0, IBM, USA). Statistical analysis of this study used p ≤ 0.05 as significant level. R software (Ver. 3.2) was used for hierarchical cluster analysis.

3. Results and discussion

3.1. Cd mobilization by Agrobacterium sp.

The concentrations of water-soluble Cd in medium without strain ranged from 7.7 to 8.1 mg/L whereas its contents in K0, K25, K50, and K100 treatments were 15.9, 22.8, 27.9 and 35.9 mg/L, respectively (Fig. 1A). After a 7-d culture period, the pH of experimental treatments decreased from 6.1 (K0) to 5.5 (K100) with an increasing K+ concentrations. No significant difference in OD600 value was found in four treatments (p > 0.05) (Fig. 1B), suggesting that the bacterial densities in medium is similar because the potassium feldspar was prepared to as K resource for the normal growth of Agrobacterium sp. Cd mobilization increased with the enhanced concentrations of K+ and the decline of pH values, indicating that K+ could induce the mobilization of insoluble Cd via the acidification of medium. Previous studies have found that medium acidification was mainly occurred with two routes: protons secreted by bacteria during the K+ absorption process or dissociation of extracellular organic and amino acids (Booth et al., 2015; Harold et al., 1970; Pinu et al., 2018). K+ is a dominant cation in the cytosol with concentrations ranged from 1600 to 8000 mg/L (Kronzucker et al., 2003). Cellular K transporters through the plasma and tonoplast membranes can maintain K balance in cytosolic by the fluxes from/to soil solution (Brito and Kronzucker, 2008). Increasing K+ concentration in medium could stimulate K+ influx and H+ efflux, facilitating solution acidification (Booth et al., 2015; Harold et al., 1970). As the activators for intracellular pyruvate kinase, glutamine synthase, cytate synthase, and fructose-6-phosphokinase, K+ promoted the activity of pyruvate kinase and the biosynthesis of pyruvate in Agrobacterium sp. (Fig. 1D).

3.2. Effects K+ and Cd mobilization on extracellular metabolites

Non-target metabolomics based on GC-MS quantified 18 metabolites in extracellular solution (Fig. 2 and Table S1). Compared with K0 treatment, the concentrations of tartaric acid, 3-hydroxybutyric acid, fumaric acid, succinic acid, valine, serine, alanine, and ribose were significantly promoted, whereas the concentrations of lactic acid, glutamic acid, galactitol, putrescine, erythritol, and glycerol were apparently decreased with an enhancive K+ concentrations, respectively (p < 0.05). There is no substantial variation in contents of glyceric, lauric, terephthalic, and stearic acids with different K+ concentrations. Based on the correlation analysis (Fig. 1C), the concentrations of monocarboxylic, dicarboxylic, and amino acids have a significantly positive correlation with water-soluble Cd contents in medium. Our previous study also found that dicarboxylic acids own a good Cd mobilization capacity compared with monocarboxylic and amino acids, and Cd mobilization ability of each organic acid in unit concentration is listed as follows: fumaric acid > tartaric acid > succinic acid > glutaric acid > lactic acid > 3-hydroxybutyrate > proline, serine, and alanine (Wei et al., 2017). Hence, according to above findings, organic acids with the better Cd mobilization capacity, especially for tartaric, fumaric and succinic acids, are promoted with increasing K+ concentrations, inducing medium acidification and Cd mobilization (Yuan et al., 2014). Although valine, serine, and alanine barely enabled Cd mobilization, the secretion of amino acids in extracellular solution increased with K+ concentrations because of the detoxification effect of these acids under the higher water-soluble Cd contents in medium (Khan et al., 2016).
3.3. De novo assembly, DEGs and functional enrichment

High quality reads of the whole genome de novo assembled into 5486 unigenes as the reference genome, and 5271 genomes were annotated in database (Table S2). Total clean reads of mRNA sequencing data of 8 samples (each treatment: 2 mixture samples) approximately ranged from 21.6 Mb to 21.8 Mb. A total of 5481 expressed genes of mRNA were found when compared with the whole genome of Agrobacterium sp., and the total mapping value was more than 78.3% (Table S3). With an increasing K\(^+\) concentrations, the number of up-regulated DEGs significantly promoted when compared with that of down-regulated DEGs (Table 1). By contrast to 0.0 mg/L K\(^+\) concentration, there were 0, 21 and 83 up-regulated and 9, 15 and 5 down-regulated DEGs in K25, K50 and K100 treatments, respectively. These results indicated that the transcriptomic analysis was in good quality, and K\(^+\) induced the genes expression in Agrobacterium sp.

DEGs among four treatments were classified into 16 GO level 2 terms (Fig. 3 and Table S4). Compared with K0 treatment, no significant difference was found in the enrichment GO terms in K25 treatment (Table S4). In K50, GO terms of cation transport and cellular homeostasis in biological processes, transmembrane proteins, cation binding enzymes in cellular components, and electron transfer and some structural molecular activity in molecular functions are enriched, respectively. In K100, the enrichment GO terms are partially listed as follows: i) cellular nitrogen compound biosynthesis, protein metabolism, amino acid, amides and macromolecule compounds, biosynthesis in biological processes; ii) intracellular small ribosomal subunit, cytoplasm, ribonucleoprotein complex, etc. in cellular components; iii) N-acetyltransferase activity, mRNA binding, N-acetyl-gamma-glutamyl-phosphate reductase, and passive transmembrane transporter activity in molecular functions. According to transcriptome KEGG pathway analysis, ribosome pathway (ko03010) is significantly enriched with the overexpressed of related genes (p < 0.05). These results suggested that the expression of genes related to cation transport, the biosynthesis of organic and amino acids, and their secretion by Agrobacterium sp. were obviously different among the four treatments.

3.4. Effects of K\(^+\) and Cd mobilization on the metabolism and transcriptome of Agrobacterium sp.

The key biological mechanisms of Cd mobilization promoted by K\(^+\) in Agrobacterium sp. included K\(^+\) induce proton efflux, organic acids strategies in glycolysis pathway and TCA, and the metabolism of glycine, serine, and threonine (GSTH) in responses to the higher water-soluble Cd levels (Guo et al., 2018). Genes involved in K\(^+\) absorption and effluxes, such as Kup, Trk, Kdp systems, and glutathione-regulated K-efflux system protein, have no significant regularity (Table S5). However, the activity P-type of ATPase in Agrobacterium sp. gradually increased with K\(^+\) contents (Fig. 1D), indicating that the H\(^+\) efflux process was strengthened in response to the enhance K\(^+\) concentrations (Beard et al., 1997). This resulted in medium acidification, and promoted Cd mobilization from the insoluble precipitate forms (Fig. 1).

The metabolomic analysis shows that organic and amino acids strategies of TCA cycle, butanolate, arginine, proline, GSTH metabolism in Agrobacterium sp. were significantly influenced by the enhance K\(^+\) concentrations (Fig. 4). In K50 and K100 treatments, the activity of pyruvate kinase increased with K\(^+\) concentrations, inducing the incremental concentrations of intracellular pyruvic acid in the glycolysis process (Fig. 1D) (Wang and Wu, 2013). According to transcriptomic KEGG analysis (Table S5), the pathway of pyruvic acid to oxaloacetate was also up-regulated with a significantly enhance expression of genes involved in encoding
acetolactate synthase (EC: 2.2.1.6). For fumaric and succinic acids, they are common intermediate products in TCA cycle, which utilized the pyruvic acid as metabolic substances. Although rare DEGs related to enzymes were found in TCA pathway, compared with K0 and K25 treatments, a relatively minor increased (~0.7 Log1.5 Ra-tio ~0.15) expressions of genes with biological functions for the biosynthesis of citrate synthase (EC: 2.3.3.1), pyruvate dehydrogenase (EC: 1.2.4.1 and 2.3.1.12), isocitrate dehydrogenase (EC: 1.1.1.42), dihydrolipoamide dehydrogenase (EC: 1.8.1.4), malate dehydrogenase (EC: 1.1.1.37), and aconitate hydratase (EC: 4.2.1.3) in K50 and K100 treatments were observed. These enzymes participate in the metabolic processes of pyruvic acid to acetyl-CoA, oxaloacetate to citrate, citrate to isocitrate, isocitrate to oxalo-succinic acid and a-ketoglutarate, and malic acid to oxaloacetate (Alteri et al., 2009; Reisch and Elpeleg, 2007). However, genes expression related to encoding succinate dehydrogenase (EC: 1.3.5.3), fumarase (EC: 2.8.1.4), and malate dehydrogenase (EC: 1.1.1.37) decreased with K50 and K100 treatments.

**Table 1**

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<th>K⁺ induced up-regulated transcripts</th>
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Fig. 2. K⁺ induced different extracellular metabolites (A) and metabolic pathway (B) in Agrobacterium sp. Red and blue color value mean up or down regulation of metabolites, respectively. PK represent pyruvate kinase. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
1.3.5.4) showed a nonsignificant decline with the enhancement of K\(^+\) concentrations. This enzyme can catalyze the metabolic processes of fumaric acid from/to succinic acid (Iverson, 2013), low expression might inhibit the biosynthesis of fumaric from/to succinic acids, inducing the accumulation of fumaric and succinic acids in intracellular of Agrobacterium sp. Tartaric acid is produced with the catabolism of oxaloacetate or hydroxy-fumaric acid. Genes correlated to encoding pyruvate...
carboxylase (EC: 6.4.1.1) and malate dehydrogenase (EC: 1.1.1.37) were substantially overexpressed in K50 and K100 when compared with that of K0 and K25 treatments, inducing an increasing biosynthesis of oxaloacetate. As presented above, the metabolic process of fumaric acid to malate might be inhibited with the down-regulated expression of correlated genes, which resulted in an accumulation of fumaric acid. The enhancive concentrations of upstream mediators to synthetize tartaric acid are most likely to an accumulation of fumaric acid. The enhancive concentrations of bacteria with pyruvic acid as the upstream mediator. Glutaric acid during the amino acid metabolism processes, and then in- increased K+ concentrations have pointed out that the P-type ATPase activity of Agrobacterium sp. is required to have a depth understand of the regulation mechanisms of K+ on organic and amino acids strategies of PSB.

It is found that secretion of amino acids (alanine, lysine, histidine) and organic acids (succinic acid, pyruvate acid) are co-transported with H+ into an extracellular solution based on a model organism of Escherichia coli (Buurman et al., 2004). That is, during the efflux process of H+, these organic and amino acids will also be secreted into medium through plasma membrane. The enhancive K+ concentrations have pointed out that the P-type ATPase activity of Agrobacterium sp. gradually increased, indicating that K+ not only altered the metabolic pathways of intracellular organic and amino acids, but also involved in the secretory pathways related to organic acids transport via plasma membrane. However, ABC transporters with biological function of organic acids and some amino acids did not present with a significant up or down regularity (Table S5). Genes expression related to encoding fructose and glutamate transporter showed a slight enhancement with the increasing K+ concentrations, inducing the secretion of those corresponding compounds from cells to the extracellular solution. In general, a qualified increasing K+ concentration could promote the strategies and exocytosis of organic and amino acids in PSB, which
promoted Cd mobilization in medium.

4. Conclusion

An appropriately increased K⁺ concentrations can promote H⁺ secretion and medium acidification via promoting P-type ATPase activity. Genes related to enzymes involved in the biosynthesis of tartratic acids overexpressed, and variance in the TCA cycle resulted in the enhanced fumaric and succinic acids concentrations, which are most likely the key processes to mobilize Cd in the insoluble forms. In turn, the increasing water-soluble Cd concentrations also stimulated GSH metabolism to produce more amino acids to detoxicate Cd intoxication in intracellular. As a result, K based fertilizers at an appropriate level is one of the potential methods for Cd contaminated soils remediation through regulation of H⁺ secretion and organic and amino acids metabolism in PSB.

Notes

The authors declare no competing financial interest.

Author contributions section

Wan-Li Li, Yao Lv, Hao-Jie Dong, and Tao He performed the Cd mobilization trial in this study. Jun-Feng Wang and Li-Li Wang have written and polished this manuscript. The experimental scheme was designed by professor Qu-Sheng Li.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.125475.

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