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Isolation of intact and pure chloroplasts from leaves of *Arabidopsis thaliana* plants acclimated to low irradiance for studies on Rubisco regulation

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Abstract

A protocol is presented for low-cost and fast isolation of intact and pure chloroplasts from leaves of plants acclimated to low irradiance. The protocol is based on a differential centrifugation of cleared leaf homogenate and omits a centrifugation on Percoll gradient step. The intactness and purity of the chloroplasts isolated from leaves of low irradiance-acclimated plants by using this protocol (confirmed by phase contrast microscopy as well as enzymatic and immunological approaches) allows plausible studies on low irradiance-dependent Rubisco regulation.

Keywords: acclimation, chloroplast isolation, differential centrifugation, low irradiance, *Arabidopsis thaliana*, Rubisco

Introduction

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), the most abundant protein in the biosphere catalyzes photosynthetic carbon dioxide fixation and photorespiratory carbon oxidation. In land plants and green algae the enzyme is an oligomeric protein composed of eight large and eight small subunits (LSs and SSs, respectively). A lot of experimental data exist indicating that Rubisco is highly regulated in response to short term fluctuations in the environment. The mechanisms involved in Rubisco regulation may occur at the level of enzymatic activity, as exemplified by the loss of the Rubisco activity which has been reported in several plant species under drought and proposed to be due to the increase in the amount of inhibitors tightly bound to the catalytic site [1,2]. Alternatively, the amount of Rubisco protein may be regulated, by changes in relative rates of synthesis and degradation of both subunits. These processes may involve mRNA accumulation [3], translational elongation [4] as well as a wealth of posttranslational phenomena including the assembly of Rubisco holocomplex [4,5] and aggregation/degradation of individual subunits [6]. As far as posttranslational phenomena are concerned these are, specifically, reactive oxygen species (ROS) generated during the exposure of plants to various stress conditions [7–9] that may drive, at least in some instances Rubisco oxidative modifications which may lead, in their turn, to a massive degradation and/or aggregation of Rubisco subunits and a loss enzymatic activity as well. In vitro oxidative treatment of purified Rubisco leads to conformational changes in LS, followed by an increase in its susceptibility to proteolysis, the disassembly of the holoenzyme and finally also SS becomes more vulnerable to proteolysis [10]. It thus may be expected that Rubisco degradation events caused by stress-related oxidative modifications occuring in vivo may start by LS degradation as well. Indeed, it was found that LS oxidative modifications (disulfide cross-linking as well as non-disulfide polymerization) and a translocation of aggregated LS molecules towards membranes, both triggered in vivo by subjecting *Chlamydomonas reinhardtii* to saline stress are accompanied by an extensive LS degradation [11]. A similar scenario, involving aggregation, membrane- translocatation and degradation of LS molecules took place under cupric ions treatment of higher plants (wheat, *Spirodela oligorrhiza*) and algae (*Chlamydomonas reinhardtii* and *C. moewusii*) as well [6]. Most probably preexisting proteases are involved as the susceptibility of oxidatively modified LS towards proteolysis correlates positively with the scale of oxidative modifications introduced to LS by stress treatment but not with possible oxidative modifications of proteases engaged [12]. Alternatively, in some instances stress-related aggregation and/or translocation towards membranes of LS molecules are not accompanied by degradation events [13,14]. Thus from the results available today it appears that regulation of both LS and Rubisco holocomplex triggered by short-term environmental fluctuations may operate through a variety of combinations of both pre- and posttranslational events. The application of new experimental models for the study of Rubisco regulation poses a challenge for future research. It is interesting in this context that a dramatic decrease in Rubisco holocomplex content was found to happen in response to shading of attached tobacco leaves without a concomitant drop in LS mRNA level [15] and one likely mechanism is through posttranslational regulation,

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possibly involving LS aggregation/degradation phenomena. Yet, attached leaves of plants acclimated to low irradiance have not been applied to studies on Rubisco regulation since then. The exploitation of this experimental model to *Arabidopsis thaliana* leaves by our group was unexpectedly retarded by low purity of chloroplasts isolated from leaves of *A. thaliana* plants acclimated to low irradiance by a widely used procedure involving a centrifugation on Percoll gradient [16]. In its turn, the same procedure gave satisfactory results when chloroplasts were isolated from leaves of *A. thaliana* control plants or the ones acclimated to moderate irradiance. The goal of the current study was to develop a protocol for high yield isolation of pure chloroplasts from leaves of *A. thaliana* plants acclimated to low irradiance, suitable for studies on low irradiance-dependent Rubisco regulation.

Material and methods

Plant material

Arabidopsis thaliana (ecotype Columbia) plants were grown in 42-mm Jiffy peat pellets on sphagnum peat moss and wood pulp (Agawa, Zielona Góra, Poland) in a growth chamber (NEMA, Netzschkau, Germany) for five weeks under short day conditions (8 h-light/16 h-darkness) with an irradiance set at moderate level of 250 µmol quanta m^{-2} s^{-1} [17] at a temperature 22/ 18°C (day/night, respectively) and constant 65% relative humidity (control plants). Then some population of plants was acclimated for 24 h either still to 250 µmol quanta m^{-2} s^{-1} (moderate irradiance-acclimated plants) or 50 µmol quanta m^{-2} s^{-1} (low irradiance-acclimated plants) with temperature and humidity kept at the level encountered during growth period before acclimation.

Isolation of chloroplasts

For chloroplast preparation the leaves of control and moderate or low irradiance-acclimated *A. thaliana* plants (12 g) were homogenized in 30 ml of ice-cold isolation buffer containing 0.3 M sucrose, 50 mM Tricine/NaOH (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, and freshly added 0.2% BSA (bovine serum albumin) by using a motor-driven blender (Homogenizer type 302, Mechanika Precyzyjna, Warszawa, Poland; two 5 s bursts at maximum speed). After filtration of the homogenate through a filter Mesh 100 (Sigma-Aldrich, St. Louis MO, USA) the cleared homogenate was centrifuged at 1000 *g* for 7 min at 4°C. The supernatant was rejected and the green pellet was carefully suspended in 7 ml of the isolation buffer and differential centrifugation step was repeated. After centrifugation the supernatant was removed and the chloroplast pellet was collected.

Alternatively, chloroplasts were prepared using Sigma Chloroplast Isolation Kit (Sigma-Aldrich, St. Louis MO, USA). The leaves (12 g) were homogenized in 48 ml of an ice-cold isolation buffer by using a motor-driven blender (Homogenizer type 302, Mechanika Precyzyjna, Warszawa, Poland; two 5 s bursts at maximum speed) and the homogenate filtered through filter Mesh 100. The filtrate was centrifuged for 7 min at 1500 *g* to sediment chloroplasts as a green pellet, which was then resuspended in isolation buffer. The chloroplast suspension was then loaded on top of linear Percoll gradient – precentrifuged at 43000 *g* for 30 min at 4°C – and centrifuged in a swing-out rotor for 6 min at 13300 *g* at 4°C (brake off). Intact chloroplasts were collected from the gradients (lower band), diluted three times in isolation buffer without BSA and centrifuged at 3300 *g* for 2 min at 4°C. The supernatant was discarded and the washed chloroplast pellet was collected.

Isolation of mitochondria

Mitochondria were prepared according to Urantowka et al. [18] with some modifications. Namely, leaves of control plants (12 g) were homogenized in 50 ml of ice-cold isolation buffer containing 0.45 M sucrose, 5 mM EDTA [Fe(III)-ethylenediaminetetraacetic acid], 1.5 mM EGTA [ethylene glycol bis (β-aminoethyl ether)-N], 5 mM MOPS [3-(N-morpholino) propanesulfonic acid]/KOH (pH 7.4), 0.5% BSA, 0.6% PVP-40, 2.5 mM PMSF (phenylmethylsulfonyl fluoride) and 5 mM 2-mercaptoethanol, by using a motor-driven blender and a homogenate was filtered through a filter Mesh 100. The cleared homogenate was then centrifuged twice at 2200 *g* for 2 min (4°C) and the supernatant after the second centrifugation step was again centrifuged for 10 min at 28000 *g* (4°C). The pellet representing crude mitochondria preparation was suspended in 10 mM potassium phosphate buffer (pH 7.2) containing 0.4 M mannitol, 1 mM EDTA, 0.5% BSA and centrifuged in a fixed angle rotor through a four-step Percoll gradient (2-ml centrifugation tube containing 50%, 29%, 26%, 18% Percoll in 0.25 M sucrose and sterile water at a 3.75:3:4:3 ratio, respectively; brake off). After centrifugation at 40000 *g* for 12 min at 4°C the mitochondria were isolated from 29/ 50% interphase. To remove Percoll the purified mitochondria were centrifuged twice in 10 mM potassium phosphate buffer (pH 7.2) containing 0.4 M mannitol, 1 mM EDTA without BSA at 23200 *g* for 10 min at 4°C.

Phase contrast microscopy

Isolated chloroplasts were viewed with phase-contrast objective to verify their intactness and chlorophyll autofluorescence was detected using broad pass-band excitation filter set (confocal laser scanning microscope LSM 510, Carl Zeiss, Jena, Germany). The phase-contrast images and the red autofluorescence of chloroplast were recorded simultaneously.

Enzyme assay for glutamate dehydrogenase (GDH)

GDH was used as a mitochondrial marker and assayed both in the mitochondria and chloroplasts. Briefly, the extracts were obtained by suspending the pellets of isolated mitochondria or chloroplasts (freshly prepared) in 50 mM phosphate buffer (pH 7.5) containing 10 mM 2-mercaptoethanol and 0.1% Triton. The suspensions were centrifuged for 15 min at 16000 *g* (4°C) and the supernatants (extracts) were used for GDH activity determination. NADH-GDH (aminating) activity was assayed as the initial rate of the 2-oxoglutarate dependent oxidation of NADH according to the method of Watanabe et al. [19]. The specific activity of the enzyme was expressed as the oxidation of 1 µmol of NAD^+ per minute per miligram of protein.

Protein determination

Protein concentration was assayed in extracts of chloroplasts or mitochondria, chloroplast lysates and in cleared leaf homogenates according to Bradford [20] using BSA as the standard.

SDS-PAGE, immunoblotting

SDS-PAGE was performed according to Laemmli [21] using 14% acrylamide resolving gel and chloroplast lysates obtained by suspending chloroplast pellets in small volume of water or

cleared leaf homogenates. After the electrophoresis, proteins of chloroplasts or cleared leaf homogenates were transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland), reacted with polyclonal antibodies raised against LS Rubisco or UGPase (Agrisera, Vannas, Sweden) and detected by applying goat anti-rabbit biotinylated immunoglobulin G (Sigma-Aldrich) and ExtrAvidin-conjugated horseradish peroxidase (Sigma-Aldrich). The quantification of immunostained signals were performed using Gelix One software (Biostep, Jahnsdorf, Germany). For each individual immunoblot the range of the linearity of immunoresponse was checked. Only blots with a linear relationship between sample dilution factor and immunoresponse were taken into consideration.

Results and discussion

When chloroplasts were isolated from leaves of *A. thaliana* low irradiance-acclimated (24 h) plants applying a method involving a centrifugation on Percoll gradient an unexpectedly small band which was formed at the lower portion of the gradient, widely regarded as representing pure and intact chloroplasts [16] exhibited an atypical, fuzzed appearance. In contrast, chloroplasts isolated from control and moderate-irradiance acclimated plants formed a typical, narrow band within the Percoll gradient, yielding 3 times more chloroplast protein per g of fresh weight (data not shown). In order to isolate a high yield of intact and pure chloroplasts from leaves of low-irradiance acclimated *A. thaliana* plants – and equally suitable for isolation of chloroplasts from leaves of control and moderate-irradiance acclimated plants – we based our protocol on established, fast procedure involving differential centrifugation of the cleared homogenate but omitting a centrifugation on Percoll gradient step. In accordance with earlier data [22] it was found by us that a proper ratio of weight of leaves vs volume of homogenization buffer, the origin and type of homogenizer as well as care and speed was essential to obtain high quality chloroplast samples. Intactness of the chloroplasts isolated by the optimal protocol, involving merely a differential centrifugation of the cleared homogenate but omitting a centrifugation on Percoll gradient was compared by using phase-contrast microscopy with the one of the chloroplasts obtained by a method involving a centrifugation on Percoll gradient (Fig. 1). The chloroplasts which appeared bright and surrounded by a clear halo were identified as intact ones while the ones found to be darker and surrounded by a less pronounced halo were regarded to be broken [23,24]. All objects visible on phase-contrast micrographs not yielding a red chlorophyll autofluorescence were described as non-chloroplast bodies. It was demonstrated that the method based on differential centrifugation allowed to obtain chloroplast preparations which gave very similar intactness, i.e. 87.5–89.2% no matter whether the chloroplasts were isolated from leaves of control, moderate-irradiance or low-irradiance acclimated plants. Furthermore, only 3.8–5.3% objects visualized by phase contrast microscopy were demonstrated to be non-chloroplast bodies. When chloroplasts isolated from leaves of control and moderate-irradiance acclimated plants have been separated on Percoll gradient the lower band contained equally intact

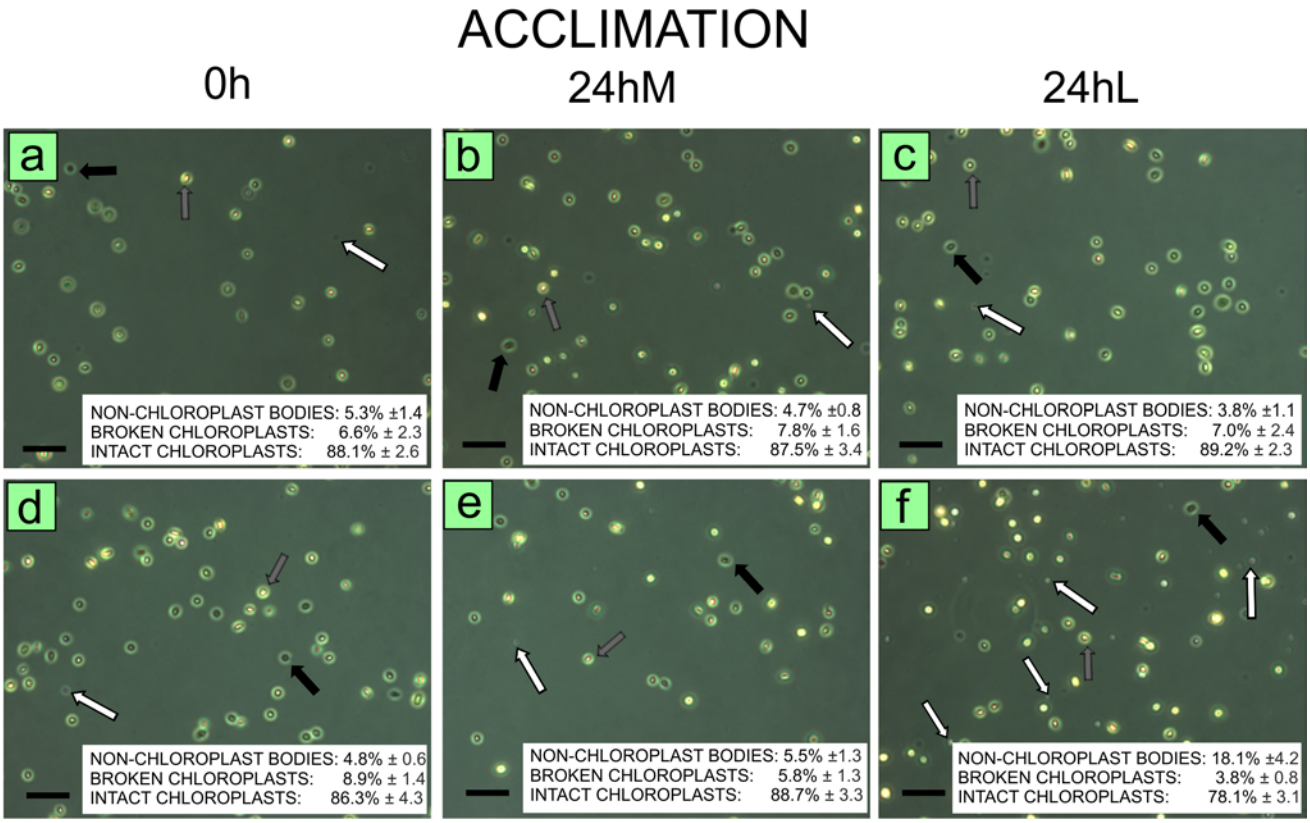

Fig. 1 Phase contrast microscopy of chloroplasts of *A. thaliana* leaves. **a,d** Micrographs of chloroplasts isolated from control plants (0 h acclimation). Plants acclimated for 24 h to moderate (**b,e**) or low (**c,f**) irradiance. Chloroplasts were isolated by differential centrifugation (**a–c**) or by using centrifugation on Percoll gradient (**d–f**). White arrows point to non-chloroplast bodies, grey arrows point to intact chloroplasts and black ones to broken chloroplasts. The insets in **a–f** show average frequencies of non-chloroplast bodies, broken chloroplasts and intact chloroplasts, quantified relative to all bodies visible on appropriate micrographs (100%). Scale bars: 30 μm.

(86.3–88.7%) chloroplast preparations, containing equally low amount of non-chloroplast bodies (4.8–5.5%). In sharp contrast to this in the case of chloroplast isolated from leaves of low irradiance-acclimated plants, the lower band collected after Percoll gradient-based centrifugation has been heavily contaminated by non-chloroplast bodies (18.1%) and chloroplast intactness fell to 78.1%.

Mitochondria are known to be popular contaminants of chloroplast preparations [25]. To estimate the extent to which chloroplast samples analysed by us were contaminated by mitochondria, measurements of GDH (a mitochondrial marker enzyme) were performed. As few as 5.0–6.5% of the specific activity found for mitochondrial sample isolated from control leaves was detected in chloroplast samples isolated using differential centrifugation (5.3% in the case of samples from leaves of low irradiance-acclimated plants; Fig. 2). In parallel, GDH specific activity of the lower band collected after Percoll gradient centrifugation of chloroplasts isolated from low-irradiance acclimated leaves was much higher (23% of the specific activity found for mitochondrial sample isolated from control leaves), corresponding to much higher contamination by non-chloroplast bodies (see Fig. 1) and this suggests that these are mainly mitochondria which contaminate this preparation.

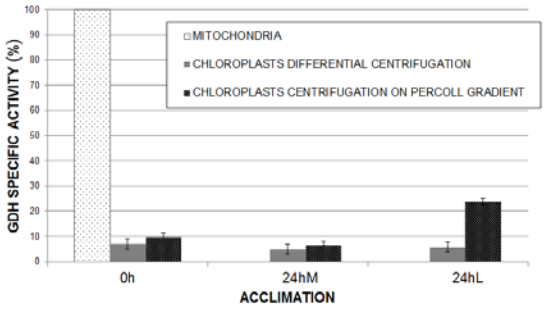

Fig. 2 GDH specific activity in chloroplasts isolated by centrifugation on Percoll gradient or by differential centrifugation from leaves of control plants (0 h acclimation) and plants acclimated for 24 h to moderate (24hM) or low (24hL) irradiation. The activity was quantified relative to the value found for mitochondrial sample isolated from control leaves (100%).

Cytoplasm is another, potential source of impurities of chloroplast preparations yet invisible on phase-contrast micrographs [26]. Thus to further validate a purity of chloroplasts isolated by differential centrifugation from various types of leaves the abundance of UGPase (a cytoplasmic marker enzyme) has been determined by immunoblotting. Again, the level of contamination was found to be marginal (5.2–6.2% of UGPase level identified in cleared leaf homogenates of relevant populations of plants; 5.4% in the case of leaves of low irradiance-acclimated plants), in opposition to what was found for lower band collected after Percoll gradient-based centrifugation of chloroplasts isolated from leaves of low-irradiance acclimated plants (14.9% relative to the value found in cleared homogenates of low irradiance-acclimated plants; Fig. 3).

Thus our fast and simple procedure allows obtaining intact and pure chloroplasts from leaves of *A. thaliana* plants acclimated for 24 h to low irradiance (and the results are very similar for chloroplasts isolated from plants acclimated to low irradiance for shorter and longer time ranges as well, data not shown). The method works equally well for chloroplasts isolated from control and moderate irradiance-acclimated plants. In opposition to this a widely used procedure involving a centrifugation on Percoll gradient works much worse in terms of purity of chloroplasts isolated from low irradiance-acclimated plants. The reason why this widely used method is not successfully applicable to isolation of chloroplasts from leaves of low irradiance-acclimated plants remains obscure.

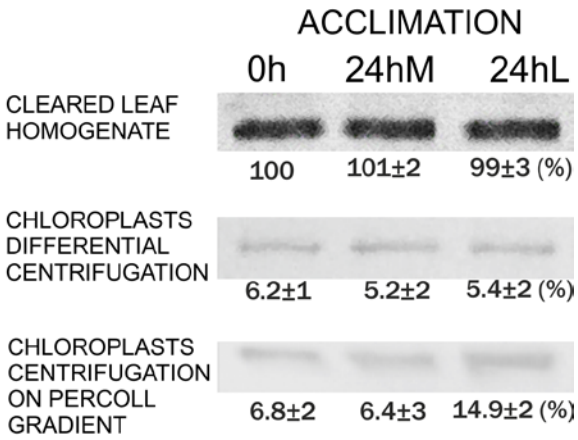

Fig. 3 Quantification of UGPase in chloroplasts isolated by centrifugation on Percoll gradient or differential centrifugation from leaves of control plants (0 h acclimation) or plants acclimated for 24 h to moderate (24hM) or low (24hL) irradiation. 2 μg protein of each chloroplast samples were introduced into gel wells. The immunoblot signals received with anti-UGPase antibodies were quantified relative to the signals identified in cleared homogenates of leaves of relevant plants (100%). The signals were determined by scanning 4 blots representing 2 biological replicates. The mean values ±*SD* are shown under respective lanes.

Next, the optimal method of isolation of chloroplasts from low irradiance-acclimated plants, involving merely a differential centrifugation of the cleared homogenate and omitting a Percoll gradient centrifugation step was applied to a plausible study of changes in intrachloroplast pool of LS Rubisco due to 24 h-long acclimation to low irradiance. It was demonstrated that LS Rubisco exhibited a pronounced decrease in its abundance inside chloroplasts during the acclimation to low irradiance conditions, down to 58% of the value found for control leaves whereas the abundance of LS Rubisco remained unchanged during 24 h of acclimation to moderate irradiance (Fig. 4). On the other hand, the decrease was not accompanied by any increase in extrachloroplast pool of LS Rubisco (data not shown) and this strongly suggests that solely the events taking place inside chloroplasts contribute to loss of LS Rubisco in low irradiance-acclimated plants. These results confirm and extend previous observations concerning a dramatic fall in the abundance of Rubisco holocomplex in shaded tobacco leaves [15]. As proteolytic phenomena may be engaged in low irradiance-dependent LS Rubisco fall, the extremely low amount of contaminations of our chloroplast preparations by mitochondria and cytoplasm is of seminal importance for the validity of potential identification of chloroplast protease involved. Namely, Miyadai et al. [27] have demonstrated that proteases of extrachloroplast origin may adhere to outer envelope during chloroplast isolation.

The experiments are in progress aimed at elucidation of molecular mechanisms of low-irradiance-dependent strong decrease in LS Rubisco amount.

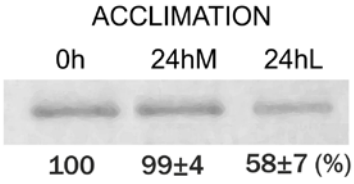

Fig. 4 Quantification of LS Rubisco in chloroplasts isolated by differential centrifugation from leaves of control plants (0 h acclimation) or plants acclimated for 24 h to moderate (24hM) or low (24hL) irradiation. 0.2 µg protein of each chloroplast samples were introduced into gel wells. The immunoblot signals received with anti-LS Rubisco antibodies were quantified relative to the signals identified in chloroplast of control plants (100%). The signals were determined by scanning 9 blots representing 3 biological replicates. The mean values ±*SD* are shown under relevant lanes.

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Authors' contributions

The following declarations about authors' contributions to the research have been made: designed the experiments: GJ, MG; performed the experiments: MG; analyzed the experimental data: MG, GJ; wrote the paper: GJ, MG.

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