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RESEARCH ARTICLE

Colorimetric and Chlorophyll Fluorescence Assays for Fast Detection and Selection of Transgenic Events of Cotton, Cowpea, Soybean and Common Bean Expressing the *Atahas* Gene

Cristiane T. Citadin[1], Mirella P. Santos[1,2], Estela R. Andrade[1,3], Thaís M. Cipriano[1], Francisco J. L. Aragão[1]*

[1]Embrapa Recursos Genéticos e Biotecnologia, Brasília 70770-900, DF, Brazil
[2]Universidade Federal do Rio de Janeiro, Macaé 27965-045, RJ, Brazil
[3]Universidade de Brasília, Campus Universitário Darcy Ribeiro, Brasília 70910-900, DF, Brazil

ABSTRACT The detection of the presence and expression of transgenes in genetically modified plants is a key step in the process of selecting promising lines. We adapted two methods developed for detecting tolerance to the herbicide imazapyr for selection of transgenic lines expressing the mutated acetohydroxyacid synthase enzyme (AHAS) from *Arabidopsis thaliana* (*Atahas* gene). This was achieved using transgenic events from cotton, cowpea, soybean and common bean, which have previously been transformed to express the mutated *Atahas* gene. In the first method, a colorimetric assay was developed that detects acetoin, an intermediate in the biosynthetic pathway of branched chain amino acids, which is accumulated in the presence of cyclopropanedicarboxylic acid (CPCA), an inhibitor of ketoacid reductoisomerase (KARI). In the presence of the herbicide, it was possible to distinguish non-transgenic from transgenic plants. Qualitative analysis of acetoin formed during the AHAS inhibition allowed to indirectly determine the *Atahas* transgene expression. The second method measured the kinetics of chlorophyll fluorescence emission. Leaf discs pre-treated with imazapyr for 24 hours were evaluated using the modulated fluorimeter for maximum quantum efficiency of Photosystem II (PSII) (Fv/Fm) and relative electron transport rate (ETR). Results showed that almost all species analyzed presented a marked decrease in Fv/Fm after treatment with imazapyr. In addition, the ETR was significantly reduced in transgenic plants treated with the herbicide. Collectively, our results showed that it is possible to identify transgenic plants expressing *Atahas* gene and infer their levels of tolerance to imazapyr at a very early stage after transformation.

Keywords Acetohydroxyacid synthase, Chlorophyll fluorescence, Colorimetric assay, Selection, Transgenic plants

INTRODUCTION

The detection of the presence of transgenes in genetically engineered (GE) plants using an appropriate method is a key step in the process of selecting promising lines. A series of analytical tools based on DNA (PCR and Southern blot), RNA (RT-PCR and northern blot) and protein (ELISA, Western blot and lateral flow immuno test) are available (Grothaus *et al*. 2006; Queiroz *et al*. 2019). However, each of these methods has its advantages and limitations with regard to differentiating between transgenic and non-transgenic plants and characterizing them at the molecular level.

Polymerase Chain Reaction (PCR) is the most commonly used method for detection and confirmation of GE plants. The method has the advantage of being effective, as it requires very few plant materials, which can be obtained early in the development of plants. It also has high sensitivity and specificity, since it is based on a pair of primer sequences which identity specific regions of the transgene, allowing for the selection of positive events for subsequent culturing and characterization (Ma *et al*. 2020).

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***Corresponding author** Francisco J. L. Aragão, francisco.aragao@embrapa.br, Tel: +55-61-34484777, Fax: +55-61-34484777

Albeit efficient, PCR only detects the presence of the transgene and does not give any insight about its expression or activity.

Our group has used the selection of transgenic plants *in vitro* in medium containing herbicide based on the insertion of a mutant gene of *Arabdopsis thaliana*, the *Atahas* gene, which codes the AHAS enzyme (acetohydroxyacid synthase or acetolactate synthase; EC 2.2.1.6) (Vianna *et al*. 2011; Cruz and Aragão 2014). The mutated form of this enzyme, unlike its native form, is not inhibited by the herbicides of the imidazolinones group. Thus, plants expressing the mutant version are tolerant to imazapyr. AHAS is the first enzyme involved in the biosynthesis of branched chain amino acids such as valine, leucine and isoleucine (Yu *et al*. 2008). As an alternative and complementary approach to PCR, we have been working to find biochemical tools that would allow for assaying the activity of this mutated enzyme, which confers tolerance to imazapyr while confirming the functional and transgenic status of the plants under study. So far, there are no ELISA nor lateral flow immune tests available since AHAS is very conserved among plant species.

One of the objectives of this study was to adapt the enzymatic activity test of AHAS (Singh *et al*. 1988; Avila *et al*. 2005), and integrate it into the laboratory routine of plant transformation, to confirm positive events of transgenic plants. This tool would allow for early and rapid evaluation of the presence, expression and activity of the AHAS enzyme. It is also possible to infer the transgene activity at the transgenic event, considering that both belong to the same DNA construct and are expected to be inserted in the same chromosomal locus.

The enzymatic activity method is a colorimetric test, for rapid diagnosis, often used to detect resistance to imidazolinones in plants, usually weeds, or as part of functional tests in transgenic plant analysis (Sato *et al*. 2009; Ogita *et al*. 2012; Xavier *et al*. 2013). In this test, cyclopropanedicarboxylic acid (CPCA) is used as an inhibitor of ketoacid reductoisomerase (KARI), an enzyme that catalyzes a key reaction of AHAS during the biosynthesis of branched-chain amino acids.

When a resistant plant is treated with a herbicide inhibitor of AHAS, in this case, imazapyr and CPCA, there is an accumulation of acetolactate, which is converted to acetoin at low pH to form a pink to red complex upon addition of creatine and naphthol. The intensity of the color formed in the reaction is proportional to the concentration of acetoin present in the mixture and can be quantified with a spectrophotometer at 530/540 nm wavelength (Osakabe *et al*. 2005).

Another simple method to detect the action of herbicides involves the study of photosynthesis. Emitted fluorescence of chlorophyll α allows the influence of environmental variations on the plant to be evaluated, as well as its sensitivity to abiotic stresses including that induced by herbicides (Murchie and Lawson 2013). The use of this technique in the screening of plants that show resistance to chemical compounds or other biotic or abiotic stresses is promising and shows advantages over conventional phenotyping techniques, since it is a simple, fast and non-invasive method (Sousa *et al*. 2014). Therefore, the application for early selection of GE plants is even more interesting since it consists of a non-destructive technique with a shorter time for obtaining results.

The measurement of chlorophyll α fluorescence offers insight into the mechanism of photosynthesis, which allows factors that may alter the plants efficiency in the use of quantum energy to be evaluated. The maximum quantum efficiency of Photosystem II (PSII) of plants is given by the ratio Fv/Fm, where Fv Is the variable fluorescence and Fm is the maximum fluorescence (Krause and Weiss 1991). The Fv/Fm ratio is used as an indicator of photosynthetic capacity in plants, and has become an important physiological feature in studies related to the action of herbicides (Kalaji *et al*. 2011). In addition, the relative rate of electron transport (ETR) is measurable and provides an index on the overall redox potential of plants. Some herbicides act by inhibiting the electron transport chain while others inhibit the synthesis of several molecules such as pigments, amino acids or proteins (Dayan and Zaccaro 2012). Imazapyr acts both by inhibiting the synthesis of amino acid and altering fluorescence parameters. The application of herbicides from the chemical group of imidazolinones in rice plants has been shown to alter the photosynthetic process of the plants as inferred from analysis of fluorescence emission in chlorophyll α (Sousa *et al*. 2014). This approach represents

a viable method for detection of resistance and/or tolerance of the imidazolinones group herbicides in plants.

The objective of the present work was to evaluate two protocols: colorimetric and chlorophyll α fluorescence to assist in the identification and early selection of positive transgenic events in a fast and efficient way, through biochemical analysis of the activity of the mutant AHAS enzyme from *Arabidopsis thaliana*.

MATERIALS AND METHODS

Plant material

For this experiment, transgenic lines of four species transformed to express the mutated *A. thaliana Atahas* gene (Fig. 1) were used: Cotton (*Gossypium herbaceum* cv. 7MH) line 20/4 (Aragão *et al*. 2005), cowpea (*Vigna unguiculata* cv BRS Nova Era) line 59 (Cruz and Aragão 2014), soybean (*Glycine max* cv. Conquista) line 8-19 (Aragão *et al*. 2000) and common bean (*Phaseolus vulgaris* cv Olathe Pinto) line 1.5 transformed according Aragão *et al*.

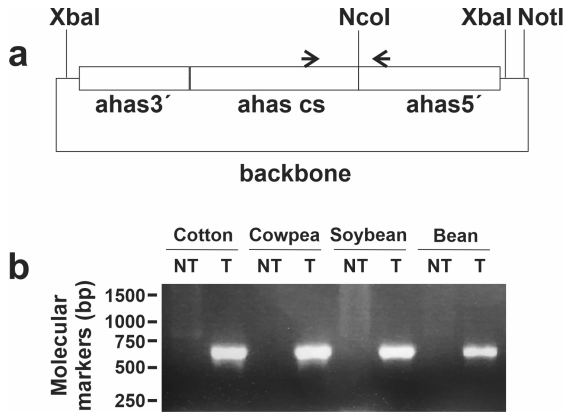

Fig. 1. (a) Diagram representing the cassette to express the *Atahas* gene in the transgenic lines of cotton, cowpea, soybean and bean. Atahas5´: *Atahas* gene promoter; *Atahas* cd: *A. thaliana* AHAS coding sequence; ahas3´: *Atahas* gene terminator). Small arrows indicate primers used for PCR screening of the transgenic lines. The backbones of the vectors are: pAC321 (Aragão *et al*. 2000) for cotton, soybean and common bean, and pCR2.1-TOPO (Invitrogen) for cowpea. (b) PCR for detection of the *Atahas* gene in the transgenic plants. T: transgenic plant, NT: non-transgenic plant.

(2000) and Rech *et al*. (2008). All lines were generated by combining resistance to the herbicide imazapyr as a selectable marker, multiple shoot induction from embryonic axes of mature seeds and biolistic techniques according Rech *et al*. (2008).

Polymerase chain reaction (PCR)

For PCR analysis of transformed plants, DNA was isolated from leaf disks according Edwards *et al*. (1991). PCR was carried out according Citadin *et al*. (2013). The primers ACTAGAGATTCCAGCGTCAC (AHASP124; within the *ahas* promoter) and GTGGCTATACAGATACCTGG (AHAS500C; within the *ahas* coding sequence) were utilized to amplify a 685 bp sequence.

Measurement of the AHAS activity

The protocol for measuring AHAS activity was based on the colorimetric enzyme assay described by Singh *et al*. (1988), with some modifications. For each plant, a leaf disc of 1.5 cm in diameter was collected, washed with water and immersed in 2 mL of pretreatment solution with 1/4 MS basal medium (Sigma Aldrich), 1mM of cyclopropanedicarboxylic acid (Sigma Aldrich) and imazapyr (2-(4.5-dihydro-4-methylethyl)-5-oxo-1Himidazol-2-yl)-3-pyridinecarboxylic acid; technical grade, (BASF) in tested concentrations of 0.4 μM for cotton, 0.7 μM for cowpea, 0.6 μM for soybean, and 0.6 μM for bean.

The same pre-treatment was carried out on the cultivars without imazapyr in the medium. The samples were maintained under fluorescent light and, after 5 hours, 10 mM of sodium pyruvate (Sigma Aldrich) was added, maintaining them for another 19 hours. In the specific case of beans, pyruvate was added 20 hours later. Leaf discs were removed from the pretreatment solution and transferred to another tube, where they were left at −20℃ for 1 hour, and then 220 μL of Triton X-100 (Sigma Aldrich) solution at 0.025% concentration was then added and incubated at 60℃ for 10 minutes. After that time, the sample was centrifuged for 3 minutes at full speed and then 110 μL of supernatant was collected, 10 μL of H_2SO_4 5% (Vetec) was added and the samples were incubated for 30 minutes more at 60ºC. From this material, 100 μL was collected and added to a clear 96-well plate, where a further

50 μL of 0.5% creatine (Sigma Aldrich) was mixed and then 50 μL of 5% 1-naphthol (Merck) dissolved in 2.5 N NaOH (JT Backer), both prepared previously at application. This was then transferred to another plate that was incubated at 37℃ for 30 minutes. The absorbance was measured in a spectrophotometer (Bio-Rad) with a 540 nm. For each treatment, triplicates of wild type (wt) and transgenic lines were used, and the changes in the visual staining as well as the absorbance values were compared with those of untransformed plants.

Measurement of chlorophyll fluorescence

To measure the kinetics of chlorophyll fluorescence emission, the third expanded leaf counted from the apex to the base of the plant was used. The leaves were kept in the dark for 30 minutes before measurements. The ADC Bio Scientific Ltd, OS5P modulated fluorimeter was used to analyze the maximum quantum efficiency of Photosystem II (PSII) (Fv/Fm) and the relative electron transport rate (ETR).

For the initial herbicide tests, two-week-old plants were grown in greenhouse at an average temperature of 25℃ and a relative humidity of 70%. The application of herbicide was carried out in two ways, by means of foliar spraying in the greenhouse or in the laboratory directly on leaf discs removed from plants. Foliar disks were maintained in Petri dishes containing 2% sucrose and 1 mM MES (2-(N-morpholino) ethanesulfonic acid buffer) pH = 6.5 containing varying doses of imazapyr, or not. Analyses were performed 24 hours after exposure to the herbicide in both cases. Doses of imazapyr used varied according to the analyzed species. The concentrations used were 200 μM, 300 μM and 500 μM for cotton, soybeans, and beans, respectively. For the cowpea, 500 μM, 600 μM and 1 mM imazapyr were used.

RESULTS

Colorimetric assay

The presence of the *Atahas* gene was confirmed in the plants used in this study by PCR analyses (Fig. 1).

The concentrations of imazapyr used for each species were 0.4 μM to cotton, 0.6 μM for soybean and common bean, and 0.7 μM in cowpea. When subjected to the colorimetric assay, wild type (wt) plants resulted in the formation of yellow to orange coloration while transgenic plants presented coloration between pink and red (Fig. 2a). This pattern was consistently maintained in cotton, cowpea and soybean. The visible results for both plants show the efficacy of this protocol on the selection of transgenic plants where it is possible to see the distinction between transgenic plants with reddish coloration and wild type plants in yellow/orange (Fig. 2a).

The data generated from absorbance readings is shown in Fig. 2b. The difference in maintenance of AHAS activity was evaluated in transgenic and wild type plants through the accumulation of acetoin, as recorded by absorbance of the reaction mixture from leaf materials treatment with and

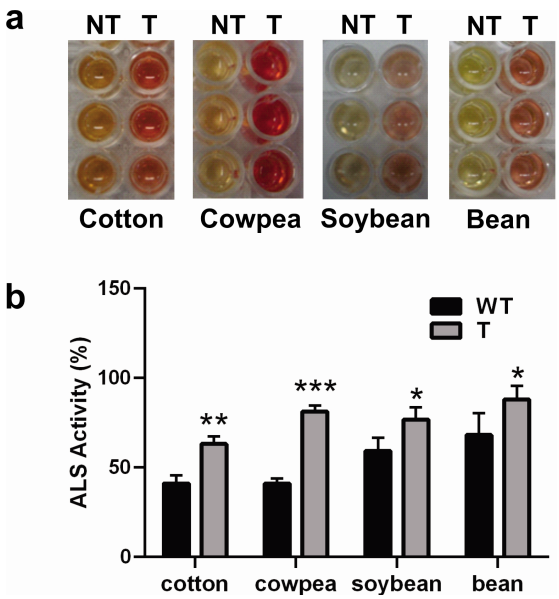

Fig. 2. Enzymatic colorimetric assays from untransformed and transgenic plants. (a) The image shows the difference in acetoin accumulation between untransformed plants (yellow/orange) and transgenic plants (pink/red). (b) Comparison of percentage of acetolactate synthase activity between WT (wild type) and transgenic plants (T) by the accumulation of acetoin in treatment with or without imazapyr in spectrophotometer at 540 nm. The asterisks represent a significant difference by the T test (*$P < 0.05$, **$P = 0.0042$ and ***$P < 0.0001$ compared to the WT of each species).

without herbicide. The best result was obtained with cowpea, where both the visual aspect and absorbance reading resulted from the maintenance of 80% activity of AHAS in the transgenic plant after treatment with herbicide. This represents twice the activity in wild type plants. This same transgenic event had previously been reported as highly tolerant to imazapyr in plant spraying tests (Citadin *et al*. 2013).

Although the results recorded in cotton, soybean and bean were not as significant as in cowpea, it was possible to observe a significant difference in maintenance of enzyme activity after application of herbicide between transgenic and wild type plants. This is clearly visible in the color change (Fig. 2a). In this experiment it was observed that the transgenic common bean line is tolerant to a high herbicide dosage of imazapyr (0.6 μM) used in the experiment (Fig. 2).

Chlorophyll Fluorescence assay

The effects of the application of imazapyr on chlorophyll α fluorescence are shown in Fig. 3. After 24 hours of treatment, there were no significant changes in plants treated with herbicide by spraying (data not shown). However, there was a significant reduction in the fluorescence characteristics analyzed when the treatment was performed on leaf discs (Fig. 3). Data from the graph demonstrate reduction of maximum quantum efficiency of photosystem II (Fv/Fm) in cotton, soybean and bean after treatment with the herbicide, when compared to the wt. However, this was not observed in cowpea. Similarly, the rate of photosynthetic ETR was reduced by treatment with imazapyr, with low values recorded for all the plants except cowpea (Fig. 3b).

DISCUSSION

One of the goals of this experiment was to establish the ideal conditions required to develop a protocol for evaluating the activity of the AHAS enzyme in each of the tested cultures. This helps in confirming the presence and expression of the transgene. For this, the adaptations of the protocol sought to reduce the scale of the experiment, in relation to the studies already reported, by relying on the visual difference in color change between samples of transgenic plants and wild type. The initial tests, following the protocol of Osakabe *et al*. (2005), were to establish the appropriate dose of the herbicide, because each wild type line may have different levels of tolerance to imazapyr. Thus, different concentrations were tested (data not shown) until ideal concentrations of 0.4, 0.6 and 0.7 μM of imazapyr were established. Even after the ideal doses of herbicide were defined, acetoin is still formed in the wild type plant. Thus, assuming that pyruvate is a substrate of acetolactate synthase (Eram and Ma 2016), it would be interesting to add it after pre-treatment with herbicide in order to inhibit the susceptible native enzymes before

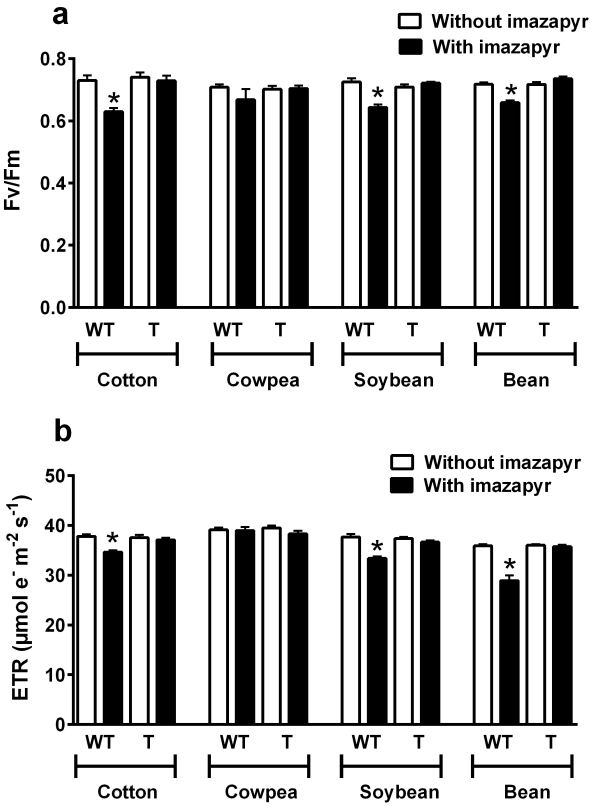

Fig. 3. Chlorophyll fluorescence parameters from untransformed and transgenic plants. The imagens show a decrease of chlorophyll a fluorescence parameters in almost all genotypes analyzed. (a) Maximum quantum efficiency of photosystem II - ratio of the variable and maximal fluorescence (Fv/Fm). (b) Relative electron transport (ETR). The asterisks represent a significant difference by the T test ($P < 0.05$).

coming into contact with the substrate, which would allow a better visual result in the wild type plants in relation to the transgenic plants. This establishes a reliable threshold within which wild type and transgenic plants are easily distinguished as can be observed (Fig. 1).

Here, we show for the first time the use of the enzymatic colorimetric assay for the detection of transgenic plants, which was previously used only for selection of herbicide tolerant genotypes. Monqueiro (2001) used an AHAS assay to differentiate susceptible and tolerant genotypes from *Bidens pilosa* and *Amaranthus guitensis* to chlorimuron-ethyl and imazethapyr herbicides. The plants were sprayed with a high dose of Ex ach herbicide and evaluated in a 2-pair leaf stage. Gerwick *et al*. (1993) distinguished susceptible and tolerant biotypes from *Xanthium strumarium* to the herbicide Imazaquim using the same assay.

In another approach, by Sato *et al*. (2009) used the colorimetric test to evaluate the activity of the rice mutant AHAS enzyme in transgenic plants of *Festuca arundinacea*, tolerant to bispyribac-sodium herbicide. In this work, the assay was conducted to complement other molecular characterization assays, but not necessarily as a way to select transgenic plants. Similarly, Ogita *et al*. (2012) characterized *Phyllostachys nigra* transformed via particle bombardment with the AHAS mutant gene from *Oryza sativa*, confirming the integration of the transgene by this assay.

In the present work, the Fv/Fm ratio, which represents the quantum yield of PSII and ETR, was evaluated as fluorescence parameters of chlorophyll α. The Fv/Fm ratio as well as the ETR, was used as they are good indicators of photoinhibitory damage in stress-prone plants, including those caused by the application of herbicides. The Fv/Fm fluorescence parameter is widely used to detect plant stress (Maxwell and Johnson, 2000). Although no changes in Fv/Fm ratio and ETR were recorded 24 hours after the application of herbicide, exposure beyond 48 hours demonstrated changes especially when higher concentrations of the herbicide were used (data not shown).

However, when the treatment was performed on leaf discs, our results show that almost all species analyzed showed a marked decrease in Fv/Fm after treatment with imazapyr. Similar results were observed by Barbagallo *et al*. (2003), who observed variations in the Fv/Fm in *Arabidopsis* treated with high concentrations of imazapyr at 4 and 8mM concentrations. In this same work, the lowest concentration used was 0.8mM, which resulted in alteration of the parameters evaluated after 48 hours.

Analysis of florescence has previously been demonstrated to be useful in detecting tolerance to herbicide belonging to the imidazolinone group in rice (Sousa *et al*. 2014). This has been confirmed by this work as demonstrated by the efficiency of chlorophyll fluorescence in the selection of plants that are tolerant to herbicide. However, in contrast to Sousa *et al*. (2014), we used this strategy to select for transgenic events. In addition, we have been able to prematurely evaluate putative transgenic events even when the explants are only regenerating. Thus, the use of leaf disc from regenerating putative explant allows a timely conclusion to be reached as to whether that plant is transgenic or not, and therefore dispensing with the requirement for the use of herbicide for selection subsequent (Sousa *et al*. 2014).

It is interesting to note that this herbicide does not act directly on the transport chain, but it inhibits the action of the AHAS enzyme which plays an important role in the synthesis of essential amino acids and important metabolic reactions for the plants. Therefore, photosynthesis is not considered a primary target of AHAS inhibitor herbicides such as imazapyr, but its rates change after application of the herbicide. Some studies also show the effect of herbicides on the emission of chlorophyll fluorescence α (Barbagallo *et al*. 2003; Silva *et al*. 2009; Dayan and Zaccaro 2012; Sousa *et al*. 2014). These herbicides are active in concentrations considered low, generating symptoms in the plant after only a few days of use (Zhou *et al*. 2007). In this way, chlorophyll fluorescence is a tool capable of rapidly assessing damage to photosystem II and selecting herbicide tolerant or genetically transformed plants for this characteristic (Barbagallo *et al*. 2003).

These results clearly demonstrate the potential for the use of chlorophyll fluorescence imaging to rapidly detect metabolic disturbances in the transgenic lines tested under the effect of imazapyr herbicide. In most of the plants tested, it is possible to use this parameter to differentiate between herbicide tolerant and sensitive plants or, in this

specific case, confirm the transgenic at the functional level of the plants containing the *Atahas* gene.

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