Scientific Research

Genomic Stability of *Palmer amaranth* Plants Derived by Macro-Vegetative Propagation

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Abstract

qPCR (quantitative polymerase chain reaction) and random amplified polymorphic DNA (RAPD) were utilized to investigate genetic stability of *Palmer amaranth* cloned plants over 10 generations. DNA from original parent *Palmer amaranth* plants (grown from seeds) was re-analyzed using qPCR, and confidence levels for determining ΔΔCt (threshold crossing) values were established. ANOVA was used to determine variation (margin of error) of these ΔΔCt values. This margin of error was applied to qPCR analysis of DNA from eight individual parent plants and their descendants (10th generation) so that possible differences in EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene copy number could be ascertained. This method (and the associated error) indicated a lack of agreement in ΔΔCt values of DNA from plants of these two generations. qPCR analysis showed that in five out of eight clones, EPSPS gene copy number varied more than the calculated error (P = 0.05). A second technique to monitor genetic stability, RAPD was used to determine possible changes in genomic DNA due to extended cloning of these regenerated plants. RAPD analysis showed that four out of the eight clones differed when the profiles of the two generations were compared. Results show that qPCR and RAPD analysis point to the fact that several *Palmer amaranth* clones experienced changes in genome structure over 10 generations. Although the glyphosate resistance trait was retained, results suggest that during cloning studies, the genetic stability of macro-vegetatively propagated lines should be monitored.

Keywords

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1. Introduction

Weeds have an estimated potential to cause nearly a 35% loss in crop yield when six crops were examined on a worldwide basis [1]. World pesticide expenditures exceeded $35 billion in 2007, with herbicides totaling over 40% ($14 billion) and herbicide sales in the US totaled nearly $5 billion in 2007 [2]. There are other obvious additional economic factors associated with weeds including, herbicide application costs and crop yield losses due to competition and contamination. Annual costs (damage and control costs) associated with alien plant species introduced into the US has been estimated at $34 billion [3].

An additional facet of weed control or weed management is the development of herbicide resistance. Currently, 238 species of weeds have become resistant to various herbicides in 65 countries, with 30 weeds documented as resistant to glyphosate [*N*-(phosphonomethyl)glycine] [4]. Glyphosate use in glyphosate-resistant (GR) crops to control weeds has exacerbated the development of weed resistance to this herbicide [5].

Palmer amaranth (*Amaranthus palmeri* S. Wats.), a dioecious plant (either male or female), has a rapid growth rate [6], can attain a height of 2 m or more [7] and produces numerous seeds [8] [9]. In addition to its weedy traits, *Palmer amaranth* has become a major glyphosate-resistant weed in the southeastern United States [10] and some biotypes possess resistance to other herbicides including: PS II inhibitors (triazine), acetolactate-synthase inhibitors, and dinitroanilines, and HPPD inhibitors [4] [11]-[15]. These growth and reproductive traits coupled with the tendency to develop resistance to herbicides make it extremely competitive and difficult to control.

Wide genetic variability within a weed population can lead to the selection of a resistant individual when the population is under high selection pressure (for example, repeated applications of the same herbicide). As a result, resistant plants in a field can dominate a population in only four to five generations (years) [16]. Glyphosate-resistance in *Palmer amaranth* (*Amaranthus palmeri*) was first reported in Georgia [17] and soon following this event, glyphosate resistant *Palmer amaranth* was discovered in several other southern states [18] [19]. Glyphosate-resistant *Palmer amaranth* is now widespread in the southern US where it has been cited as one of the most troublesome weeds of agronomic crops [20].

Multiple factors are involved in the distribution of *Palmer amaranth*, including the spread of seeds by equipment, animals, wind and water. Resistance traits can be spread through pollen (genetic exchange), and since *Palmer amaranth* is an obligate out-crosser (cross-pollinator), exchange of genetic traits can proceed rapidly. Pollen can travel up to 1000 feet from a resistant male plant to susceptible female plants [21]. Some populations of *Palmer amaranth* contain multiple copies (5 to 160) of the EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene, which was originally reported as the mechanism of resistance to glyphosate [22]. This resistance trait has also been found in other biotypes of this species [23]-[26].

Studies of populations of a given weed versus analysis of individual plants when addressing mechanism of resistance or other certain physiological, biochemical and molecular biological questions pose an important issue. In *Palmer amaranth*, results have indicated a wide range or variation in resistance in biotype populations and wide diversity of individuals within populations of this weed [27]. A macro-vegetative propagation method was used to facilitate long-term study on selected cloned lines of this weed so that in-depth examination of individual plants could be achieved [26]. RAPD (random amplified polymorphic DNA) has proven to be an efficient method to detect genetic variations [28] [29] and RAPD markers have been successfully applied to detect the genetic similarities or dissimilarities in micro-propagated material in various plants [30]. Quantitative polymerase chain reaction (qPCR) is another technique used to amplify and quantify a specific RNA/DNA sequence. qPCR enables both detection and quantification of one or more specific sequences in an RNA/DNA sample. Quantification can provide an absolute number of RNA/DNA sequence copies or a relative amount of copies when normalized to RNA/DNA input or additional normalizing genes [31]. This technique has been useful to determine gene copy number in various studies of herbicide resistant Palmer [22] [26].

Due to the increasing spread and economic cost associated with the spread of glyphosate-resistance *Palmer amaranth* more biochemical, physiological and molecular biological studies related to growth and reproduction, mechanisms of herbicide resistance and methods of controlling this weed are important. Knowledge gained through these studies will lead to more effective control of this serious weed problem. Continued propagation of selected cloned plant lines can provide genetically characterized individual plant material to carry out such studies. The objectives of this study were to use qPCR and RAPD techniques to examine the genomic stability of individual *Palmer amaranth* plants descended via macro-vegetative propagation (cloning) over 10 generations from parents previously characterized to have a range of tolerance or resistance to glyphosate [26].

2. Materials and Methods

2.1. Plant Growth and Culture

Seeds of the *Palmer amaranth* source were planted in a commercial potting mix Jiffy-mix (Jiffy Products of America, Inc., USA) contained in plastic trays (12 cm by 12 cm). These trays were placed into larger sub-irrigated trays placed on greenhouse benches. Greenhouse temperatures ranged from 28°C to 32°C with 40% - 90% relative humidity (RH). The photoperiod was 12 - 14 h with 1650 $\mu Em^{-2} \cdot s^{-1}$ photosynthetically active radiation measured at midday with a light meter (LI-COR, Inc., USA). The plants were thinned about one week after emergence (6 - 8 per tray) and the young seedlings were supplied with NPK (Miracle-Gro; The Scotts Company LLC, USA) on a weekly cycle. Two weeks after emergence, plants were selected and transplanted into large plastic pots (160 mm diam.) containing the soil mixture as described above and grown to the appropriate stage as needed for testing.

2.2. Plant Macro-Vegetative Propagation (Cloning)

To test and characterize individuals from this population in a variety of tests over a long time period, selected individual parent plants (grown from seeds) were cloned as described elsewhere [26]. Parent plants and their successive cloned lines used in the present study were previously named using an alpha-numeric code (R-5, R-6, R-7, R-9, G-1, G-3, G-4, G-8). Briefly, excised petioles from branch points (~10 - 20 mm long) of mature plants were immediately placed in deionized water. Each excised plantlet was removed from the water, blotted (absorbent paper towels), the cut end coated with rooting hormone powder (Hormex; 1.6%) (Brooker Chemicals, USA), and planted in moistened vermiculite/peat/loam mixture contained in plastic trays (6 cm × 14 cm). The planted cuttings (8 to 10 plantlets per tray) were maintained at 21°C - 23°C under low continuous light (~150 $\mu Em^{-2} \cdot s^{-1}$) for 6 to 7 days to allow root initiation and growth, and then transferred to a greenhouse (28°C - 34°C, a 14/10 day/night cycle at ~1650 $\mu Em^{-2} \cdot s^{-1}$ during the day) or growth chamber at similar environmental conditions. Plants propagated by this method were grown to the desired growth stage, re-cloned and/or used for testing.

2.3. DNA Extraction and qPCR

DNA was isolated from fresh leaves of *Palmer amaranth* according to a modified method of the DNeasy Plant Mini Kit (QIAGEN Inc., USA), quantified spectrophotometrically by means of a Nano Drop^{TM} 2000C and analyzed for quality (lack of fragmentation or degradation) using gel-electrophoresis. qPCR was conducted using Power Syber-Green Master Mix (Applied Biosystems, USA) on an ABI-7500 Real Time PCR System (Applied Biosystems, USA). The qPCR thermocycler program consisted of an initial 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, with a final extension of 10 min at 72°C. To determine gene copy number of EPSPS, gene specific primers for EPSPS and ALS (acetolactate synthase) were utilized: EPSPSF (5'-CAACAGTTGAGGAAGGATCTG-3', EPSPSR (5'-CAGCAAGAGAGAATGCCAT-3') and ALS, ALSF (5'-GCTGCTGAAGGCTACGCT-3'), ALSR (5'-GCGGGACTGAGTCAAGAAGTG-3') [22] and primer efficiency was determined [31]. $\Delta\Delta Ct = [(Ct_{EPSPS} - Ct_{ALS})$ resistant $- (Ct_{EPSPS} - Ct_{ALS})]$ sensitive was determined relative to the low copy number control ALS gene, with known monogenic inheritance in other *Amaranthus* species [32].

2.4. RAPD Analysis

Five arbitrary 10-base primers (Ready-To-Go RAPD Analysis Beads; GE Healthcare, USA) were used for polymerase chain reaction (PCR). Primer sequences were: P1-5'-GGTGCGGGAA-3'; P2-5'-GTTTCGCTCC-3'; P3-5'-GTAGACCCGT-3'; P5-5'-AACGCGCAAC-3'; and P6-5'-CCCGTCAGCA-3'. Amplification reactions were performed with one reaction bead, 50 ng template DNA, and 25 pmol of the primer, brought to a volume of 25 µl with molecular grade water. Replicate DNA amplification was performed in a PTC-200 Peltier thermal cycler (St. Bruno Quebec, Canada) programmed for 45 cycles as follows: first cycle of 5 min at 95°C, 45 cycles each of 1 min at 95°C, 1 min at 36°C, 2 min at 72°C. The amplification products were separated by electrophoresis in 2% (w/v) agarose gel with 0.5× TAE buffer and stained with fluorescent DNA dye (AMRESCO EZ-vision; AMRESCO, USA).

2.5. Amplified DNA Marker Scoring

Electrophoretic gel images were captured with Bio-Rad Image Lab software version 4.1 build 16 and evaluated for presence and size utilizing Carestream Molecular Imaging Software, 5.0 2.30. Amplified DNA band profiles on gels were scored as present or absent for each macro-propagated plant. DNA bands of low visual intensity (not readily discernible) were considered as ambiguous markers and were not scored. The data were analyzed using locus-to-locus gel readings and the rates of *in vitro* DNA polymorphism were determined and presented as number of bands in agarose gels for each clone.

2.6. Statistical Analysis

Analysis of variance (ANOVA) was performed using SAS Statistical Software to determine if current generation relative ΔΔCt was different from parent generation relative ΔΔCt. ANOVA was performed using a general linear mixed model with fixed effects for: generation (parent vs current), type (resistant vs susceptible), clone within type (8 resistant clones plus 2 susceptible clones), generation × type and clone × generation within type. Random effects estimate sources of variability in data with Rep (random block effect representing differences in parent data between stored DNA and DNA at time of extraction), Rep[*] generation and residual error (3 technical measurements on each sample). Least Significant Differences (LSD) at $P \leq 0.05$ were calculated and used to compare: 1) Ct difference between each resistant clone and susceptible clone (C3-9); 2) Ct difference between each resistant clone and susceptible clone (C3-7); and 3) Ct difference between each resistant clone and the mean of susceptible clones (C3-9 and C3-7).

3. Results and Discussion

In our investigations of glyphosate resistance in *Palmer amaranth* plants of resistant and susceptible populations, plants grown from seed were tested using whole plant spraying (destructive) and leaf disc bioassay (nondestructive) techniques [27] [33]. Selected plants were then chosen for macro-vegetative propagation (cloning) [26] to retain consistent genetic lines of these individuals for extensive investigations. This method has enabled the maintenance of many genetically distinct lines of *Palmer amaranth* asexually, without introduction of new diverse genetic material experienced when plants cross-breed. *Palmer amaranth* can self-cross or cross with similar species [34] [32], thus altering the genome each generation. The mechanism of glyphosate resistance we are primarily investigating is increased EPSPS gene copy number [22] and an important technique to estimate increased gene copy number is by qPCR.

The present study utilized qPCR and RAPD analysis to examine the genetic stability of successively cloned *palmer amaranth* plants. To determine the error within the qPCR method, duplicate assays were compared. Genomic DNA from the parent of each of the eight lines cloned was initially analyzed at extraction time, and a second test performed on the same extract (stored −80˚C) one year later (**Figure 1**). The average difference in ΔΔCt values between the fresh and stored parent DNA was 0.235 ± 0.042 SEM. Changes in ΔΔCt values outside of this error were arbitrarily considered to reflect real changes in the EPSPS gene copy number when applied to other qPCR analyses comparing cloned plants tissue to that of their respective parents.

ANOVA was used to estimate the ΔΔCt values (determined by qPCR) of each 10th generation *Palmer amaranth* plant relative to its parent. **Table 1** depicts the ΔΔCt of the 10th generation minus the ΔΔCt of the parent generation. P is the percent or probability of being wrong at the rejection of the null hypothesis: parent generation ΔΔCt value EPSPS gene is equal to ΔΔCt value EPSPS gene of the new generation. qPCR results comparing the parent to the 10th generation clone indicated that 5 out of eight clones had a significantly different EPSPS gene copy number (**Table 1**). qPCR has been used to demonstrate that elevated EPSPS gene copy number in *Palmer amaranth* populations confers resistance to glyphosate [22]. However, due to the exceptionally high copy number found in this weed (>100), even a small error in ΔΔCt can greatly skew the gene copy number value. It has been suggested that generally between 30 - 50 EPSPS gene copy numbers are required for a plant to survive typical field rates of glyphosate [35]. All plants used in the present study had copy numbers greater than this value range and they all (parent plants and 10th generation plants) exhibited resistance to glyphosate. In another study, individual plants with a wide range of EPSPS gene copy numbers within a population exhibited varying degrees of susceptibility to glyphosate which did not always correlate with copy number, suggesting the possibility of other resistance mechanisms [26]. Tests on another population of *Palmer amaranth* with low EPSPS
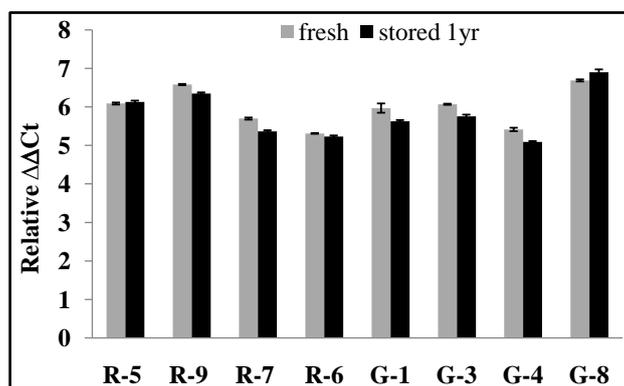
Figure 1. Average difference in qPCR ΔΔCt values between fresh and stored original parent DNA. Error bars are ±1 SEM.

Table 1. ΔΔCt differences between original parent and 10th generation clone. Asterisks indicate clones with altered EPSPS gene copy number.

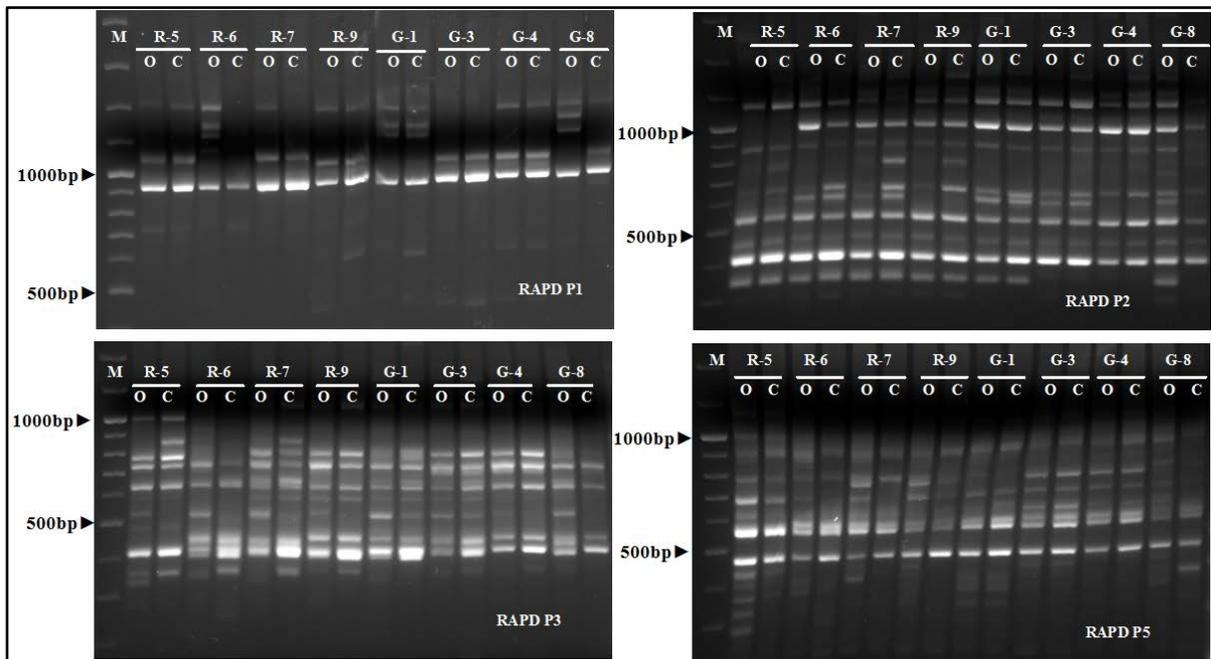| Clone	R-5*	R-6	R-7	R-9*	G-1*	G-3*	G-4	G-8*
ΔΔCt	0.8466	0.1334	0.4272	−0.6991	−0.5719	−0.6169	0.2857	−0.8466
P	0.0068	0.5439	0.0742	0.0092	0.0243	0.0172	0.2099	0.0031

gene copy numbers showed that individual plants exhibited a wide degree of susceptibility to glyphosate, also suggesting alternative resistance mechanisms to this herbicide [33].

RAPD analysis is an easy and economical way to evaluate the genetic fidelity of propagated plants. For example, it has been utilized to test genetic stability in prickly pear (*Opuntia* spp.) [36] and turmeric (*Curcuma longa*) [37]. In another study, five primers were used to distinguish 13 polymorphisms that occurred across 6 of 23 axillary branching-derived plants from *Populus deltoides* clones [38]. RAPD markers were useful to evaluate genetic stability of regenerated soybean plants, obtained through somatic embryogenesis [39]. This method has also been utilized to develop markers in *Amaranthus* species [40].

In the present study, RAPD analysis was used to determine possible genetic variance in *Palmer amaranth* propagated over ten cloned generations. Five primers produced 34 reproducible and polymorphic banding patterns, and the number of bands produced ranged from 5 - 9 scoreable bands, depending on primer used. Gel images (**Figure 2**) of RAPD profiles of primers P1, P2, P3 and P5 show the banding patterns of the original parent (O) and 10th generation clone (C). Primer P6 did not show any differences in banding patterns and therefore is not depicted. The presence or absence of a band differing from the parent suggests that the genome has changed during the ten generations covered in this study. A summation of the gel image data is presented in tabular form (**Table 2**). Changes in the banding pattern indicates some alterations in the genome, however these changes may or may not reflect changes of the EPSPS gene. **Table 3** summarizes the results of the qPCR and RAPD analyses for each cloned *Palmer amaranth* line. When the 10th generation clones were compared to their corresponding original parent, R-5 and G-8 showed changes with both methods, whereas R-5, R-6, R-7 and G-8 showed changes utilizing the RAPD method and R-5, R-9, G-1, G3 and G-8 indicated genomic changes with qPCR. The one exception was the 10th generation clone G-4 which showed no changes using either method. This could be due the fact that no changes occurred, changes occurred but were below the sensitivity of specific qPCR method conditions, or changes may have occurred outside the genomic regions that were analyzed by RAPD.

Modifications of the qPCR method, such as incorporation of gene specific molecular beacons for the target and reference genes, could increase sensitivity (and costs). Greater numbers of primers than included in the RAPD kit would have increased coverage of the genome, thereby increasing the ability to detect genomic alterations and may have resulted in a closer agreement between the qPCR and RAPD results observed in the present study. Nevertheless, the results of this study suggest that some genomic alterations occurred during the 10 generations of cloning in these *Palmer amaranth*s, but not to a degree that compromised resistance to glyphosate. It is possible that during the process of macro-propagation transposon activity or other mechanism(s) may be re-

Figure 2. Genetic variation in *Palmer amaranth* clones relative to parent plants investigated by RAPD analysis. Lane M represents a 100 bp ladder. Clones: R-5, R-6, R-7, R-9, G-1, G-3, G-4, G-8. Original parent = O; Clone = C. RADP primers P1, P2, P3 and P5 are defined in the Methods and Materials.

Table 2. RAPD band pattern comparison of 10th generation clone relative to original parent RAPD band pattern.

	R-5	R-6	R-7	R-9	G-1	G-3	G-4	G-8
Primer 1	same	different	same	same	same	same	same	different
Primer 2	same	same	different	same	same	same	same	different
Primer 3	different	different	different	same	same	same	same	different
Primer 5	same	same	different	same	same	same	same	different
Primer 6	same	same	same	same	same	same	same	same

RAPD banding profile rating: "same" = no changes in the banding pattern between original parent and 10th generation clone; "different" = band absent or present as a different size.

Table 3. Summary of qPCR and RAPD analysis of changes in genome profile[*].

Clone line	qPCR	RAPD
R-5	+	+
R-6		+
R-7		+
R-9	+	
G-1	+	
G-3	+	
G-4		
G-8	+	+

sponsible for altering EPSPS gene copy number and the genome. Alterations in the banding patterns could arise via transposon activity as suggested to occur in a glyphosate-resistant *Palmer amaranth* from Georgia, and re-

search to elucidate this mechanism controlling the DNA-mediated amplification is being pursued [41] [42]. Further investigation will be required to determine the cause of the alteration of the *Palmer amaranth* genome and to determine the mechanism of EPSPS gene copy increase.

4. Conclusions

Overall, the RAPD primers used to examine the genome of 8 cloned lines revealed that 4 of the 8 lines had genomic changes after 10 generations, which suggests that in plant cloning studies, it is prudent to monitor genetic stability of the propagated lines. Cloning by macro-vegetative propagation can be utilized for evaluating plants during long-term studies, and multiple clones can be used to provide replicates in non-lethal or in lethal tests where plants may be sacrificed since back-up cloned plants are available for continued propagation of each cloned line. Long-term micro-propagation has been utilized for conservation and preservation of plant resources under *in vitro* conditions and to produce numerous plants (clones) with identical genetics. This method has been used to propagate disease-free planting material and has commercial implications [37]. However, some *in vitro* transfers over long periods can induce somaclonal genetic variations [43]. There are also cases of low frequency somaclonal genetic variations from axillary-branch raised plants, such as *Opuntia ficus-indica* [36]. However, an axillary-branch technique was found to be a highly reliable and genetically true-to-type method of plant propagation [36].

Some variation occurred in *Palmer amaranth* after successive cloning over 10 generations and transposon activity may be a possible cause. The fact that some changes occurred in the genetic profiles may be important in characterizing the stability and primal cause(s) involved in the phenomenon of elevated EPSPS gene copy number. While qPCR analysis indicated that 5 of the 8 clone lines underwent changes in the EPSPS gene copy number, the two lines without changes may contain altered copy numbers at levels not detectable due to the ΔCt error associated with the exceptionally high copy number of glyphosate-resistant *Palmer amaranth*. Also, the clone lines that did not appear to have altered genomes determined by RAPD analysis may contain changes that were not within the DNA sequences amplified by the primers utilized. Nevertheless, both of these methods indicate changes in EPSPS copy number when progeny plants were compared to parent plants. Further studies will be necessary to characterize the nature of the variation in genetic profiles observed in these macro-vegetatively propagated weeds.

Acknowledgements

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