Summary  Twenty percent of of the world’s flowering plants produce recalcitrant seeds (i.e., seeds that cannot withstand drying or freezing). We investigated whether the embryonic axis from the normally recalcitrant seeds of silver maple (Acer saccharinum L.) can be made tolerant to desiccation (10% water content) and low temperature (–196 °C, cryopreservation) by pretreatment with ABA or the compound tetcyclacis, which enhances endogenous ABA concentrations. Pretreatment of axes with both ABA and tetcyclacis increased germination after desiccation and freezing to 55% from a control value of zero. Pretreatment of axes with ABA and tetcyclacis increased the ABA content of the axes, as measured by enzyme-linked immunoassay, and stimulated the synthesis of storage and dehydrin-like proteins, believed to have a role in the desiccation tolerance of orthodox seeds.

Keywords: abscisic acid, recalcitrant, storage, temperature, tetcyclacis.

Introduction
Silver maple (Acer saccharinum L.) is a key native species in many North American swamp ecosystems (USDA Forest Service 1974), providing food for squirrels and beavers (Nixon and Ely 1969, Reichard 1976) and a nesting site for wood and goldeneye ducks and many other birds (Prince 1968, Gabbe et al. 2002). North American wetlands (e.g., Bernert et al. 1999, Lehtinen et al. 1999) are in widespread decline, and conservation work is hampered by the difficulty in storing the recalcitrant (i.e., intolerant of desiccation and freezing) seeds of silver maple (Roberts 1973, King and Roberts 1979). Silver maple seeds exhibit a loss in viability when desiccated to water contents below 30%, do not withstand low temperature storage (–196°C) (Becwar et al. 1983), and cannot be stored at 4°C for longer than 18 months (Jones 1920, USDA Forest Service 1974).

Daws et al. (2004) proposed the existence of “phenotypic recalcitrance,” which is recalcitrance contingent on environmental conditions during seed development. These authors found that, in the northern part of its range, Aesculus hippocastanum L. produced seeds that were less developed and exhibited greater sensitivity to desiccation when shed from the tree, than did trees of the same species in the southern portion of its range. This raises the question of whether it is possible, through environmental manipulation, to induce orthodox seed storage behavior in a recalcitrant seed.

There is evidence to suggest that seed recalcitrance occurs when seeds fail to undergo the final stages of orthodox seed development (Finch-Savage 1992, Farrant et al. 1993). These stages include a maturation phase, leading to tolerance of desiccation, followed by a drying phase during which seeds become metabolically quiescent. During maturation, a number of key events occur, including the synthesis and deposition of stored reserves (storage proteins, carbohydrates and lipids) and late embryogenic abundant (LEA) proteins such as dehydrins (reviewed by Thomas 1993), a class of hydrophilic proteins. Dehydrins are believed to play an essential role in the stabilization of membranes and proteins during dehydration (reviewed by Close 1996). Thus, understanding factors that affect dehydrin accumulation may help explain how seeds become desiccation tolerant.

Correlated with dehydrin accumulation in orthodox seeds is the presence of the plant growth regulator abscisic acid (ABA). Moreover, exogenous ABA stimulates the accumulation of dehydrins (Karssen 1995, Kermode 1995, Pammenter and Berjak 1999), thus suggesting a causal role of ABA in the development of orthodox seeds. In addition, ABA appears to have a regulatory role in embryo development, including the onset and breaking of dormancy (Hetherington and Quarano 1991).

Further evidence of a role of ABA in orthodox seed development is the finding that, in species that normally produce orthodox seeds, both ABA anabolic mutants, in which ABA synthesis in the embryonic axis is blocked (Rock and Zeevart 1991), and ABA-insensitive mutants (McCarty et al. 1991) produce seeds with a recalcitrant phenotype. As with other recalcitrant seeds, these mutant seeds can germinate precociously (before seed development is completed) or viviparously (on the maternal plant) (Koornneef et al. 1982, Brown 1995, Robertson 1995).
The plant growth inhibitor tetcyclacis inhibits ABA catabolism (Zeevaart et al. 1990, Davis and Curry 1991) leading to substantial increases in endogenous ABA concentrations (Hauser et al. 1990, Zeevaart et al. 1990, Belefant and Fong 1991) and may therefore be useful in studying the role of ABA in seed development and germination. The objective of this study was to assess the impact of ABA and tetcyclacis pretreatment on desiccation and freezing tolerance of silver maple embryonic axes (i.e., embryos with cotyledons removed).

Materials and methods

Collection of seeds and isolation of embryonic axes

Silver maple seeds were collected from a single tree in New Brunswick (45° N and 66° W), Canada, on June 7, 1997. The developmental stage of the seeds was standardized relative to anthesis (DAA = days after anthesis), where 0 DAA corresponds to the day when anthesis was first evident to the naked eye. Seeds were taken at four different developmental stages: 25, 30, 35 and 40 DAA. The 40 DAA collection represented mature seeds that were just starting to be dispersed naturally from the tree. (Mature seeds, as described here, specifically refers to the stage considered mature for recalcitrant seeds, not orthodox seeds, i.e., they germinate under normal conditions.) The 40 DAA seed collection excluded fallen seeds, to ensure that all were from the same tree.

Within 3 h of seed collection, ovules were isolated and surface sterilized in a 1% sodium hypochlorite solution for 5 min, rinsed three times in sterile water and blotted on sterile filter paper. Cotyledons were excised and the embryonic axes frozen at –20 °C for molecular analyses. All experimental treatments and tolerance tests were conducted with axes from the 40 DAA seed collection.

ABA and tetcyclacis pretreatments

Immediately following excision, axes were placed in 10-cm petri dishes containing 10 ml of woody plant medium (Lloyd and McCown 1981) plus one of the following six combinations of ABA (Sigma, St. Louis, MO) and tetcyclacis (diluted from BASF, Ludwigshafen, Germany): (1) 0 µM ABA + 0 M tetcyclacis (Control); (2) 20 µM ABA (20 µM ABA); (3) 60 µM ABA (60 µM ABA); (4) 10−6 M tetcyclacis (TC); (5) 20 µM ABA + 10−6 M tetcyclacis (20 µM ABA + TC); and (6) 60 µM ABA + 10−6 M TC (60 µM ABA + TC). Tetcyclacis was dissolved in acetone before addition to the medium. The final concentration of acetone in the medium was < 0.5%. The petri dishes were placed in a Conviron growth cabinet (26 °C, 12 h daily illumination from cool white fluorescent light (80 µmol m−2 s−1)) and then randomly allocated to four groups that were subjected to: (1) desiccation; (2) cryopreservation; (3) desiccation and cryopreservation; or (4) freezing at –20 °C, for isoelectric focusing (IEF) of proteins (Treatments 1 and 5), Western blotting (Treatments 2–6) and ABA quantification (Treatments 1–6). All axes in the control treatment germinated 4 days after being placed on woody plant medium (i.e., the root and shoots had elongated, the shoot area had turned green and the embryonic leaves had expanded). The other axes did not germinate during the 2-week treatment. The axes in the control treatment were frozen at –20 °C after being placed on woody plant medium for 2 and 6 days and then analyzed for proteins by IEF.

Determination of optimum desiccation rate for embryon axes

We investigated the effect of three rates of desiccation on germination of excised embryonic axes. In the fastest desiccation treatment, axes were placed in desiccators containing activated silica gel. In the intermediate desiccation rate treatment, axes were first placed in a desiccator containing sodium bromide (NaBr·2H2O, 58% relative humidity) followed by calcium chloride (CaCl2·6H2O, 31% relative humidity). In the slowest desiccation treatment, axes were placed first in a desiccator containing stirred saturated solutions of sodium phosphate (Na2HPO4·12H2O, 95% relative humidity), then ammonium chloride (NH4Cl, 79% relative humidity), sodium bromide (NaBr·2H2O, 58% relative humidity) and, finally, calcium chloride (CaCl2·6H2O, 31% relative humidity). Axis water content was equilibrated in each relative humidity regime before transfer to the next. The water content of the axes was determined on three replicates, each comprising 1 g of axes (International Seed Testing Association oven-drying method (ISTA 1985)) and expressed on a fresh mass basis. After desiccation, axes were placed in petri dishes containing woody plant medium in a Conviron growth cabinet (26 °C, 12 h daily illumination from cool white fluorescent light (80 µmol m−2 s−1)) and % germination and root and shoot growth of three replicates of 15 axes recorded.

For the experiment combining ABA and tetcyclacis pretreatments with desiccation, pretreated embryonic axes were dried to a water content of 10% at the intermediate rate. In addition, untreated 40 DAA embryonic axes (controls) were also desiccated. Following desiccation, the axes either underwent cryopreservation or were tested for germination capacity as described above (three replicates and 15 embryos). At the end of the two week germination test, axes were scored for shoot and root growth, if any. Additional pretreated and desiccated axes were frozen at –20 °C for measurement of ABA content.

Cryopreservation of embryon axes

Axes were cryopreserved by placing them in polypropylene vials (25 axes per 2.5-ml vial) (Nalgene, Rochester, NY) in a programmable freezer (Kryo 10, Planar Products, Sunbury-on-Thames, U.K.) and reducing the temperature from 2 to 0 °C at a rate of 5 °C min−1, and from 0 to –40 °C at a rate of –0.33 °C min−1. After 4 h at –40 °C, the vials were submerged in liquid nitrogen and placed in liquid nitrogen freezers for 24 h and for 1, 6, 12 and 24 months. To thaw the axes, vials were placed in a water bath at 40 °C for 5 min. The thawed axes were tested for germination capacity as described above (either three replicates of 15 embryos (24 hour cryopreservation treatment) or five replicates of 10 axes (1, 6, 12 and 24 months of cryopre-
servation treatments). Root and shoot growth during the germination test were monitored daily.

**Biochemical analyses**

Analyses of protein synthesis by IEF were conducted only on axes from the 20 µM ABA + TC pretreatment, which resulted in the highest desiccation tolerance (see Table 1). Analyses were conducted immediately following the pretreatment and at 2 and 6 days following germination. Unpretreated control axes were also analyzed. Western blotting, for the detection of dehydrin, was performed on mature unpretreated seeds and on axes from each of the pretreatments ABA or tetcyclacis, or both. Quantification of ABA was performed on all axes, including those taken at different stages of seed development (0, 25, 35, 40 DAA), as well those that underwent ABA + tetcyclacis pretreatments, with or without desiccation and cryopreservation.

**Assessment of actively synthesized protein**  Axes (0.1 g fresh mass) were homogenized in extraction buffer (1.0 M sodium chloride, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 62.5 mM Tris-HCl, pH 6.8) at 4 °C with a mortar and pestle that had been cooled with liquid nitrogen. After centrifugation at 16,000 g for 5 min, the supernatant was collected and heated at 100 °C for 5 min. Protein was quantified with the DC protein assay (Bio-Rad, Rockford, IL) and a bovine serum albumin standard.

For the examination of in vivo protein synthesis, samples (0.1 g fresh mass of axes) were incubated with 4.0 MBq [35S]-methionine (ICN Biomedical, Irving, CA) in distilled water for 2 h at 24 °C. Samples were rinsed with distilled water and proteins were isolated in extraction buffer as previously described. Incorporation of radiolabeled methionine was determined by cold acetone precipitation (Gifford and Bewley 1984).

**Isoelectric focusing**  Isoelectric focusing was performed with a Mini Isoelectric Focusing Unit (Bio-Rad) according to a modified procedure described by O’Farrell (1975). Gels were prefocused for 30 min at 200 V. After focusing, gels were extruded and incubated in a sample buffer (2% SDS, 10% glycerol, 0.0625 M Tris-HCl, pH 6.8) for 20 min. The pH gradient across the gel was determined using Bio-Rad IEF standards. For the second dimension, IEF gels were overlaid on an SDS 14% polyacrylamide gel (Laemmli 1970) under dissociating conditions (with SDS). Each well was loaded with 5 × 10^5 cpm of [35S]-methionine. The proteins were visualized according to fluorographic procedures described by Gifford and Bewley (1984).

**Western blotting**  Protein was isolated and quantified as described above, with the modification that 50 mM dithiothreitol was added to the extraction buffer. One-dimensional SDS-PAGE under dissociating and reducing conditions was performed using 14% polyacrylamide gels (Laemmli 1970). Twenty µg of protein was loaded per well. Proteins were transferred to a nitrocellulose membrane with a mini trans-blot electrophoretic transfer cell according to the manufacturer’s instructions (Bio-Rad). The nitrocellulose was probed with the primary antibody immunoglobulin G (IgG) against dehydrin in 1:5000 (v/v) dilution (a gift from Dr. T.J. Close, Department of Botany and Plant Sciences, University of California, Riverside, CA, USA), according to the procedure of Towbin et al. (1979). Binding of IgG to the membrane was visualized with anti-rabbit IgG conjugated to peroxidase (Jackson Immuno Research Laboratory, West Grove, PA) and the ELC chemiluminescence kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions.

**ABA quantification**  Abscisic acid was extracted from embryonic axes according to the procedure of Raikhel et al. (1987). Abscisic acid was quantified by an enzyme-linked immunosassay, Phytoptect-ABA (Idetek, Sunnyvale, CA), using a monoclonal antibody to 2-cis-(S)-ABA according to the manufacturer’s instructions. Recovery of ABA was 93% (± 5% standard error) of the 3H counts.

**Results**

**Effects of desiccation and cryopreservation on germination**

There was a rapid decline in germination percentage with desiccation at all desiccation rates (Figure 1). However, root growth was best at the intermediate desiccation rate (Table 1). The critical water content (King and Roberts 1979) at which viability was greatly reduced was 16–19% for the slow and medium desiccation rates and 23% for the fast desiccation rate (Figure 2).

Germination of desiccated embryonic axes pretreated with 20 and 60 µM ABA + TC was 97 and 63%, respectively (Table 1). Germination of axes desiccated after pretreatment with

![Figure 1](http://heronpublishing.com)
TC was only 22% (Table 1). All axes that germinated after desiccation formed viable seedlings (results not shown). Axes subjected to desiccation and cryopreservation failed to germinate unless pretreated with 20 or 60 µM ABA + TC (43 and 55% germination, respectively) (Table 2). Root growth was generally greater than shoot growth following cryopreservation (Table 2). In the desiccated and cryopreserved axes pretreated with 20 µM ABA + TC, germination was 50 to 54% following 1 to 24 months of storage (Table 3). Germination percentage was lower in the axes pretreated with 60 µM ABA + TC, ranging from 31 to 39% after 1 to 24 months of storage.

**Isoelectric focusing and Western blotting**

Protein synthesis was examined in unpretreated axes and axes pretreated with 20 µmol ABA + TC (i.e., with no desiccation or cryopreservation: Treatments 1, 2 and 6 days on woody plant medium). Axes from this pretreatment were chosen because they were the most tolerant to desiccation, as indicated by the % shoot and root growth following desiccation (Table 1). Synthesis of 55-kDa proteins, which are likely storage proteins (Ziegenfus and Clarkson 1971, Brown 1995), was evident in the mature seeds (40 DAA) and in the pretreated axes (Figure 3). Following 2 days of incubation on woody plant medium, the synthesis of these proteins decreased substantially and after 6 days, the axes were fully germinated and 55-kDa proteins were no longer being synthesized.

Western blotting was conducted with extracts of axes from each of the pretreatments with ABA or tetcyclacis or both, as

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### Table 1. Germination of embryonic axes from *Acer saccharinum* seeds collected 40 days after anthesis. Axes were pretreated for 2 weeks with abscisic acid (ABA) or tetcyclacis (TC), or both, and then desiccated to 10% water content at the intermediate rate. The “Control” comprised the axes placed on medium without ABA or tetcyclacis for 2 weeks, and the “Control (desiccated at excision)” were axes desiccated immediately after they were isolated from seed (no 2-week imbibition pre-treatment). Without pretreatment or desiccation, germination was 98%, and both roots and shoots elongated (results not shown). Values are the means of three replicates of 15 axes ± SE. Within a column, values followed by different letters are significantly different (P < 0.05) based on a Duncan Waller’s test of the means.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>% Germination of axes desiccated to 10% water content</th>
<th>% Growth after desiccation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Shoots</td>
</tr>
<tr>
<td>Control (desiccated at excision)</td>
<td>0 ± 0.0 a</td>
<td>0 ± 0.0 a</td>
</tr>
<tr>
<td>Control</td>
<td>0 ± 0.0 a</td>
<td>0 ± 0.0 a</td>
</tr>
<tr>
<td>TC</td>
<td>22 ± 2.6 b</td>
<td>22 ± 2.5 b</td>
</tr>
<tr>
<td>20 µM ABA</td>
<td>0 ± 0.0 a</td>
<td>0 ± 0.0 a</td>
</tr>
<tr>
<td>20 µM ABA + TC</td>
<td>97 ± 1.2 c</td>
<td>97 ± 1.2 c</td>
</tr>
<tr>
<td>60 µM ABA</td>
<td>0 ± 0.0 a</td>
<td>0 ± 0.0 a</td>
</tr>
<tr>
<td>60 µM ABA + TC</td>
<td>63 ± 1.7 d</td>
<td>63 ± 1.7 d</td>
</tr>
</tbody>
</table>

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### Table 2. Germination of desiccated (to 10% water content) abscisic acid (ABA) and tetcyclacis (TC)-treated axes from 40 DAA seeds after 24 h of cryopreservation. Without pretreatment or desiccation, germination was 98%, and both roots and shoots elongated (results not shown). Values are the means ± SE of three replicates, with 15 axes per replication. Within a column, values followed by different letters are significantly different (P < 0.05) based on a Duncan Waller’s test of the means.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Germination of axes after desiccation and cryopreservation</th>
<th>% Growth after cryopreservation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shoots</td>
</tr>
<tr>
<td>Control (cryopreserved)</td>
<td>0 ± 0.0 a</td>
<td>0 ± 0.0 a</td>
</tr>
<tr>
<td>Control (desiccated and cryopreserved)</td>
<td>0 ± 0.0 a</td>
<td>0 ± 0.0 a</td>
</tr>
<tr>
<td>TC</td>
<td>0 ± 0.0 a</td>
<td>0 ± 0.0 a</td>
</tr>
<tr>
<td>20 µM ABA</td>
<td>0 ± 0.0 a</td>
<td>0 ± 0.0 a</td>
</tr>
<tr>
<td>20 µM ABA + TC</td>
<td>55 ± 2.3 b</td>
<td>55 ± 2.3 b</td>
</tr>
<tr>
<td>60 µM ABA</td>
<td>0 ± 0.0 a</td>
<td>0 ± 0.0 a</td>
</tr>
<tr>
<td>60 µM ABA + TC</td>
<td>43 ± 3.3 b</td>
<td>43 ± 3.3 b</td>
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</tbody>
</table>
well as untreated control seeds. Dehydrin polyclonal antibody was found to have cross-reacted with two polypeptide with molecular masses of 44 and 32 kDa for the ABA + TC treatments and for the TC-only treatment, respectively (Figure 4). The bands were the strongest in the 20 and 60 µM ABA + TC treatments. The preimmune antiserum (negative control) did not cross-react with any polypeptides (results not shown).

ABA content of embryonic axes

The ABA content of axes peaked at 35 DAA at 75 pmol axis\(^{-1}\) and then declined to 62 pmol axis\(^{-1}\) for the untreated 40 DAA embryonic axes (Figure 5A). Abscisic acid content of axes slowly declined after seed shedding, with an ABA content of 25 pmol axis\(^{-1}\) 15 days after shedding. A further decline in ABA content of axes occurred during germination. The control axes, which germinated within 4 days of incubation, had an ABA content of 7.5 pmol axes\(^{-1}\), which declined to 2 pmol axis\(^{-1}\) after desiccation.

Desiccation of untreated axes caused an approximately threefold decrease in ABA content (Figure 5). Only the 20 and 60 µM ABA + TC treated axes, which tolerated cryopreservation and exhibited high shoot growth after desiccation, maintained a high ABA content before and after desiccation (Tables 1 and 2, Figure 5B). The TC and 20 and 60 µM ABA-treated axes, which did not tolerate desiccation, had lower ABA contents before desiccation than the axes from untreated seeds (Figure 5). Generally, the TC-treated axes with or without ABA had a high ABA content before desiccation, similar to the amount in untreated axes. However, the

<table>
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<th>Duration (months)</th>
<th>% Germination after cryopreservation</th>
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<tr>
<td></td>
<td>20 µM ABA + TC</td>
</tr>
<tr>
<td>1</td>
<td>50 ± 3.1</td>
</tr>
<tr>
<td>6</td>
<td>52 ± 2.0</td>
</tr>
<tr>
<td>12</td>
<td>54 ± 1.8</td>
</tr>
<tr>
<td>24</td>
<td>50 ± 3.1</td>
</tr>
</tbody>
</table>

Figure 3. Two-dimensional isoelectric focusing gel of proteins synthesized in axes (A) from 40 DAA seeds, (B) after a 2-week 20 µM abscisic acid (ABA) + tetcyclacis (TC) treatment, (C) 2 days after germination and (D) 6 days after germination. Proteins were separated in the second dimension under non-reducing conditions.
addition of ABA to the TC-containing medium was required to maintain the high ABA content after desiccation.

Discussion

Tolerance to desiccation and cryopreservation

Axes subjected to desiccation or cryopreservation, or both, following pretreatment with 20 or 60 µM ABA + tetcyclacis were capable of germination with both root and shoot elongation. Both exogenous ABA treatment and treatment with tetcyclacis enhanced tissue ABA concentrations. Our results thus suggest that ABA has a role in determining the desiccation tolerance of silver maple seeds. Superior shoot growth in the 20 µM ABA + tetcyclacis treatment versus the 60 µM ABA + tetcyclacis treatment, indicates an optimum ABA concentration of less than 60 µM. As only one concentration of tetcyclacis was tested (10⁻⁶ M) our experiment sheds no light on the optimum concentration of this compound. Moreover, it is unclear how tetcyclacis exerts its effect. Beside inhibiting ABA catabolism, this compound is reported to block a critical step in the biosynthesis of gibberellic acid (Sponsel 1995). There is evidence that gibberellic acid is involved in the regulation of seed maturation, as well as germination and dormancy (Hetherington and Quatrano 1991). Tetcyclacis may, therefore, exert its effect on desiccation tolerance of silver maple, at least in part, through an effect on gibberellin metabolism.

Although the ABA pretreatment increased the tolerance of the roots to desiccation, shoot desiccation tolerance required the addition of tetcyclacis, suggesting that there is a tissue-specific response to this agent. The existence of a tissue-specific response to ABA has been reported previously for silver maple seeds, with higher concentrations of ABA leading to lower shoot growth relative to root growth. (Marshall et al. 2000). Further studies are needed to determine the concentration of ABA within the axes that yields the greatest number of embryonic axes with high root and shoot growth. Nevertheless, the results presented here indicate that the overall concentration of ABA, regardless of whether it is externally applied or endogenously produced, is the most important factor in determining shoot and root tolerance to desiccation and cryopreservation.

Biochemical analysis

The role of ABA in inducing desiccation tolerance in silver maple embryonic axes was evident from biochemical analysis. For example, the synthesis of putative seed storage proteins was induced in axes shortly following treatment with 20 µM ABA + tetcyclacis. This finding further supports the view that normal events that occur during seed maturation in orthodox seeds are induced by ABA in silver maple embryos. Consistent with this conclusion was the detection of putative dehydrin homologues in axes that survived desiccation when pretreated with 20 or 60 µM ABA + tetcyclacis. Dehydrins are known to be critical to the development of desiccation tolerance in orthodox seeds (Close 1996).
Our results suggest that, by treatment with ABA, silver maple embryonic axes can be induced to undergo a process of seed maturation characteristic of orthodox seeds. Thus long-term storage of silver maple, as well other recalcitrant seeds, appears attainable. Further studies are required to determine the concentrations of ABA and tetcyclacis required for optimum results.

Acknowledgments

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