Role of chlorophyllase in chlorophyll homeostasis and post-harvest breakdown in *Piper betle* L. leaf

Supriya Gupta1*, Sanjay Mohan Gupta2+ and Nikhil Kumar1*

1Betel Vine Biotech. Laboratory, 2Plant Gene Expression Laboratory, National Botanical Research Institute, Lucknow-226 001, India

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*Piper betle* L., a dioecious shade-loving perennial climber is one of the important Pan-Asiatic plants. More than hundred landraces having marked variation in leaf chlorophyll (Chl) content are in cultivation in India. In this study, role of chlorophyllase (Chlase) in Chl homeostasis and post-harvest breakdown was investigated in two contrasting *P. betle* landraces Kapoori Vellaikodi (KV) with light green and Khasi Shillong (KS) with dark green leaves. The two landraces showed negative correlation between Chl content and Chlase activity in fresh as well as stored leaves. Accumulation of chlorophyllide a (Chlid a) was correlated with the level of Chlase activity, which was higher in KV than KS. The overall response of abscisic acid (ABA) and benzylaminopurine (BAP) was similar in KV and KS, however, the time-course was different. ABA-induced Chl loss was accompanied by rise in Chlase activity in KV and KS and the delay in Chl loss by BAP was accompanied by reduction in Chlase activity. While there were significant differences in Chlase activity in KV and KS, only minor differences were observed in the enzyme properties like pH and temperature optima, *K*<sub>m</sub> and *V*<sub>max</sub>. No landrace-related differences were observed on the effect of metal ions and functional group reagents/amino acid effectors on Chlase activity. These results showed that despite significant differences in Chl content and Chlase activity between landraces KV and KS, the properties of Chlase were similar. The findings show that in *P. betle* Chlase is involved in Chl homeostasis and also in Chl degradation during post-harvest storage and responds to hormonal regulations. These findings might be useful in predicting the stability of Chl during post-harvest storage and also the shelf-life in other *P. betle* landraces.

**Keywords:** *Piper betle*, Degradation, Chlorophyllase, Chlorophyllide, Leaf senescence, Hormones.

The loss of chlorophyll (Chl) which results in the commonly observed yellowing or appearance of fall colors in leaves of some of the deciduous trees in temperate regions is the most common sign of leaf senescence in planta or during post-harvest. Chl is present in thylakoid membranes as Chl-protein complexes and initiation of its breakdown is preceded by extensive proteolysis<sup>1</sup>. The first step in Chl breakdown is removal of phytol tail catalyzed by chlorophyllase (Chlase). Though the loss in chlorophyll is considered as the major event during senescence, it largely remains unexplored due to lack of detection of catabolites<sup>1</sup>. Chl degradation is important for development and survival as it prevents cellular damage by the photodynamic action of free Chl and its degradation products formed in the course of breakdown<sup>2</sup>.

Chl catabolism is a highly controlled sequential process, involving a series of enzymes which convert Chl into the primary fluorescent catabolites (pFCCs) and finally into non-fluorescent catabolites (NCCs)<sup>1</sup>. Initial few steps of Chl degradation are common to all plants and involve several enzymatic steps, leading to the production of colourless compounds pFCCs<sup>3</sup>. These pFCCs undergo species-specific modifications before being transported from the chloroplast and stored in the vacuole as NCCs<sup>4</sup>.
Leaf senescence in plants is the final stage of development which is regulated by developmental, environmental and hormonal factors. Many plant hormones have been implicated in regulation of leaf senescence and different plant species may have different hormonal dependence. In general, cytokinins (e.g. benzylaminopurine) are considered as a negative regulator of senescence as they delay the onset and progression of senescence. Hormones with a potential role in the induction of senescence include abscisic acid (ABA), ethylene, salicylic acid and jasmonic acid (JA). Levels of these hormones increase during senescence activating different groups of senescence-enhanced genes on each pathway, however, the role and importance of each in the senescence process is not always clear.

Though the role of Chlase in Chl catabolism has been emphasized in several studies, however, in Arabidopsis, there are reports contrary to this. Role of chlorophyllase is also shown in Chl homeostasis in Gingko biloba and turnover in Arabidopsis and Synechocystis. Thus, there is a lack of unanimity on the role of chlorophyllase warranting further studies in other taxa, so that a consensus is reached on its role in homeostasis and/or in degradation of Chl.

Piper betle is a Pan-Asiatic plant being used by more than 600 million people globally on daily basis. More than hundred landraces are being currently grown in different parts of India and have marked differences in the leaf Chl content and in post-harvest shelf life. Understanding the Chl stability during post harvest storage is of significant importance in this plant. In the present study, two contrasting landraces of P. betle with significant differences in leaf Chl content and Chlase activity have been used to investigate the role of Chlase in Chl homeostasis and regulation of Chl degradation. The results presented here on P. betle show the involvement of Chlase in Chl homeostasis and breakdown during post-harvest storage.

Materials and Methods

Plant material

Two Piper betle L. landraces Kapoori Vellaikodi (KV) and Khasi Shillong (KS) with marked differences in leaf Chl content were collected from different locations in India; KS from Shillong (Meghalaya) and KV from Sirugamani (Tamilnadu). The vines were grown in the botanical garden of the National Botanical Research Institute, Lucknow under fully protected cultivation at 30-40% of incident photosynthetically active radiation (PAR, 300-600 μ mol m⁻² s⁻¹).

Chl and Chlase extraction and assay

Total Chl content in leaf was estimated in 10 cm² leaf discs according to Lichtenthaler. Chlase in fresh and stored treated leaves was extracted by homogenizing the leaf sample (1 g/5 ml) in cold extraction buffer containing 100 mM potassium phosphate (pH 7.0), 50 mM KCl, 5 mM sodium diethyl dithiocarbamate (DECA), 1 mM diethylenepentamine acetic acid (DPTA), 0.24% Triton X-100 and 15% (m/m) pre-swollen (in the extraction buffer) insoluble polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 15000 x g for 15 min and the supernatant was used as chlorophyllase enzyme. Chlase was assayed according to Tsuchiya et al. as modified for P. betle by Kumar et al. using Chl a as substrate. The reaction was followed as change in Chl a over zero time. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 nmol Chl a s⁻¹ at 40°C. Protein in the enzyme extract was precipitated by adding 5% cold trichloroacetic acid and assayed according to Lowry et al. using bovine serum albumin as standard.

Properties of chlorophyllase

Effect of pH, temperature and substrate concentration on chlorophyllase

The pH and temperature optima of chlorophyllase in mature leaves of landraces KV and KS were studied using desalted enzyme prepared from acetone powder. Leaves were homogenized in -20°C acetone, quickly filtered and re-extracted thrice or more to remove any green colour. Chlase in acetone powder was solubilized for more than 1 h by gentle stirring in cold 100 mM cold phosphate buffer (pH 7.0) containing 50 mM KCl, 5.0 mM DECA, 1.0 mM DPTA, 0.24% Triton X-100 and 5% (m/m) pre-swollen PVP. Solubilized enzyme was centrifuged at 12000 × g for 15 min and the supernatant was subjected to 0-70% ammonium sulphate precipitation. The harvested precipitate was solubilized and desalted on G-25 column, equilibrated with same buffer and used for further studies. Effect of pH on Chlase was studied by using pH range (3.0-9.0) and temperature (15-70°C) for 15 min in dark and estimating Chlid a
as described above. The buffers used for studying optimum pH were acetate buffer (pH 3.0-5.0), citrate phosphate buffer (pH 5.0-6.5), potassium phosphate buffer (pH 7.0-8.0) and Tris-HCl buffer (pH 8.0-9.0). Effect of temperature was studied from 10-70°C using multi-temperature bath. Kinetic properties were studied by using different concentrations of the substrate Chl a.

Metal ions and functional group effectors

Effect of metal ions and functional group reagents/amino acid modifiers on Chlase activity in KV and KS leaves was studied using desalted enzyme as described above. Extracts were incubated in dark for 24 h at 4°C in the presence of 5.0 mM various metal ions (Mg²⁺, Hg²⁺, Co²⁺, and Fe³⁺) and 30 mM functional group reagents/amino acid modifiers phenyl methane sulfonyl fluoride (PMSF), sodium ethyl mercurithiosalicylate (SEMTS), diethyl pyrocarbonate (DEPC), dithiothreitol (DTT); N-ethyl maleimide (NEM) and β-mercaptoethanol (βME). Chlid a was estimated as described above.

Application of senescence inducer ABA and retardant BAP and changes in Chl and Chlase activity

Harvested leaves from both landraces were washed with distilled water, patted dry and soaked in ABA (1.0 mM) or BAP (200 µM) in phosphate buffer (pH 7.0) for 6 h at 25°C in dark and control leaves were treated the same manner in phosphate buffer. The leaves after treatment were stored at 25°C in dark in plastic boxes lined with filter paper moistened with hormone solutions or plain buffer and changes in Chl, chlorophyllase and Chlid were monitored at different time intervals. During storage, the leaves were shuffled everyday to check the build-up of any entrapped microclimate and to remove leaves with signs of rotting.

Sample preparation for HPLC

Samples for HPLC were prepared by grinding 250 mg leaf tissue from treated and control leaves at different time intervals in 5 ml cold (-20°C) 100% acetone (HPLC Grade). Extract was centrifuged at 12000 x g for 15 min in cold and the supernatant was dried in stream of N₂. The residue was dissolved in 1 ml HPLC grade acetone and clarified by centrifugation. The supernatant was passed through 0.45 µm syringe filter (Millipore, MA) before loading on HPLC column.

Pigment standards for HPLC

Chl a and b were prepared from fresh spinach leaves²¹. Chlid a was prepared¹⁸ by treating Chl a with Chlase isolated from KV leaves. The reaction was performed for 15 min at 40°C in dark and the extract (0.5 ml) was phase separated in the centrifuge tubes containing 5 ml of phase separation mixture, acetone/hexane/10 mM KOH (2: 3: 0.2, v/v), and mixed by vortexing for 30-40 s. The tubes were centrifuged at 12000 x g for 5 min. The lower phase after drying was used for determination of retention time of Chl a.

HPLC analysis

Reversed-phase C-18 column (Lichro CART 250-4, RP C-18 Particle size, 5 µ) with Varian Prostar liquid chromatography solvent delivery system was used for pigment separation. For HPLC analyses, 25 µl of sample was injected and pigments were eluted at 1 ml/min flow rate at room temperature (ca 25°C) using a linear gradient for 5 min from 100% solvent A (methanol containing 0.5 M ammonium acetate; 80:20) to 100% solvent B (methanol: acetone; 80:20) and solvent B was maintained until all the pigments were completely eluted in about 40 min, followed by further 10 min in solvent B before next sample was loaded. The pigments were detected by fluorescence emission using Merck, Hitachi L-7480 fluorescence detector set at excitation λ 440 nm and emission λ 660 nm. Pigments were identified by their retention time (tₑ) and quantitated by peak area (Fig. 1).

Results

Chl content and Chlase activity

P. betle landraces KV and KS showed marked differences in leaf color, due to differences in total Chl content which was negatively correlated with the Chlase activity. The total Chl content in KS (564 ± 10.8 mg m⁻²) was about two-fold higher than KV (274 ± 15.6 mg m⁻²), whereas the Chlase activity was nearly five-fold lower in KS, as compared to KV. The

![Fig. 1—Elution profile of chlorophyll b, a and chlorophyllide a](image-url)
specific activity of desalted Chlase prepared from acetone powder was 0.23 and 0.05 units mg⁻¹ protein, respectively for KV and KS. Negative correlation between leaf Chl content and Chlase activity in both the landraces was validated by Spearman's correlation test. Spearman's rank correlation coefficient (r_s) values for KV and KS were 0.143 and 0.60 respectively, which were found significantly lower than critical value (r_s)_{0.05(2),0} = 0.886.

Effect of ABA and BAP on total Chl, Chl alp, Chlase, Chlase/Chl alp and Chl id alp during post-harvest storage

Time-course of Chl degradation and changes in Chl alp/b during post-harvest senescence was studied in excised leaves of KV and KS treated with ABA and BAP (Fig. 2a-d). Application of ABA in both the landraces led to decrease in total Chl content and rise in Chlase activity. Significant loss (70%) of Chl in KV was observed by day 6 PT, while for the same duration in control the loss in Chl was only 20% over initial (Fig. 2a). A similar pattern was also observed in KS, but over a longer duration of storage. Unlike KV, where the loss in Chl due to ABA at day 5 PT was more than 50% of the initial, in KS 50% Chl loss was observed at day 10 PT. Further storage of ABA-treated KS leaves up to day 20 PT led to 85% Chl loss in, while in control it was only 60% (Fig. 2c).

The ratio of Chl alp showed increase with the progression of senescence and the rise was more in ABA-treated leaves than the control in both the landraces. Rapid loss of Chl in KV was also accompanied by sharp changes in alp ratio. The alp ratios changed from 2.7 to 3.8 in control, while in ABA-treated leaves it was 5, indicating the extent of change due to ABA. The ratio in KS control ranged from 2.3-2.9 which rose to 3.7 in ABA-treated leaves with the increase in Chl loss. BAP treatment delayed senescence as seen by longer retention of Chl than control in both the landraces (Fig. 2b and d). At day 21 PT, the loss of Chl in BAP-treated KV leaves was only 54% of the initial, while in control it was 65%. A similar response was also observed in KS, where the leaves could be stored beyond day 30 PT BAP treatment in KS led to higher retention of Chl (40%) in treated leaves than control (16%) at day 30 PT. It was also evident from the data that the shelf life of KV was shorter than KS and ABA caused rapid senescence in KV than KS.

Time-course of changes in Chlase activity in excised P. betle leaves treated with ABA and BAP were also monitored (Fig. 3a-d). ABA caused increase in Chlase activity in KV and KS, however, there was marked difference in ABA response between KV and KS. ABA treatment in KV led to complete yellowing
and senescence in 6-7 days, though the KS leaves survived for longer duration, nearly three-times more than KV. The decrease in Chl levels due to ABA was accompanied by increase in Chlase activity (Fig. 3a and c) and BAP led delay in Chl loss was accompanied by lower Chlase activities (Fig. 3b and d). As the rise in Chlase activity was correlated with the loss in Chl, changes in the ratio of Chlase activity/Chl a (Fig. 4a-d) showed the relationship between the enzyme unit (EU) and its substrate Chl a. There was sharp rise in the ratio due to ABA (Fig. 4a and c) in KV and KS, however, it was less marked in KS, indicating landrace-specific response. In BAP treatment (Fig. 4b and d), the changes in ratio was less marked in both the landraces, however, in KV it was still more than KS, showing quantitative differences in the response between the two landraces.

Time-course of appearance of Chlid a in KV and KS leaves is shown in Fig. 5a-d. Initial amounts of Chlid a was low in both the landraces, however, it was more in KV than KS. Rapid loss in Chl in KV due to ABA was not accompanied by high accumulation of chlid a as it was more in control at day 7 PT (Fig. 5a). In slow de-greening KS, the accumulation of Chlid a was more in ABA than control (Fig. 5b). Accumulation of Chlid a was also observed in BAP-treated (Fig. 5b and d) KV and KS leaves, however, it was slower than ABA and the landrace-specific differences were well marked. Chlid a/Chl a was high in KV than KS (Fig 6a-d) and the rise was observed in both the landraces during post-harvest storage. Senescence promoting hormone ABA (Fig. 6a and c) led to rise in Chlid a/Chl a in KV and KS leaves, though the differences between the landraces remained. Effect of BAP on Chlid a/Chl a (Fig. 6b and d) was more pronounced in KV than KS. Thus, the overall effect of hormone did not override the landrace-specific differences.

Fig. 4—Effect of ABA (a, c) and BAP (b, d) on changes in the ratio of chlorophyllase enzyme unit and chlorophyll a (EU/chlorophyll a) in excised P. betle leaves of landrace KV (4a, b) and KS (2c, d) stored in dark at 25 ± 1°C. [■] control and ( ) hormone-treated

Fig. 5—Effect of ABA (a, c) and BAP (b, d) treatment on time course of appearance of chlorophyllide a in excised P. betle leaves of landrace KV (a, b) and KS (c, d) [■] control and ( ) hormone-treated

Fig. 6—Time-course of changes in chlorophyllide a/chlorophyll a in excised P. betle leaf-treated with ABA and BAP in landrace KV (a, b) and KS (c, d) [■] control and ( ) hormone-treated
Properties of Chlase

**Effect of pH, temperature and substrate concentration**

Nearly five-fold difference in Chlase activity between KV and KS necessitated investigations on its properties. Minor differences in temperature and pH optima of Chlase in KV and KS were observed. The enzyme was active over a broad pH (6-9) and temperature (40-60°C) range. Maximum activity of Chlase in KV and KS was respectively, at pH 7.0 ± 0.2 and 6.1 ± 0.4. The optimum temperature for Chlase was respectively, 48 ± 0.6 and 52 ± 0.8°C for KS and KV. The substrate affinity of Chlase to Chl α was studied in the concentration range of 0.05-1.0 µM Chl α. The observed $K_m$ (µm) and $V_{max}$ (µm/min) were within range of reported values in other species. The $K_m$ value for Chl α was about four-fold higher in KV (20) as compared to KS (5), while $V_{max}$ was two-fold higher in KV (400) than KS (200).

**Effect of metal ions and functional group effectors**

The effect of metal ions and functional group modifiers on Chlase is shown in Table 1. Marginal inhibition in Chlase activity was observed with CoCl₂, MgCl₂ and FeCl₂ and significant inhibition (about 50%) was observed only in case of HgCl₂, indicating the involvement of -SH group in the enzyme activity. Effect of functional group modifiers PMSF and SEMTS caused >30% inhibition in Chlase activity in both the landraces, suggesting the involvement of serine residue in the enzyme activity. DEPC, a histidine residue modifier also decreased the activity up to 50% in both the landraces, indicating a greater role for histidine in the catalytic activity. These findings suggested involvement of SH, serine and histidine residues in Chlase activity. Thus, there were no landrace-based differences in the role metal ions and functional group effectors.

**Discussion**

Synthesis and degradation of Chl in the turnover process is well known. Chl is also synthesized and degraded in the leaf several times before the leaf enters in senescence phase, where it is finally degraded. Chlase is the primary enzyme that initiates degradation of Chl and has been extensively studied at the biochemical and molecular level. Studies have revealed that expression of Chlase is responsive to seasonal changes, wounding and high light stress as well as hormones such as ethylene and methyl jasmonate.

ABA-induced degradation of Chl is believed to be a natural element in the control of leaf senescence induced by a number of biotic and abiotic factors. It is well known that ABA-induced senescence and accompanying Chl degradation is effectively delayed by cytokinin. In P. betle, ABA treatment led to an increase in Chlase activity and Chl loss, however, the extent of rise in Chlase and rate of Chl loss differed significantly between KV and KS. The increase in the ratio of Chl alb during senescence in both the landraces suggested that Chl b was converted to Chl a, before it was catabolized and this conversion contributed to the rise in the ratio. It has been reported in barley leaves that the induction of senescence is accompanied by a marked increase in Chl b reductase activity. Later, it has been shown that senescence of barley leaves in the presence of D₂O leads to the formation of C7-hydroxy Chlid a as a stable intermediate and Chlid a is first product of Chl catabolism catalyzed by Chlase. The ratio of Chl alb showed increase with the progression of senescence and the rise was more in ABA-treated leaves than the control in both the landraces, suggesting that the mechanism of degradation of Chl was same in both the landraces.

Involvement of Chlase in Chl catabolism has been shown in several plants. Expression of native and post-translationally modified Chlase in squash lead to comparable amounts Chlase protein in the leaf tissue. However, accumulation of Chl bid a/Chl a in these leaves where post-translationally modified protein is

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### Table 1—Effect of metal salts (5 mM) and functional group effectors (30 mM) on chlorophyllase activity in P. betle landraces Kapoori Vellaikodi (KV) and Khasi Shillong (KS)

<table>
<thead>
<tr>
<th>Metal salts/functional group modifiers</th>
<th>KV Activity (%)</th>
<th>KS Activity (%)</th>
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<tbody>
<tr>
<td>HgCl₂</td>
<td>43</td>
<td>41</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>100</td>
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<tr>
<td>FeCl₂</td>
<td>96</td>
<td>100</td>
</tr>
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</tr>
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</tr>
<tr>
<td>DTT</td>
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<td>98</td>
</tr>
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<td>NEM</td>
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<td>54</td>
<td>50</td>
</tr>
<tr>
<td>βME</td>
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<td>97</td>
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100% KV ≈ 1.07 ± 0.153 µM/ml; 100% KS ≈ 0.07 ± 0.015 µM/ml
expressed to 90-fold more than that of leaves expressing full-length Chlase. The rise in ChlId/Chl a was more in landrace KV with higher Chlase activity than KS with low Chlase activity. Degreening in response to ABA with increased Chlase activity was also accompanied by increase in ChlId/Chl a.

Induction of Chlase has been shown by ethylene and jasmonate; however, the role of ABA remains unexplored. Our findings have shown that the rise in Chlase in response to ABA and its suppression by BAP could be due to their role in induction. The results presented here are also in agreement with the findings in slow and fast-degreening litchi (Litchi chinensis Sonn.) fruits, where slow-degreening litchi ‘Feizixiao’ fruits have shown less peak area of ChlId and pheophorbide than fast-degreening litchi ‘Nuomici’ fruits. The higher Chlase activity is also reported in fast-degreening ‘Nuomici’ than slow-degreening ‘Feizixiao’. Thus, our observation on KV and KS are in agreement with the findings reported on litchi fruit. Inter-generic variations in Chl catabolism, especially the terminal product of catabolism are also reported, however, there is lack of information on inter-specific variations.

Differences in the properties of Chlase, such as pH and temperature optima were observed between the two landraces. The results are comparable with the reported properties of Chlase in Ailanthus and Chlorella protothecoides. Earlier, temperature optima have been reported in Phaseolus vulgaris and wheat leaves. Our findings in P. betle showed that there could be differences within species. Further, inhibition of Chlase by PMSF (a serine protease inhibitor), as well as by DEPC (a histidine modifier) implied the involvement of both serine and histidine residues in the activity. These findings are in agreement with earlier reports which showed the presence of catalytic triad in Chenopodium album chlorophyllase comprising of Ser162, Asp191 and His262. Other properties such as inhibition of Chlase activity by Hg ions, SEMTS and thiol modifiers suggested the involvement of SH in Chlase activity. However, the enzyme neither required SH activators nor showed inhibition by βME and DTT, indicating no involvement of S-S or lack of exposed SH, which would require protection. It is plausible that SH affected by mercurials was not involved in the catalytic site and might be buried inside, hence not accessible to βME, DTT and NEM, as was also reported in an earlier study. Thus, the SH might have a role in maintaining proper orientation of hydrophobic catalytic site of Chlase, as established by site-directed catalytic site of Chlase, as established by site-directed catalytic site of Chlase, as established by site-directed mutations C234A and D264N in Chenopodium album which result in severe loss of activity. These residues, although not involved in the active site might be necessary in correct protein folding essential for catalytic activity. The Km value of P. betle Chlase was within the range of reported values (1-70 μM) from other plants.

Thus, the major difference in Chl content in fresh leaves of P. betle landraces KV and KS could be attributed to Chlase activity. It was also apparent that in P. betle Chlase has a role in Chl homeostasis and also in Chl breakdown during post-harvest senescence and the enzyme was regulated by ABA and BAP. As the properties of Chlase did not show any major difference between KV and KS, it was plausible that the expression of Chlase might differ in KV and KS which needs to be examined further. Since P. betle is one of the economically important Pan-Asiatic plant, it should be investigated in greater details for better utilization of this natural resource.

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References