POLYAMINE MEDIATED GENOTYPE INDEPENDENT SOMATIC EMBRYOGENESIS IN PAPAYA (CARICA PAPAYA L.)

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Abstract

A highly repeatable genotype independent, two-steps somatic embryogenesis protocol was developed. Immature zygotic embryos excised from 90 to 120 days old fruit of Pusa Delicious, CO 7 and Red Lady induced somatic embryos in half strength MS medium fortified with 10 mg l−1 2,4-D and polyamine spermidine at 100 µM. Embryos were matured with 45 mg l−1 polyethylene glycol (PEG) and germinated on hormone free medium. Embryogenesis frequency among different cultivars ranged from 5.7 to 72 per cent. Rooted plantlets were successfully acclimatized in autoclaved coco peat fortified with MS salt mixture under shade net condition. The plants were established successfully in field.

Key words: Genotype, Carica papaya L., polyamine, somatic embryogenesis, PEG.

Introduction

Papaya (Carica papaya L.) belongs to family Caricaceae (Badillo, 2000; Van Droogenbroeck et al., 2002). Major papaya producing countries are Brazil, Mexico, Nigeria and India (Jayavalli et al., 2011). Papaya has become an important fruit crop having immense nutritive and commercial value. It is a fair source of iron and calcium, a good source of vitamins A and B, and an excellent source of vitamin C. The latex of papaya plant has commercial use, as it is the source of proteolytic enzymes, papain and chymopapain.

Papaya suffers serious losses due to Papaya Ring Spot Virus (PRSV) disease (Kumari et al., 2015) across the country and Papaya Leaf Curl Virus (PaLCuV) disease in northern as well as southern part of India. There is an urgent need to develop transgenic papaya conferring resistance against these viruses in India. PRSV resistant transgenic papaya has been successfully developed and commercialized in Hawaii, USA (Gonsalves and Ferreira, 2003 and Gonsalves et al., 2004). A robust in vitro regeneration system which holds good for major genotypes of papaya is pre-requisite for development of transgenic variety in papaya. Earlier in vitro regeneration protocols have been proposed but they were all genotype-dependent with low regeneration frequency rate (Fitch, 1993, Anandan et al., 2010; Cabral et al., 2008; Farzana et al., 2008 and Cai et al., 1999). Present paper, therefore, describes a genotype-independent and highly repetitive polyamine mediated in vitro regeneration system of papaya.

Materials and Methods

Cultivars and explant

Three cultivars of papaya were chosen for the study; Red Lady, being the most popular cultivar in the country; Pusa Delicious, being the leading gynodioecious cultivar from north India and CO 7, being the most popular south Indian cultivar. The trees were grown inside a shade net house. Each tree was selfed to harvest seeds in homozygous state.

Three types of explants were chosen for the study viz., immature zygotic embryos excised from unripe fruit; hypocotyls and root apices from in vitro grown seedlings. All the plants were subjected to different bioregulants in order to induce embryogenesis.

Preparation of explants

Sterilization and inoculation

The fruits were washed in running tap water and then soaked in 1.0 per cent Sodium Hypochlorite (NaOCl)
solution containing 1 drop of Tween-20 for surface sterilization for 1 hour and then washed with autoclaved distilled water 5 times.

**Immature zygotic embryo**

The fruits were bisected under aseptic conditions; the testa of seeds was removed using forceps and scalpel and then white and plump immature seeds were scooped out. Excised immature zygotic embryos were inoculated onto induction medium; under dark condition for 10-12 weeks for somatic embryogenesis.

**Hypocotyl**

The hypocotyl portion of *in vitro* grown plantlets was bisected and cut into small pieces and inoculated onto the culture media.

**Root apices**

Similarly, root apices of *in vitro* grown papaya plants were also cut into small pieces and inoculated onto inoculation medium.

**Nutrient formulations for somatic embryogenesis**

Explants were inoculated on to induction medium containing half strength MS media supplemented with differential regime of 2,4-D (2,4-dichlorophenoxyacetic acid) (5, 10, 15 and 20 mg l$^{-1}$), 400 mg l$^{-1}$ glutamine, additional 1 mg l$^{-1}$ MS vitamin with 6 per cent sucrose, 0.08 per cent agar. The pre-embryonic calli (PEC) were subjected to proliferation medium fortified with different polyamines *viz*., spermidine, spermine and putrescine at concentrations ranging from 50, 100 and 200 µM for 48 hours under dark conditions and then retracted from the media and again kept onto fresh induction medium devoid of any polyamine in dark, for observing the impact of polyamine in augmenting embryogenesis. The somatic embryos were removed from proliferation medium after 90-120 days and inoculated on MS medium fortified with different osmoticums *viz*., poly ethylene glycol (PEG), sorbitol and mannitol (15, 30 and 45 mg l$^{-1}$) along with 500 mg l$^{-1}$ activated charcoal for a week. The dehydrated embryos were further subjected to regeneration medium containing 100 mg l$^{-1}$ IAA (indole-3-acetic acid), 4 mg l$^{-1}$ BAP (6-benzylaminopurine), 100 mg l$^{-1}$ glutamine and 100 mg l$^{-1}$ casein hydrolysate for 30-60 days. 1 cm long papaya shootlets with 4 true-leaves stage were withdrawn from regeneration media and inoculated on rooting medium containing half strength MS medium with 500 mg l$^{-1}$ activated charcoal, 2 per cent sucrose, 0.08 per cent agar and different IBA (indole-3-butyric acid) concentrations (2, 3 and 4 mg l$^{-1}$) for 7 days. After the emergence of root primordial, the shootlets were removed from rooting medium and incubated in light on basal MS medium for growth and proliferation.

**Acclimatization**

Healthy rooted plantlets having 4-6 open leaves were inoculated on autoclaved coco peat fortified with 4.31 gm l$^{-1}$ MS salt. When leaves start touching the cap of bottles, they were removed and gradually shifted to shade net house with 90 per cent humidity.

**Culture conditions and experimental layout**

All the experiments have been laid down in Completely Randomized Design (CRD) and each treatment was replicated thrice. The culture conditions comprised of 25±2°C temperature, 50-55% RH (relative humidity) and 3000 lux light/dark regime of 16/8 hours.

**Statistical analyses**

The experiments were repeated at least thrice with minimum of 10 explants in each set of experiment. Analysis of variance (ANOVA), CD (critical difference) value and SEm (standard error mean) were computed using OP-STAT software.

**Results and Discussion**

**Embryogenic potential of different papaya cultivars**

For assessment of different cultivars’ response for somatic embryogenesis, a total of 100 immature zygotic embryos of each cultivar have been inoculated per treatment and each treatment was replicated thrice. All the cultivars of papaya responded to somatic embryogenesis with differential frequencies. Although Pusa Delicious was found to be the most responsive cultivar wherein callus formation took comparatively lesser time (41.6 days) with more number of explants (72 per cent) inducing callus. Each explant produced 19 globular embryos in 12 weeks’ time (fig. 1). Not only the embryogenic clumps were heavier (257.1 mg/clump) but also the weight of individual embryo was higher (234.2 SE/clump) as compared to the other cultivars. Growth value (197.7) and Growth index (196.7) were also very high in Pusa Delicious over Red Lady and CO 7. Over all, Pusa Delicious was found to be a highly responsive cultivar. The zygotic embryo cotyledons of Pusa Delicious opened within 7 days after transfer to induction medium exposing the meristem, where a cluster of somatic embryos, about 2-3 mm in diameter, developed in 8 weeks. Whereas Red Lady cultivar was late in showing embryogenesis, the somatic embryos regenerated from this tissue within 4 weeks were non-friable and loose layered, unlike the previous clusters of early stage embryos that resulted in increased mass of tissue. Approximately 19.6 per cent explants of Red Lady formed
callus which was followed by 5.7 per cent recorded in CO 7, which was found to be the least responsive cultivar in terms of somatic embryogenesis (table 1).

Regeneration potential of cultivars depends upon the endogenous level of plant growth hormone. Sahi, which is an established cultivar of papaya in Bangladesh, was found to be highly regenerative under \textit{in vitro} condition (Azad and Rabbani, 2005). Tainung No. 2 which is popular cultivar in Taiwan has also been found highly regenerative under \textit{in vitro} condition. Thus, it could be concluded from the data that somatic embryogenesis can be induced in all papaya cultivars irrespective of their genotype following the current study.

\textbf{Effect of explant on embryogenesis}

Among three explants used (immature zygotic embryo, hypocotyl and root apices), the immature zygotic embryos took least time for callusing (45 days) and gave the highest number of embryos/ explant (19). A total of 9 embryos regenerated into plantlets using immature zygotic embryos (fig. 2). Hypocotyl demonstrated low frequency embryogenesis (16 embryos/ explant) which lead to development of 7 plants. Root apices were swollen and formed loose yellowish callus at the cut end. However, it failed to form any embryo (table 2).

Cotyledons, leaves and hypocotyls are found to be the best explant for embryogenesis in tomato (Madhulata \textit{et al.}, 2006). However, immature zygotic embryo is the most preferred explant in papaya (Cai \textit{et al.}, 1999; Fitch, 1993 and Kumari \textit{et al.}, 2015). Shoot tips and leaves were also employed as explant (Fitch \textit{et al.}, 1990) with little success. Litz and Conover (1978 a, b) reported micropropagation of papaya through shoot explants as well.

\textbf{Effect of 2,4-D on somatic embryogenesis}

The results showed 10 mgl\textsuperscript{-1} 2,4-D coupled with 400 mgl\textsuperscript{-1} glutamine fortified on half strength MS medium induced maximum embryos in minimum time (fig. 3). It also reduced the total time taken for callusing. The weight

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|c|c|}
\hline
\textbf{Variety} & \textbf{Days taken for callusing} & \% callusing & \textbf{No. of SE} & \textbf{Weight of each embryonic clump} & \textbf{Weight of SE/Clump} & \textbf{Growth Value} & \textbf{Growth Index} \\
\hline
Pusa Delicious & 41.6 & 72.0 & 19.0 & 257.1 & 234.2 & 197.7 & 196.7 \\
Red lady & 77.0 & 19.6 & 4.6 & 37.7 & 27.7 & 28.9 & 27.9 \\
CO-7 & 67.6 & 5.7 & 3.6 & 14.5 & 10.4 & 11.2 & 10.2 \\
SEm± & 0.858 & 2.327 & 0.576 & 2.638 & 2.870 & 1.861 & 1.038 \\
C.D. (p 0.05) & 3.027 & 8.208 & 2.031 & 10.125 & 10.125 & 6.565 & NS \\
\hline
\end{tabular}
\caption{Response of different papaya cultivars for somatic embryogenesis.}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{Type of Explant} & \textbf{Days taken for callusing} & \textbf{No. of embryos formed} & \textbf{No. of embryos/ explant} & \textbf{No. of plant regenerated} \\
\hline
Immature zygotic embryos & 45 & 21 & 19 & 9 \\
Hypocotyl & 156 & 16 & 13 & 7 \\
Root apices & 143 & 0 & 0 & 0 \\
SEm± & 1.414 & 2.944 & 2.804 & 2.236 \\
C.D. (p 0.05) & 4.989 & 10.385 & 9.174 & NS \\
\hline
\end{tabular}
\caption{Effect of explant on embryogenesis.}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|c|c|}
\hline
\textbf{2,4-D(mgl\textsuperscript{-1})} & \textbf{Days taken for callusing} & \% callusing & \textbf{No. of somatic embryos} & \textbf{Weight of embryonic clump (mg)} & \textbf{Weight of somatic embryo/clump (mg)} & \textbf{Growth value} & \textbf{Growth index} \\
\hline
5 & 56.33 & 33.61 & 7.33 & 0.18 & 0.14 & 138.10 & 137.10 \\
10 & 41.35 & 71.03 & 18.66 & 0.32 & 0.29 & 253.15 & 252.15 \\
15 & 66.00 & 32.08 & 12.33 & 0.21 & 0.18 & 161.79 & 160.79 \\
20 & 77.00 & 19.63 & 4.66 & 0.038 & 0.031 & 29.00 & 28.00 \\
Control & 41.35 & 71.03 & 18.66 & 0.32 & 0.29 & 253.15 & 252.15 \\
SEm± & 0.98 & 6.68 & 0.83 & 0.015 & 0.009 & 11.23 & 11.23 \\
C.D. (p 0.05) & 3.26 & 22.13 & 2.76 & 0.048 & 0.030 & 37.19 & 37.19 \\
\hline
\end{tabular}
\caption{Effect of 2,4-D on somatic embryogenesis of papaya.}
\end{table}
of embryos and embryonic clump was also significantly higher at this concentration (table 3). The results are in accordance with Fitch (1993) and Cai et al. (1999), who also found that 2,4-D at 10 mg l⁻¹ is prerequisite for embryo induction in papaya. The observations made during embryogenesis of papaya cv. Pusa Delicious revealed that 7-8 explants implanted on petriplate without dipping their radicle induced faster embryogenesis. The fused dicotyledons of embryos unfolded in 5-6 days and callusing started from the radicle. Within 3 weeks the explant was overlapped with callus forming a slimy layer of tissues beneath. Morphogenesis started from 4th week leading to production of numerous threads-like yellowish-white structures called somatic embryos. The rate of formation of embryos increased when embryogenic calli cut into pieces and subcultured on fresh medium. The embryos emerging from the slimy growth of tissues beneath the embryogenic calli were soft and non brittle as compared with those emerging on the embryogenic calli.

Lad et al. (1997) reported that embryogenic nucellar cultures of mango were established on 4.5 µM 2,4-D. Somatic embryogenesis occurred directly from the nucellar explants at low frequencies. 2,4-D in combination with glutamine leads to induction and proliferation of adventive embryos. Zouine and El Hadrami (2006) found that glutamine with 2,4-D proved better for embryo production. The proliferation of embryogenic suspension culture in two cultivars (Jihel and Bousthami Noir) of Phoenix dactylifera L. was tested on liquid media with or without 2,4-D and with different glutamine concentrations (3.35 × 10⁻⁴, 6.7 × 10⁻⁴ and 13.4 × 10⁻⁴ M). The liquid medium with 0.1 mg l⁻¹ 2,4-D and 6.7 × 10⁻⁴ M glutamine has clearly improved the proliferation of somatic embryos.

Fig. 1: Somatic embryogenesis in papaya cv. Pusa Delicious: (a) Immature zygotic embryo, (b) Somatic embryo, (c) germination of embryos, (d) Rooting of shootlet, (e) Acclimatization of plantlets, (f) Acclimatized plants in poly bags and (g) Establishment of plants in field.
Effect of polyamine on proliferation of somatic embryos

Quick somatic embryogenesis was induced on PEC (pre embryonic calli) of papaya inoculated on ½ MS medium fortified with 10 mg l⁻¹ 2,4-D, 400 mg l⁻¹ glutamine, 1 mg l⁻¹ MS vitamin, 6 per cent sucrose, 0.8 per cent agar and 100 µM spermidine. It is clear from the data (table 4) that threefold increase in embryogenesis was observed under the influence of 100 µM spermidine (70.6 embryos/explant) with significant increase in weight of embryonic clump (899 mg) followed by 75 µM spermidine (53.3 embryos/explant). All three polyamines (PAs) viz., spermine, spermidine and putrescine augmented production of embryos over control. Increasing concentration (50, 75, 100 µM) of polyamines enhanced embryo production as well (fig. 4). However, it is interesting to note that callusing was reduced in explants treated with polyamine suggesting direct embryogenesis. Explants exposed to 100 µM spermidine were least (7.03 per cent) callused whereas maximum callusing was observed in tissues devoid of any polyamine during the entire course of investigation.

PAs play a pivotal role in in vitro morphogenesis and improved shoot and root induction (Bajaj and Rajam,
1996 and Rajam et al., 1998). PAs have never been used to augment regeneration frequency in papaya; as current study is the first report of the same. However, PAs have been reported as an important determinant for regulation of somatic embryogenesis (Minocha and Minocha, 1995, Minocha et al., 1999 and Rajam et al., 2004).

**Effect of osmoticum on conversion of embryos**

Somatic embryos do not germinate unless they are mature enough. Various chemicals and stresses have been suggested to enable somatic embryos to mature and thereby germinate at high frequencies. The data revealed that polyethylene glycol (PEG) at 45 mg l\(^{-1}\) (table 5) was found to be the most suitable osmoticum to convert globular embryos into cotyledonary embryos and their subsequent germination and regeneration into plantlets (28 per cent) (fig. 5).

It seems that dehydration of embryos leads to conversion in papaya Zhang et al. (2005) found that somatic embryos of *Eruca sativa* developed into mature embryos on MS medium in the presence of 45 mg l\(^{-1}\) PEG.
Table 4: Influence of polyamines on induction of embryogenesis.

<table>
<thead>
<tr>
<th>Polyamine</th>
<th>Concentration (μM)</th>
<th>% Explant callused</th>
<th>No. of somatic embryos/ explant</th>
<th>Weight of embryonic clump (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>0</td>
<td>72.66</td>
<td>18.60</td>
<td>328</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>25.70</td>
<td>22.0</td>
<td>363</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>19.20</td>
<td>25.0</td>
<td>380</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>11.50</td>
<td>26.6</td>
<td>400</td>
</tr>
<tr>
<td>Spermine</td>
<td>0</td>
<td>72.66</td>
<td>18.6</td>
<td>328</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>20.00</td>
<td>27.0</td>
<td>376</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>14.30</td>
<td>35.3</td>
<td>425</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>10.60</td>
<td>40.6</td>
<td>484</td>
</tr>
<tr>
<td>Spermidine</td>
<td>0</td>
<td>72.66</td>
<td>18.6</td>
<td>328</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>16.00</td>
<td>45.3</td>
<td>463</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>11.60</td>
<td>53.3</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.03</td>
<td>70.6</td>
<td>899</td>
</tr>
<tr>
<td></td>
<td>SEm±</td>
<td>6.042</td>
<td>6.924</td>
<td>6.664</td>
</tr>
<tr>
<td></td>
<td>C.D. (p 0.05)</td>
<td>17.741</td>
<td>20.329</td>
<td>19.567</td>
</tr>
</tbody>
</table>

Table 5: Effect of different osmoticums on conversion of embryos.

<table>
<thead>
<tr>
<th>Osmoticum</th>
<th>Concentration (mg/l)</th>
<th>No. of cotyledons</th>
<th>% embryos matured</th>
<th>% plants regenerated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly Ethylene Glycol (PEG)</td>
<td>15</td>
<td>158.00</td>
<td>47.00</td>
<td>12.66</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>181.66</td>
<td>58.66</td>
<td>17.66</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>216.66</td>
<td>81.00</td>
<td>28.33</td>
</tr>
<tr>
<td>Mannitol</td>
<td>15</td>
<td>49.00</td>
<td>8.33</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>59.33</td>
<td>8.00</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>77.33</td>
<td>8.66</td>
<td>0.66</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>15</td>
<td>46.33</td>
<td>6.33</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>60.33</td>
<td>6.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>75.00</td>
<td>6.66</td>
<td>0.33</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>20.22</td>
<td>2.89</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>SEm±</td>
<td>5.14</td>
<td>1.55</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>C.D. (p 0.05)</td>
<td>15.30</td>
<td>4.63</td>
<td>1.89</td>
</tr>
</tbody>
</table>

Table 6: Effect of IBA on in vitro root induction in papaya.

<table>
<thead>
<tr>
<th>Auxin (mg/l)</th>
<th>No. of roots</th>
<th>No. of secondary roots</th>
<th>Length of roots (cm)</th>
<th>Fresh weight of roots (gm)</th>
<th>Dry weight of roots (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>½ MS + IBA 1.0</td>
<td>1.667</td>
<td>3.667</td>
<td>4.55</td>
<td>0.083</td>
<td>0.004</td>
</tr>
<tr>
<td>½ MS + IBA 2.0</td>
<td>3.0</td>
<td>2.667</td>
<td>4.63</td>
<td>0.061</td>
<td>0.015</td>
</tr>
<tr>
<td>½ MS + IBA 3.0</td>
<td>1.33</td>
<td>5.0</td>
<td>5.48</td>
<td>0.133</td>
<td>0.007</td>
</tr>
<tr>
<td>Control (½ MS)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>SEm±</td>
<td>1.232</td>
<td>1.622</td>
<td>0.772</td>
<td>0.018</td>
<td>0.006</td>
</tr>
<tr>
<td>C.D. (p 0.05)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
After desiccation, somatic embryos developed into plantlets by culturing the mature somatic embryos on half strength MS medium containing 0.24 μM IBA. Partial desiccation of mature somatic embryos improves conversion frequencies (Hammatt and Davey, 1987) but efforts to duplicate the developmental environment of zygotic embryos should further improve the maturation of somatic embryos (Finkelstein and Crouch, 1986). Factors which determine the ability of embryos to convert into plants include the synthesis and accumulation of storage compounds, especially storage proteins and the acquisition of desiccation tolerance (Blackman et al., 1992; Kermode, 1995). The importance of water relations in controlling embryo maturation was proposed by Fischer et al. (1987) and has been supported by evidence from both embryo culture experiments (Xu et al., 1990) and in situ studies (Saab and Obendorf, 1989).

**In vitro rhizogenesis and acclimatization**

2 cm long microshoots having 4 healthy leaves were subjected to half strength MS medium fortified with 2 mg l⁻¹ IBA + 500 mg l⁻¹ activated charcoal, produced 3 primary roots/shoot (fig. 6). *In vitro* rhizogenesis reduced with decreasing or increasing IBA concentration. The fresh and dry weights for all the treatments ranged from 0.061 to 0.13 and 0.004 to 0.015, respectively. Hence, this is apparent from the data that 2 mg l⁻¹ IBA is optimum for profuse rooting in papaya (table 6).

Yu et al. (2001) reported 2.5 μM IBA is optimum for rooting under dark condition as the photooxidative nature of IBA causes delayed growth of plant. Many authors have reported IBA or NAA (1-naphthaleneacetic acid) as suitable auxins for inducing roots in papaya. Higher frequency of rooting occurred when papaya plantlets were subcultured on media containing IBA and NAA (Winnaar, 1988).

**Conclusion**

Micropropagation in papaya has been attempted through shoot tip, somatic embryos and callus culture. However, somatic embryogenesis remains a preferred pathway for genetic manipulation. Several authors have reported somatic embryogenesis in papaya with low frequency regeneration system. Polyamines have been demonstrated to be useful in regeneration in few crops. The current study reports a three-fold increase in somatic embryogenesis in papaya with use of 100 μM spermidine.

**Acknowledgement**

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**References**


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