Marker-Assisted Breeding for Papaya Ringspot Virus Resistance in *Carica papaya* L.

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Abstract

There have been numerous attempts to transfer *Papaya ringspot virus*-Type P (PRSV-P) resistance from wild *Vasconcellea* relatives (*Vasconcellea pubescens, Vasconcellea stipulata, Vasconcellea cauliflora* and *Vasconcellea quercifolia*) to *Carica papaya*. Success has been limited by the high degree of genetic divergence and thus incompatibility between *Vasconcellea* species and *C. papaya*. This has resulted in infertility of intergeneric F₁ hybrids and failure to perform backcrosses which are essential to transfer PRSV-P resistance to *C. papaya*. However, there has been success in producing intergeneric hybrid populations of *C. papaya* x *V. pubescens*; *C. papaya* x *V. parviflora*; and intrageneric populations of *V. pubescens* x *V. parviflora*. The aim of this research was to develop a resistant *V. parviflora* population containing the PRSV-P allele from *V. pubescens* and then transfer the resistance into *C. papaya*.

In this research, F₂ and F₃ populations have been produced from the *V. pubescens* x *V. parviflora* [F₁]. Individuals selected for their allele of the PRSV-P resistance gene i.e. homozygous resistant (RR) and heterozygous (Rr) were backcrossed to *V. parviflora* (rr) or outcrossed to *C. papaya* (rr) and their seedlings evaluated for morphological characteristics and PRSV-P resistance. Dominant traits were transferred from all species to progeny, e.g., leaf vein number (7) from *V. pubescens* (RR); pink flower colour from *V. parviflora* (rr); petiole colour (red-green) and plant size from *C. papaya* (rr). Other traits were intermediate in hybrids, e.g., flower shape and fruit size.
When the wild species or their hybrids were outcrossed to *C. papaya* (rr), inheritance patterns did not always follow Mendelian ratios, suggesting abnormal pairing of chromosomes or preferential elimination of the *Vasconcellea* genes. For example, all flowers were female in crosses between *C. papaya* (rr) x *V. pubescens* (RR) and the cream flower colour of *C. papaya* (rr) was dominant over the pink colour of *V. parviflora* (rr). However the PRSV-P resistance gene from *V. pubescens* (RR) was transferred into *V. parviflora* (rr) from their F2 hybrids and pollen fertility was obtained in hybrids between *C. papaya* (rr) and *V. parviflora* (rr), thus *V. parviflora* (rr) is proposed as a bridging species between the other two.

A co-dominant CAPS marker has been developed which is closely linked to PRSV-P resistance in *V. pubescens* (RR). This marker was used to facilitate these intergeneric and intrageneric hybridisation programmes and was used at the *in vitro* stage after embryo rescue of wide crosses.
Statement of Originality

I hereby certify that research presented in this thesis has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself. The original crosses of *V. pubescens* x *V. parviflora*, *C. papaya* x *V. pubescens* and *C. papaya* x *V. parviflora* were done before the commencement of my candidature for this degree.

Student: Christopher O'Brien ........................................ Date ........................
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I would like to express my appreciation to my supervisors, Professor Rod Drew and Dr Mamhoud Azimi for their support, guidance and encouragement throughout my research.

I would like to thank my parents and wife for their moral support.

I would like to acknowledge and thank fellow researchers for allowing me to utilise plant material obtained from our previous research work.
Chapter 1 Literature review

Carica papaya L.  Vasconcellea parviflora

Vasconcellea quercifolia  Vasconcellea pubescens
1.1 Overview of *Carica papaya* L. (papaya)

### 1.1.1 Taxonomy

*C. papaya* is often referred to as papaya, papaw, paw paw or the melon tree. It is a member of the Caricaceae family and belongs to the genus *Carica*. In Australia, red and pink-fleshed cultivars are known as ‘papaya’ as opposed to yellow-fleshed fruit known as ‘paw paw’ or ‘papaw’ (Papaya Australia, 2007).

Originally the Caricaceae family was thought to comprise 31 species in three genera, namely *Carica*, *Jacaratia* and *Jarilla* from tropical America and one genus, *Cylicomorpha* from equatorial Africa (Nakasone and Paull, 1998). The Caricaceae family currently contains *Cylicomorpha* and five South and Central American genera, *Carica*, *Jarilla*, *Jacaratia*, *Vasconcellea* and *Horovitzia* (Badillo, 1971). A recent taxonomic revision reclassified some *Carica* species into the genus *Vasconcellea* (Badillo, 2000, 2001).

The two most important genera of the Caricaceae family are *Vasconcellea* and *Carica* (Badillo, 2000) with *C. papaya* being the only member of the genus *Carica* (Badillo, 2000). The genus *Carica* is monotypic (van Droogenbroeck et al., 2004).

### 1.1.2 Origin

The wild papaya, *C. peltata* Hooker et Arnott, downgraded to a synonym for *C. papaya* by Badillo (1967), existed in Central America (Purseglove, 1968) but was often ignored (Leon, 1987; Samson, 1989) and poorly documented. However, the genetic origin of cultivated papaya is not clear (Jobin-Décor et al., 1997). It probably originated in the lowlands of Central America and since the time of Columbus spread to the tropics and became well established in tropical and sub-tropical regions (Chay-Prove et al., 2000). Seeds were distributed to the Caribbean and south-east Asia...
(Philippines) during Spanish exploration in the 16th century and later to India, the Pacific and Africa (Villegas, 1997). It was introduced into Hawaii in the early 1800s by the Spanish explorer Don Francisco Marin and in 1948 became an export crop of Hawaii (Fitch, 2005). It was deliberately introduced into Australia as a horticultural crop for fruit production more than a century ago (Garrett, 1995).

1.1.3 Botany

*C. papaya* has a single stem with a crown of leaves at the apex; however, some trees may become multi-stemmed if damaged (Villegas, 1997). They are erect, small, soft-wooded and fast-growing trees (Magdalita, 2003) with soft, hollow, green or deep-purple cylindrical stems, 10-30 cm in diameter, with prominent leaf scars and spongy-fibrous tissue. It is a short-lived, herb-like tufted tree growing up to 10 m in height (Morton, 1987; Du Puy and Telford, 1993; OECD, 2005), surviving for about five to ten years; however, commercial plantations are often replanted every two years (Chay-Prove et al., 2000).

Papaya leaves emerge directly from the upper part of the stem in a spiral on nearly horizontal green or tinged-purple petioles, 25 to 100 cm long, forming a loose open crown. New leaves grow at a rate of 1.5 to 4 per week and the life expectancy of a leaf is 2.5 to 8 months. The leaf blade, deeply divided into 5 to 9 main lobes, varies from 25 to 75 cm wide and has prominent yellowish ribs and veins. The leaf shape is palmate-lobed with an acute apex and obtuse base (Colombo et al., 1989). Colleters are present at leaf bases and along vein margins and their secretions may aid in protection against desiccation and/or protection against insect predators (Ronse Decraene and Smets, 1999).

Papaya flowers are borne on inflorescences appearing in the leaf axils. Male flowers are ivory-white in colour and are smaller than female flowers, each approximately 3 cm long and borne on pendulous panicles 60 to 90 cm long (Nakasone and Paull, 1998). Female flowers are also ivory-white in colour and in clusters of 2 to 3 or singular on short petioles (Nakasone and Paull, 1998). Bisexual/hermaphrodite
flowers are intermediate between the two unisexual forms with temperature affecting the bisexual tree’s floral morphology during the early stages of inflorescence development (Nakasone and Paull, 1998). Figure 1.1.3.1 illustrates morphological characters of *C. papaya*.

**Figure 1.1.3.1** Morphological characters of *C. papaya* (from Badillo, 1993).
(a) anthers, (b) leaf form, (c) petiole length, (d) male flower, (e) leaf form, (f) male inflorescences, (g) usual stigma, (h) female flower with stigma, (i) female flower, (j) cross section of ovary and (k) leaf form.
Table 1.1.3.1 compiled by the Office of the Gene Technology Regulator (2008) summarises flower characteristics.

**Table 1.1.3.1** Characteristics of the major flower types in papaya (from Office of the Gene Technology Regulator, 2008).

<table>
<thead>
<tr>
<th>Character</th>
<th>Male (Staminate)</th>
<th>Female (pistillate)</th>
<th>Hermaphrodite: elongate</th>
<th>Hermaphrodite: intermedia</th>
<th>Hermaphrodite: pentandria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflorescence</td>
<td>Pedunculate Pendulous Panicle</td>
<td>Solitary or few flowered cymes, stalk 3.5-5 cm long</td>
<td>Short peduncled cluster, stalks less than 25 cm</td>
<td>Short peduncled cluster, stalks less than 25 cm</td>
<td>Short peduncled cluster, stalks less than 25 cm</td>
</tr>
<tr>
<td>Corolla</td>
<td>Trumpet-shaped, 2.5 cm long, 5 lobes, light yellow/cream</td>
<td>5 almost free petals (fused at base); petals fleshy, yellow/cream</td>
<td>5 partially united petals, fused from ¼ to 3/4 of their total length</td>
<td>Undefined: petals may be fused up to 2/3 of their length or free</td>
<td>5 almost free petals; petals fleshy, yellow/cream</td>
</tr>
<tr>
<td>Stigma</td>
<td>None</td>
<td>5, fan-shaped on a short style</td>
<td>5, fan-shaped</td>
<td>5, fan-shaped</td>
<td>5, fan-shaped</td>
</tr>
<tr>
<td>Ovary</td>
<td>Rudimentary non-functional ovary (or pistillode)</td>
<td>Ovoid oblong, 2-3 cm long, central cavity, numerous ovules</td>
<td>Elongate</td>
<td>Distorted, irregular shape</td>
<td>Ovoid oblong, 2-3 cm long, 5-furrowed</td>
</tr>
<tr>
<td>Stamen</td>
<td>10 in 2 whorls alternating with petal lobes</td>
<td>None</td>
<td>10 borne at the throat of the corolla in 2 clusters (5 long, 5 short)</td>
<td>2-10, some or all of which are distorted</td>
<td>5 attached by long filaments near base of ovary</td>
</tr>
<tr>
<td>Shape of fruit</td>
<td>NA</td>
<td>Spherical/ovoid</td>
<td>Cylindrical to pear-shaped</td>
<td>Carpelodic (cat face)</td>
<td>Ovoid and lobed</td>
</tr>
</tbody>
</table>
In nature, *C. papaya* plants are often dioecious with male and female flowers on separate plants (de la Cruz Medina et al., 2002). However, commercial cultivars are usually inbred gynodioecious, having bisexual/hermaphroditic flowers on some plants and only female flowers on other plants of the same species. In Australia, many commercial orchards have populations of out-crossing dioecious plants (Kim et al., 2002) resulting in plants with female or male flowers only.

*C. papaya*, a dicotyledonous, polygamous diploid species with a small genome of 372 Mbp/IC (Arumuganathan and Earle, 1991), has nine pairs of chromosomes (Bennett and Leitch, 2005) and three sex types: female (Figure 1.1.3.2), male (Figure 1.1.3.3) and hermaphrodite (Magdalita, 2003).

![The female papaw plant](image)

**Figure 1.1.3.2** Female *C. papaya* plant (from Chay-Prove et al., 2000).
The morphology of hermaphroditic fruit is affected by environmental influences, and carpellody or abortion of carpels can cause production losses in geographic regions with pronounced seasonal changes. Thus, commercial exploitation of gynodioecious (hermaphroditic) papaya lines is restricted to tropical regions with uniform environmental conditions, while in sub-tropical regions the more stable female phenotype of dioecious lines is the basis of papaya production (Manshardt and Drew, 1998). Both sex expression and fruit development are influenced by genetic traits (Storey, 1976). *C. papaya* fruit are climacteric, exhibiting a characteristic rise in ethylene production and respiration during fruit ripening that stimulate the
development of ripening attributes such as colour, texture, aroma and flavour (Tucker and Grierson, 1987; Abeles et al., 1992).

Papaya fruit are generally 7 to 35 cm long, borne axillary on the main stem, usually singly, sometimes in small clusters and are often pear-shaped. Female plants produce good quality, medium to large spherical fruit with a large seed cavity. Hermaphroditic plants produce good quality, small to medium elongated fruit with a smaller seed cavity, and male plants with bisexual flowers may produce a few, elongated, poor quality fruit (Crane, 2005). The skin colour ranges from dark green to bright yellow when ripe (Magdalita, 2003). The flesh colour can range from pale yellow to red and is often soft and juicy. The centre of the fruit cavity is five-angled and filled with shiny greyish-black seeds often in rows. An individual seed is spherical-shaped, about 5 mm in diameter and is enclosed in gelatinous sarcotesta. The embryo is median and straight with ovoid, flattened cotyledons surrounded by a fleshy endosperm. Fruit weight can vary, up to 9 kg depending on the cultivar (Magdalita, 2003); however, preferred weight of commercial fruit is between 500 g and 1 kg.

1.1.4 Importance

*C. papaya* is cultivated mainly in tropical countries and economically is the most important species within the Caricaceae family. It is considered the fourth most important tropical fruit crop worldwide. Its fresh fruit is economically and socially significant due to its international market acceptance (Gertrudes et al., 2002). Papaya is grown in many countries where *Papaya ringspot virus* Type P (PRSV-P) is present: the Philippines, Thailand, Vietnam, Hawaii, the Caribbean Islands, Taiwan, Brazil and Africa. PRSV-P is one of the major limiting factors for papaya production. Worldwide, 6,634,580 tonnes of papaya fruit were produced in 54 countries (FAO, 2007). Table 1.1.4.1 lists the top 10 papaya-producing countries in 2005.
Table 1.1.4.1 Top 10 papaya-producing countries in 2005 (FAO, 2007)

<table>
<thead>
<tr>
<th>Country</th>
<th>2005 Production (kilo tonnes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>1,573.82</td>
</tr>
<tr>
<td>Nigeria</td>
<td>834.04</td>
</tr>
<tr>
<td>India</td>
<td>783.38</td>
</tr>
<tr>
<td>Mexico</td>
<td>709.48</td>
</tr>
<tr>
<td>Indonesia</td>
<td>646.65</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>259.17</td>
</tr>
<tr>
<td>Congo</td>
<td>215.98</td>
</tr>
<tr>
<td>Peru</td>
<td>171.06</td>
</tr>
<tr>
<td>Colombia</td>
<td>137.66</td>
</tr>
<tr>
<td>Philippines</td>
<td>132.00</td>
</tr>
</tbody>
</table>

Mexico, Malaysia and Brazil are the major exporters of papaya (FAO, 2007). In 2005 Mexico exported 81.88 kilo tonnes, Malaysia 46.74 kilo tonnes, Brazil 40.12 kilo tonnes and the Netherlands 9.23 kilo tonnes (FAO, 2007). Not all countries export their fruit; as a result, statistical data underestimate *C. papaya*’s economic importance worldwide. In south-east Asian countries, daily papaya consumption ranks second only to banana (OECD, 2005). In Australia the average consumption of raw papaya fruit is around 135 g per person per day (FSANZ, 2002).

Vegetables, fruit and fresh garden products are very important throughout our lives constituting an appreciable source of essential elements (Hardisson et al., 2001). The ripe papaya fruit is eaten fresh. Nutritionally it provides a good source of essential mineral elements and antioxidant nutrients such as carotenes, vitamin A, vitamin C and flavonoids; vitamin B folate and pantothenic acid; potassium and magnesium; and fibre (Nakasone and Paull, 1998). The approximate content per 100 g edible portion of papaya is water 86.6 g, protein 0.5 g, fat 0.3 g, carbohydrates 12.1 g, fibre 0.7 g, ash 0.5 g, potassium 204 mg, calcium 34 mg, phosphorus 11 mg, iron 1 mg, sodium 3 mg, vitamin A 450 mg, vitamin C 74 mg, thiamine 0.03 mg, niacin 0.5 mg and riboflavin 0.04 mg (Villegas, 1997).
Papaya is used in drinks, jams, candies and as a dried fruit (Villegas, 1997). The leaf sap can be used as meat tenderizers and to reduce the cloudiness of beer (Purseglove, 1974). Young leaves are cooked and eaten like spinach in Indonesia and male flowers boiled and eaten as a vegetable in New Guinea (Morton, 1987). Green fruit are used in a variety of savoury Asian salads and dishes including pickles and chutneys, and for canning in sugar syrup (Morton, 1987). Papaya seeds can be dried and ground for use like pepper (Papaya Australia, 2007). In some countries seeds are used as a vermifuge and abortifacient. Women in India, Pakistan, Sri Lanka and other parts of the world have long used green papaya as a folk remedy for contraception and abortion. Medical research in animals has confirmed the contraceptive and abortifacient capability of papaya (Lohiya et al., 2002). Carpaine, an alkaloid present in papaya can be used as a heart depressant, amoebicide and diuretic (Villegas, 1997).

Papaya has many pharmaceutical and industrial applications as it contains several proteins and alkaloids (El Moussaoui et al., 2001). Papain is an important proteolytic enzyme produced in the milky latex of the plant particularly in green, unripe papaya fruit. In food biotechnology, papain is used in chewing gums, in the preparation of fish protein concentrates for animal feed and for production of dehydrated pulses and beans (Morton, 1987). It is used in the textile industry, for degumming silk and softening wool (Villegas, 1997) and for tanning leather. It is used in drug preparations for various digestive ailments, in the preparation of vaccines, for deworming cattle, in the treatment of gangrenous wounds and hard skin, for reducing swelling, fever and adhesions after surgery and dissolving membranes in diphtheria (Morton, 1987; Cornell University, 2001; Mezhlumyan et al., 2003; Practical Action, 2006). It is a component in soap, shampoo, lotions, skin care products and toothpaste (Morton, 1987). Zaire, Tanzania, Uganda and Sri Lanka are the principal papain producers whilst the United States, Japan, United Kingdom, Belgium and France are the principal importing countries (Practical Action, 2006). In 2001, global world production of papain was in the order of 900 metric tonnes per year (AfricaBiz Online, 2001).
1.2 Overview of *Vasconcellea* species

1.2.1 Taxonomy

*Vasconcellea* is the most important genus of the Caricaceae family and is the largest genus with 21 species. It was part of the genus *Carica*; however, recently it was rehabilitated as being a proper genus (Badillo, 2000). *Vasconcellea* species are often called ‘highland papayas’ or ‘mountain papayas’ (National Research Council, 1989) as they resemble papaya and are treated as their close relatives. Highland papayas tend to be smaller, less succulent and quite different in taste to the common papaya (National Research Council, 1989). Due to easy natural hybridisation among species of the *Vasconcellea* genus, correct species identification can be difficult (Scheldeman et al., 2002). Thus, the hybrid *V. x heibornii* with its different varieties has not been fully described (Badillo, 1993).

1.2.2 Origin

The genus *Vasconcellea* originated in South and Central America (Badillo, 1993). Ecuador holds 16 of the 21 *Vasconcellea* species (Badillo, 1983, 1997, 1999). Ecologically they prefer higher altitudes and cluster together in regions with mild temperatures. They can be found in the wild in a broad ecological range from dry coastal tropical lowlands over the humid sub-tropical forests to temperate regions. They are located in the Andean highlands from Colombia to Peru with a hot spot in southern Ecuador and northern Peru (Table 1.2.2.1) (Badillo, 1993; van den Eynden et al., 1999; Scheldeman et al., 2002; Badillo, 2000).
Table 1.2.2.1 *Vasconcelllea* species (from Badillo, 1993; van den Eynden et al., 1999; Scheldeman et al., 2002; Badillo, 2000). Sp = Spanish, Po = Portuguese

<table>
<thead>
<tr>
<th>Species</th>
<th>Sub-Species</th>
<th>Status</th>
<th>Common Name</th>
<th>Origin</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. quercifolia</em> (St. Hil.) A.DC.</td>
<td></td>
<td>Wild</td>
<td>Sp: higuera del monte, sacha higuera, higueron, calasacha, gargatea, orto karalau, mammon del monte Po: mamaosinho</td>
<td>Peru, Brazil, Argentina, Bolivia, Paraguay, Uruguay</td>
<td>Edible fruit</td>
</tr>
<tr>
<td><em>V. pubescens</em> Lenne &amp; Koch. synonym includes <em>V. pubescens</em> (Solms-Laub.) V. Badillo</td>
<td></td>
<td>Wild, cultivated</td>
<td>Sp: toronche, chamburo, papaya de tierra fria, siglalon</td>
<td>Colombia, Venezuela to Bolivia</td>
<td>Edible fruit</td>
</tr>
<tr>
<td><em>V. parviflora</em> A.DC.</td>
<td></td>
<td>Wild</td>
<td>Sp: papaya de monte, coral, papayillo, yucca del campo</td>
<td>Ecuador, Peru</td>
<td>Edible fruit</td>
</tr>
<tr>
<td><em>V. x heilbornii</em> (V. Badillo) V. Badillo</td>
<td></td>
<td>Wild, cultivated</td>
<td>Sp: toronche, chihualcan, babaco, chamburo</td>
<td>Ecuador</td>
<td>Edible fruit</td>
</tr>
<tr>
<td><em>V. stipulata</em> (V. Badillo) V. Badillo</td>
<td></td>
<td>Wild</td>
<td>Sp: toronche, siglalon Silvestre</td>
<td>Ecuador, Peru</td>
<td>Edible fruit</td>
</tr>
<tr>
<td><em>V. weberbaueri</em> (Harms) V. Badillo</td>
<td></td>
<td>Wild</td>
<td>Sp: mausha</td>
<td>Ecuador, Peru</td>
<td></td>
</tr>
<tr>
<td><em>V. candicans</em> (A. Gray) A.DC.</td>
<td></td>
<td>Wild</td>
<td>Sp: chungay, toronche chicope; mito</td>
<td>Southern Ecuador to Northern Peru</td>
<td>Edible fruit</td>
</tr>
<tr>
<td><em>V. microcarpa</em> (Jacq.) A.DC.</td>
<td>4 subsp., baccata, macrocarpa, pilfera, heterophylla</td>
<td>Wild</td>
<td>Sp: col de monte, sapiro, lechose de monte, higuillo Po: mamao rana</td>
<td>Panama, Venezuela, Colombia, Ecuador, Brazil, Peru, French Guyana</td>
<td>Edible fruit</td>
</tr>
<tr>
<td>Species</td>
<td>Sub-Species</td>
<td>Status</td>
<td>Common Name</td>
<td>Origin</td>
<td>Uses</td>
</tr>
<tr>
<td>---------------------------------</td>
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<td>------------------------------------------</td>
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<td>-------------------------------</td>
</tr>
<tr>
<td>\textit{V. monoica} (Desf.) A.DC.</td>
<td>Wild</td>
<td>Sp: col de monte, peladera, col de Montana, yumbo papaya, brenjena, toronche, chamburo</td>
<td>Ecuador, Peru, Bolivia</td>
<td>Edible fruit, edible leaves</td>
<td></td>
</tr>
<tr>
<td>\textit{V. palandensis} (V. Badillo et al.) V. Badillo</td>
<td>Wild</td>
<td>Sp: papillo</td>
<td>Southern Ecuador</td>
<td></td>
<td>Edible fruit</td>
</tr>
<tr>
<td>\textit{V. cauliflora} (Jacq.) A.DC.</td>
<td>Wild</td>
<td>Sp: tapaculo, papayo de Montana, zonzapote</td>
<td>Southern Mexico to northern part of South America</td>
<td>Edible fruit</td>
<td></td>
</tr>
<tr>
<td>\textit{V. chilensis} (Planch. ex A.DC.) A.DC.</td>
<td>Wild</td>
<td>Sp: palo gordo, monte gordo</td>
<td>Central Chile</td>
<td>Fodder</td>
<td></td>
</tr>
<tr>
<td>\textit{V. crassipetala} (V. Badillo) V. Badillo</td>
<td>Wild</td>
<td></td>
<td>Colombia</td>
<td>Edible fruit</td>
<td></td>
</tr>
<tr>
<td>\textit{V. glandulosa} A.DC.</td>
<td>Wild</td>
<td></td>
<td>Peru, Brazil, Bolivia, Argentina</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{V. goudotiana} Triana et Planch.</td>
<td>Wild cultivated</td>
<td></td>
<td>Colombia</td>
<td>Edible fruit</td>
<td></td>
</tr>
<tr>
<td>\textit{V. horovitziana} (V. Badillo) V. Badillo</td>
<td>Wild</td>
<td>Sp: papayuela de bejuco, badea del monte</td>
<td>Ecuador</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{V. longiflora} (V. Badillo) V. Badillo</td>
<td>Wild</td>
<td>Sp: col de monte</td>
<td>Colombia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{V. omnilingua} (V. Badillo) V. Badillo</td>
<td>Wild</td>
<td>Sp: col de monte</td>
<td>Ecuador</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{V. pulchra} (V. Badillo) V. Badillo</td>
<td>Wild</td>
<td>Sp: col de monte</td>
<td>Ecuador</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{V. sphaerocarpa} (Garcia-Barr. et Hern.) V. Badillo</td>
<td>Wild</td>
<td>Sp: papaya de monte, higuillo negro, higuillo, papayo silvestre, papayuela</td>
<td>Colombia</td>
<td>Edible fruit</td>
<td></td>
</tr>
<tr>
<td>\textit{V. sprucei} (V. Badillo) V. Badillo</td>
<td>Wild</td>
<td></td>
<td>Ecuador</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2.3 Botany

*Vasconcellea* species are all semi-lignose shrubs to tree-like having an erect habit except for *V. horovitziana*, a climber. The stem is medullose. Leaves are generally entire or lobed although heart-shaped (*V. candidans*) and compound palm-shaped leaves (*V. palandensis*) can be found. Plants are dioecious possessing only female or male flowers having no bisexual flowers (Horovitz, 1954); however, some may be monoeocious or hermaphroditic. Flowers are typically 5-merous with a small calyx and tubular corolla. Male flowers show diplostemonous stamens with filaments free or fused above the corolla mouth. Female flowers show an incomplete 5-locular ovary with 5 entire or branched stigmata. Male inflorescences are always multi-flowered usually with long-peduncles and panicles. Female inflorescences are single-flowered with short peduncles. Fruit are baccate with crested seeds often embedded in gelatin-like sarcotesta (Scheldeman et al., 2002). To date there have been no reports of natural pollination within *Vasconcellea* species.

1.2.4 Importance

Some *Vasconcellea* species are regarded as unexploited crops because of their tasty, high quality fruit and as a source of proteolytic enzymes (National Research Council, 1989). Their fruit have a wide array of flavours and qualities. However, some are tasteless and many have to be cooked with sugar to make them palatable. A few (*V. pubescens*, *V. x heilbornii*, and *V. goudotiana*) are appealing as fresh fruit. To date, *Vasconcellea* species are not as economically important as *C. papaya* yet *V. pubescens* and *V. x heilbornii* ‘Babaco’ are currently cultivated on a commercial scale. Babaco’s high fruit yields are gaining importance, with Ecuadorian/Andean fruit production generating 632 metric tons of fruit in 1996 (Soria and Viteri, 1999). Babaco is cultivated in countries such as New Zealand, Italy, Spain and South Africa. *V. pubescens* is grown commercially in Chile and is being sold as “ababai”. On a local scale some species are important, being consumed raw or prepared into juices, preserves or candies. A few species show commercial potential for fruit and papain production, for their organoleptic characteristics for juices and marmalades, as well as sources of useful traits for genetic improvement of *C. papaya* (Scheldeman et al.,
2002). Some *Vasconcellea* species possess useful traits such as high quality fruit, high level of proteolytic enzymes and cold tolerance which may be useful in papaya breeding programmes for papaya improvement (National Research Council, 1989).

An important use of *Vasconcellea* species is the extraction of the proteolytic enzyme complex papain used in food and pharmaceutical industries. In some species, dried latex activity is up to 15 times higher than in some *C. papaya* cultivars (Scheldeman et al., 2002). Some reports have been made of its medicinal values, e.g., the use of the fruit to treat arterial sclerosis and the use of latex to cure skin mycosis (FAO, 1992).

As discussed previously, the genus *Vasconcellea* contains 21 species making it the most important and diverse genus in the Caricaceae family (Scheldeman et al., 2002). *Vasconcellea* species vary in leaf shape, leaf size, fruit colour, fruit shape, and flower colour and flower shape, making each species uniquely different from *C. papaya* (Badillo, 2000) and potentially useful for breeding. *V. pubescens*, *V. quercifolia*, *V. stipulata* and *V. cauliflora* are known to be resistant to PRSV-P providing a valuable source of resistance genes for application in *C. papaya* breeding programmes. Other *Vasconcellea* species have exhibited resistance to Phytophthora (*V. goudotiana*) and pawpaw dieback (*V. parviflora*) (Drew et al., 1998).

The diversity of the genus *Vasconcellea* at the species level presents interesting commercial and scientific possibilities. To take full advantage of this diversity there is an urgent need for conservation action to prevent the increasing threat of human population pressures on the land which is resulting in deforestation and loss of wild *Vasconcellea* stands. People need to understand the importance of these wild *Vasconcellea* species to safeguard them against further losses especially in southern Ecuador and surrounding areas. Multi-location field collections should be established to protect diverse collections of *Vasconcellea* accessions for future use in research programmes (Scheldeman et al., 2003).
1.2.5 Brief synopsis of Vasconcellea species

_V. pubescens_

_V. pubescens_, a native to southern Ecuador, grows in cool, moist habitats at 3,000 m in elevation (Manshardt and Drew, 1998) and is the most common and widespread species of highland papaya. It is a monoecious or dioecious herbaceous tree reaching a height of 10 metres. Leaves are palmate-lobed and pubescent with 7 main veins. The plant can be distinguished by the coating of hairs on the underside of the leaves (National Research Council, 1989). Petioles are green in colour and are pubescent. Flowers are green in colour and small. Male flowers are multiple and attached by long inflorescences (Figure 1.2.5.1). Male trees can produce hermaphroditic flowers in some seasons. Female flowers are borne in clusters of 8-12 on short inflorescences.

**Figure 1.2.5.1** Morphological characters of _V. pubescens_ (from Badillo, 1993). (a) Male flower, (b) leaf, (c) anthers, (d) male inflorescences, (e) female flower, (f) seed, (g) leaf form, (h) fruit and (i)-(k) leaf form.
Fruit are ovoid, smaller than *C. papaya* and turn yellow or purple when ripe (Badillo, 1993). The flesh is yellow and the mass of spiky seeds is enclosed in the central cavity of the fruit. The fruit has a strong and characteristic aroma (Moya-Leon et al., 2004) both pleasing and penetrating. The fruit are 15-20 cm long, weigh about 130 g and have medium papain content (National Research Council, 1989).

Fruit vary greatly in sweetness with some eaten fresh; however, most are cooked. They are suitable for stuffing with fruit, vegetables, or other fillings because their firm flesh holds its shape during cooking (National Research Council, 1989). In Chile, there is a small commercial industry based on canned fruit of *V. pubescens* (Manshardt and Drew, 1998), in addition it is cropped for juice, jam and processed sweets (Carrasco et al., 2008). It produces latex with a high level of papain which is used as a meat tenderizer and in the pharmacological industry to treat arteriosclerosis (Sanchez, 1994).

Moya-Leon et al. (2004) investigated *V. pubescens*’ ripening physiology as there were suggestions it was a climacteric fruit. They found that during ripening there was rapid degreening of the fruit skin followed by the climacteric ethylene rise confirming a climacteric nature. The degreening rate was faster than for several *C. papaya* cultivars (Zhang and Paull, 1990). Maximum rates of ethylene production reached values between 1.82 and 3.24 µmol kg⁻¹ h⁻¹, being higher than the rates reported for *C. papaya* cultivars of between 0.14 and 0.40 (Zhang and Paull, 1990; Paull, 1996). When ethylene perception was blocked by 1-methylcyclopropene (1-MCP; 0.3 µl l⁻¹ for 16 hours at 20°C), the increase in ethylene evolution was avoided and softening and colour development was partially delayed, indicating that treatment of *V. pubescens* with 1-MCP delayed the onset of the climacteric phase, extending the short shelf-life of the fruit and increasing its marketability.
**V. parviflora**

*V. parviflora* is a native of southern Ecuador and northern Peru and is found on the Andean Pacific slope between 0 and 1800 m in elevation. It grows in warm, dry to very dry environments having the same climatic preference as *C. papaya*. It can be dioecious or monoecious, showing both pistillate and staminate flowers (Badillo, 1993). It is a semi-lignose, herbaceous, slow-growing tree that reaches a height of 3 metres. Leaves are palmate lobed or sub-orbicular in shape (Badillo, 1993) with 5 veins. Flowers are bright pink to purplish in colour and small in size. Male flowers are multiple on long inflorescences while females are on short inflorescences in clusters of 8-14. Fruit are elongated, small in size, approximately 2 cm wide and 1 cm long (Figure 1.2.5.2) and orange in colour when ripe. Fruit are edible (Badillo, 1993, 2000; van den Eynden et al., 1999; Scheldeman et al., 2002;).

![Morphological characters of V. parviflora](image)

*Figure 1.2.5.2* Morphological characters of *V. parviflora* (from Badillo, 1993).
(a)- (b) Leaf form, (c) gynoecium, (d) leaf form, (e) group of fruit, (f) leaf form, (g) seed, (h) male flower, (i)-(j) leaf form, (k) anthers, and (l) male inflorescences.
**V. quercifolia**

*V. quercifolia*, a native of Central and South America including Argentina and Brazil and to the lower reaches of the Andes (Badillo, 1967, 1971), has great adaptability, resists drought and reproduces by seed with mainly male plants being produced. Agamic reproduction, by cutting or grafting, is poor and not practical for commercial production (Colombo et al., 1989). The monoecious herbaceous trees bear very sweet yellow fruit and can be cultivated in sub-tropical climates (Colombo et al., 1989). It has many branching apices unlike the single apex of *C. papaya*. It is sub-ligneous. The leaf shape is ovate-lanceolate with an obtuse base and acute apex (Colombo et al., 1989). The number of main veins is 5. During the colder months, *V. quercifolia* is deciduous. Flowers are light green in colour and 3-5 mm in size. Male flowers (Figure 1.2.5.3) are multiple and on long inflorescences while female flowers are on short inflorescences in clusters of 8-12.

**Figure 1.2.5.3** Morphological characters of *V. quercifolia* (from Badillo, 1993). (a)- (b) Leaf form, (c) anthers, (d) male flower, (e)-(h) leaf form, (i)-(j) female flower, (k) seed, (l)-(o) leaf form, (p)-(q) fruit, (r)-(v) leaf form, and (w) female flower.
Fruit are small in size and ridged having a high sugar content (up to 17 °Bx). They can be used for direct consumption as juice, having a unique taste of sweet pulp and spicy seeds. They are rich in papain (Colombo et al., 1989).

Colombo et al. (1989) investigated *V. quercifolia* (formerly *Carica quercifolia* Solms-Laub) as a new cultivar capable of yielding low-cost products for easy commercialization in Sicily. Its biorhythm and phenology correlated with anatomy demonstrated its compatibility with the ecological characteristics of the Sicilian coastal zone. Its hardiness, especially with regard to its resilience under dry conditions, made it suitable to grow on the ‘Oleo-Ceratonion’ strip extending from 0 to 200 m above sea level. Colombo et al. (1989) found the biorhythm of *V. quercifolia* was seasonal and similar to temperate climate species though it retained certain tropical aspects such as its 30% increase in new shoot growth between the summer pause and winter dormancy, the leaf growth rhythm, the organization of the adult leaf vascular system showing the adaptation to lack of water as well as the duration of flowering and fructification.

*V. quercifolia*’s excellent fruit yield (up to 35 kg per plant), fruit organoleptic properties, the high level of extractable papain and vitamin C content contribute to its potential market value (Colombo et. al., 1989).

**V. x heilbornii**

*V. x heilbornii*, commonly called ‘Babaco’, originated in the central highlands of Ecuador and is considered to be a naturally occurring hybrid of *V. stipulata* and *V. pubescens*. It thrives in a cool sub-tropical climate, free of frost, and is much more tolerant of cool, damp winters than *C. papaya*. It will grow in warmer regions; however, high temperatures and low humidity can result in sunburnt fruit and cause immature fruit drop. In California it grows in the coastal areas of the southern part of the state and as far north as the San Francisco Bay area (Badillo, 1993; van den Eynden et al., 1999; Scheldeman et al., 2002; Badillo, 2000).
The babaco, a small, semi-lignose, herbaceous shrub, grows to 2-7 metres in height with an erect softwood trunk lined with leaf scars. It rarely branches; however, shoots often appear around the base. It has moderately large, palmate leaves with prominent ribs and veins on long hollow petioles that radiate from the trunk. Flowers are variable green, white to yellow, usually solitary on the end of a long pendulous stalk that arises from every leaf axil with flowers forming on the newly developed trunk during the growth phase of the tree. Plants are female, very rarely monoecious or male (Badillo, 1993; van den Eynden et al., 1999; Scheldeman et al., 2002; Badillo, 2000).

Babaco fruit set parthenocarpically and are five-sided, rounded at the stem end and pointed at the apex with some weighing up to 2 kg. They are eaten fresh or stewed. A typical characteristic of the fruit is the absence or very low content of (mostly) empty or non-viable seeds (Badillo, 1993; van den Eynden et al., 1999; Scheldeman et al., 2002; Badillo, 2000). The flesh is very juicy, slightly acid and low in sugar with a strawberry, pineapple and papaya flavour. The smooth thin skin is edible. The babaco is grown commercially in Ecuador and as an export fruit in New Zealand. In southern California fruit are sold in farmer and speciality markets.

**V. stipulata**

*V. stipulata* originated in the Andes of central and southern Ecuador and northern Peru between 1600 and 2500 m in elevation. It is a semi-lignose, dioecious plant growing to 10 metres with typical stipules that convert to spines after leaf fall. Individual plants vary from one another (National Research Council, 1989). Leaves are lobed with flowers variable white, yellow to reddish. Male and female flowers are borne on separate plants (National Research Council, 1989). Fruit are variable 10-lobed, especially when immature, ovoid-oblong to oblong, yellowish green to orange at maturity, 6-15 x 3-8 cm, with many seeds, sometimes smooth or corky. Many fruit are soft-skinned and long; others are firm and squat. Pulp is used to prepare preserves and candies. It has a strong pleasant aroma and is very rich in papain. The raw juice tends to irritate the skin (Badillo, 1993; van den Eynden et al.,
1999; Scheldeman et al., 2002; Badillo, 2000). This species could be grown as a source of papain (National Research Council, 1989).

**V. cauliflora**

*V. cauliflora* is found from the south of Mexico to the north of South America, growing only in open areas and along the forest edge. It is rare in the Panama Canal area. It is a small tree, with a single, straight, unbranched trunk with large, highly lobed leaves. No other *Vasconcellea* tree has such convoluted leaves. Flowers are dioecious with males producing white flowers on long stalks on the trunk near the leaves. The female flowers are on shorter stalks. Fruit are large, green and inedible (Badillo, 1993; van den Eynden et al., 1999; Scheldeman et al., 2002; Badillo, 2000). A monogenic dominant resistance gene to papaya mosaic virus exists in *V. cauliflora* (Capoor and Verma, 1961).

*V. cauliflora* has known resistance to PRSV-P. Research efforts have concentrated on hybridising *V. cauliflora* with *C. papaya* to produce hybrid plants. However, resultant hybrids lack vigour, rarely survive until flowering and if they do flower, fail to produce any viable pollen that could be used for further backcrossing to *C. papaya* (Chen et al., 1991; Horovitz and Jimenez, 1967; Khuspe et al., 1980; Litz and Conover, 1983; Magdalita et al., 1996; Manshardt and Wenslaff, 1989a).

**V. monoica**

*V. monoica* originated in the sub-tropical parts of the eastern Andean slope between 600 and 1700 m in elevation of Ecuador, Peru and Bolivia and is a ‘dwarf’ papaya (National Research Council, 1989). It is a semi-lignose, monoecious shrubby plant growing to 3 metres. It has lobed leaves, white flowers and commonly has male and female flowers together on the same inflorescence; the flowers are usually self-fertilized. It crosses easily with *V. pubescens*, often yielding hybrids that bear much fruit of good flavour (National Research Council, 1989).
Fruit are ovoid with little aroma, shiny orange at maturity, 7-8 x 4-6 cm with large-horned seeds and a high papain content (National Research Council, 1989). The fruit are edible and can be cooked or fried, or prepared with milk (Badillo, 1993; van den Eynden et al., 1999; Scheldeman et al., 2002; Badillo, 2000). They have been likened to stewed apricots when cooked with lemon and sugar. Their firmness makes them suitable for drying and candying and they freeze well. The young seedlings and mature leaves are cooked as greens. In Ecuador and eastern Peru the people like the fruit so much they protect and nurture even wild specimens (National Research Council, 1989).

**V. weberbaueri**

*V. weberbaueri* originated in the Andes of southern Ecuador and northern Peru and is semi-lignose. It grows to 10 metres in height, with lobed leaves and green-yellowish flowers. Plants are dioecious with fruit pyriform 5-lobed, orange at maturity, and 3-8 x 4-6 cm in size. Fruit are edible. It has resistance against *Fusarium* fungi and *Meloidogyne* nematodes (Badillo, 1993; van den Eynden et al., 1999; Scheldeman et al., 2002; Badillo, 2000).

**V. candicans**

*V. candicans* originated in the higher parts of the Andean Pacific slope of southern Ecuador and Peru and is a semi-lignose and tree-like plant, growing to 8 metres. Plants are dioecious with typical heart-shaped leaves, hairy below with greenish to purplish flowers. Fruit are ellipsoidal, yellow green at maturity, 10-18 x 4-6 cm with many seeds. Fruit are edible and can be eaten raw, roasted or cooked into a stew (Badillo, 1993; van den Eynden et al., 1999; Scheldeman et al., 2002; Badillo, 2000).
V. microcarpa

*V. microcarpa* grows wildly in Panama, Venezuela, Colombia, Ecuador, Brazil, Peru and French Guyana and is a semi-lignose, tree-like plant growing to 3 metres, with variable leaves that are entire or lobed. Flowers are cream-yellow and fruit are ovoid, orange at maturity, 2.5 cm long and having few seeds. The fruit and leaves are edible (Badillo, 1993; van den Eynden et al., 1999; Scheldeman et al., 2002; Badillo, 2000).

V. palandensis

*V. palandensis* originated in the humid forest of south-eastern Ecuador at 1800 m in elevation and is a semi-lignose, tree-like dioecious plant growing to 6 metres. Leaves are palmately compound with green-yellowish flowers. Fruit are almost spherical with acuminate apex, orange at maturity, 7-8 x 7-8 cm, and are edible, with the pulp surrounding the seeds also being eaten (Badillo, 1993, van den Eynden, 1999; Scheldeman et al., 2002; Badillo, 2000).

V. goudotiana

*V. goudotiana* occurs in humid forests between 1500-2300 m in elevation; however, it can tolerate some dryness. It is mostly dioecious having an upright habitat but often branches at the base and grows to 8 metres. Male and female flowers are sometimes intensely red or purple, with red or green petioles. It bears heavily and some fruit are quite sweet with an apple-like taste while others are astringent and barely edible even when cooked. The fruit are usually five-angled, pale yellow, with occasional shades of purple, red, or orange growing up to 20 cm long and weighing up to 200 g (National Research Council, 1989).
1.3 Papaya ringspot virus (PRSV-P)

1.3.1 Overview

Papaya is grown commercially throughout the world, but has a narrow gene-pool and is susceptible to many diseases (Drew et al., 1998), none more devastating worldwide to the papaya industry than PRSV-P which is a potyvirus. Papaya leaf distortion virus and papaya distortion mosaic virus are thought to be related potyviruses. Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai)), a major crop in the southern United States, is affected by Papaya Ringspot Virus-Type-W which affects all agriculturally important species of the Cucurbitaceae and is significant because of its destructiveness (Provvidenti, 1993). Isolates of both PRSV strains are closely related serologically, biologically and in sequence homology of the coat protein and other genes. The major distinguishing feature is the inability of type W to infect papaya. Type W only infects cucurbits and type P infects both cucurbits and *C. papaya* (Gonsalves, 1998; Persley, 2004). There are different races of PRSV-P with each region having a unique race. A race found in Hawaii is different from that found in Thailand or Florida, thus making it difficult to control the virus although many control methods have been attempted (Brown, 2000).

The potyvirus group is the largest of the 34 plant virus groups and families (Ward and Shukla, 1991). This group contains at least 180 definitive and possible members (30% of all known plant viruses) causing significant losses in agricultural, pastoral, horticultural and ornamental crops (Ward and Shukla, 1991). PRSV is a member of the *Potyviridae* family, genus *Potyvirus* (van Regenmortel et al., 2002). Potyvirus virions are non-enveloped (Langenberg and Zhang, 1997) filamentous particles, 680 to 900 nm long and 11 to 15 nm wide (Dougherty and Carrington, 1988; Riechmann et al., 1992). The definitive morphological structure is composed of approximately 2000 copies of capsid protein (CP) (Martin and Gelie, 1997) which encapsulates a single-stranded, positive sense RNA genome approximately 10 kb in length which has a 5’ terminal-linked protein (VPG) (Hari, 1981; Riechmann et al., 1989; Murphy
et al., 1990) and a 3’ poly-A tail (Hari et al., 1979; Takahashi et al., 1997). The positive sense genome can act directly as a messenger RNA, with the 5’ non-coding region functioning as an enhancer of translation (Carrington and Freed, 1990). Virions are found in all parts of the host plant in cytoplasm and in cell vacuoles. Inclusions present in infected cells are amorphous X-bodies and pinwheels [i.e., scrolls (Edwardson’s type 1 inclusions)] that do not contain virions (Purcifull et al., 1986).

1.3.2 Distribution

PRSV-P occurs in nearly every region where papaya is grown and is severe in Thailand, Taiwan, the Philippines, the southern region of the People’s Republic of China, the Caribbean Islands, South America, the United States and Africa. It has reduced papaya production significantly in Hawaii, the Caribbean, Brazil and south-east Asia (OECD, 2003).

The virus was discovered in Hawaii during the 1940s and destroyed papaya production on Oahu Island in the 1950s. In the early 1960s the papaya industry relocated to the Puna district of Hawaii where the virus was not present. However, in 1992 it invaded the district and in 1994 the virus was out of control (Gonsalves, 2004). In 1982 it was detected in the Philippines. In 1991 it was first identified in south-east Queensland and in suburban Brisbane (Thomas and Dodman, 1993; Chay-Prove et al., 2000), where it spread to commercial plantations in south-east Queensland. As a result of this infection in the south, the Australian industry is now centred in tropical North Queensland. PRSV-P was confirmed in Saipan, Northern Mariana Islands and Guam in 1994 (Kiritani and Su, 1999). Since 1994 occasional observations of ringspot symptoms on papaya have been made on the islands of Tahiti and Moorea and in the Cook Islands (Davis et al., 2005). Papaya was introduced to the southern tropical provinces of Iran in the 1990s and in 2000 severe leaf distortions and mottling were observed on the trees in the Hormozgan Province (Pourrahim et al., 2003).
1.3.3 Symptoms and effects

PRSV-P derives its name from the striking symptoms that develop on the fruit. These consist of concentric rings and spots or C-shaped markings which are a darker green than the background green fruit colour. Symptoms persist on the ripe fruit as darker orange-brown rings (Persley, 2004). Initially, the disease appears as oil streaks on stems and petioles and as it progresses, mottling of leaves becomes evident. The disease causes leaf chlorosis and distortions, water soaked lesions on the stems and petioles and ringspotting on the fruit (Conover, 1964) (Figure 1.3.3.1).

Figure 1.3.3.1 PRSV-P symptoms on papaya leaves (top left and top right). PRSV-P symptoms on papaya fruit (bottom left) and papaya petioles (bottom right).
Plants infected with PRSV-P lose much photosynthetic capacity and display stunted growth, deformed and inedible fruit. Fruit often show uneven bumps. It reduces tree vigour, fruit set, yield and quality (Gonsalves, 1998). It ultimately results in the death of the plant (Manshardt, 1992). When young plants are infected, fruit will never develop (Gonsalves, 1998). The productive life of an infected papaya plant is reduced from three years to one or less (Manshardt, 1992). The symptoms of the virus are more severe in cool weather (Conover, 1964). Sugar content in affected fruit is reduced (Gonsalves, 1998).

### 1.3.4 Transmission

PRSV-P is a microparasite, needing a vector to transmit infection from host to host. The virus is sap-transmissible. It relies on biotic or living vectors and is spread quickly by aphids having mouthparts that pierce the leaves. Two types of aphids, *Myzus persicae* and *Aphis gossypii*, are responsible for the spread of PRSV-P (Jensen, 1949; Conover, 1964; Zettler et al., 1968). They transfer the virus from an infected plant to a healthy plant. PRSV-P is not spread by any other insect and cannot survive in the soil or dead plant material. The virus is not seed-transmitted (Gonsalves, 1998). However, the disease can be spread by humans from one plant to another (Taylor, 1997). It can also be spread by the movement of infected papaya plants and cucurbit seedlings (Persley, 2004).

Aphids spread the disease in a non-persistent manner. Amorphous inclusion protein (AI) is required as the helper component for transmission (Purcifull et al., 1986). The amount of primary infection increases as the distance from the infected papaya trees decrease. Secondary infection spreads rapidly and trees can be infected in three to four months when young orchards are located close to infected plants and during periods when populations of winged aphid flights are high (Gonsalves, 2005). The virus can be carried by aphids from cucurbits to papayas (Persley, 2004).
1.3.5 Control

PRSV-P is very difficult to control and once a plant has become infected there is no effective treatment (Westwood, 1999). The best way to eliminate the virus is by discontinuing papaya production in areas that have it. However, the following controls are used to try and manage the virus.

- Quarantine - controlling the movement of papaya plants and cucurbit seedlings utilising quarantine measures.
- Roguing - removing and destroying infected plants from the field as soon as they are discovered. This is not effective once the disease becomes established.
- Isolation – using barriers has been reported to be useful (Gonsalves, 2005), but in other cases proved to be ineffective (Pohronezny and Litz, 1993).
- Insecticides and netting - using insecticides during aphid outbreaks, and using aphid proof netting in the early stages of plant growth (first 3 months). This is costly and is rarely used; however, it is used for commercial production in Taiwan.
- Breeding cultivars for tolerance/resistance (Conover et al., 1986; Manshardt and Drew, 1998) although roguing methods still need to be employed (Gonsalves, 1998), breeding of intergeneric hybrids followed by backcrossing programmes (Drew et al., 1998)
- Genetic engineering originally developed by Fitch et al. (1992) has been applied commercially in Hawaii (Gonsalves et al., 2006); however, in other countries it has not been commercialised because of resistance to genetically modified organisms and reluctance by many governments to register them for commercial use.
- Cultural practices - planting a non-host crop, such as corn, around the papaya trees and even between rows; consequently, aphids flying into an orchard can land and feed on the alternative crop and lose their ability to transmit the virus due to the non-persistent mode of transmission (Gonsalves, 2005).
• Cross protection – using a mild strain of PRSV-P to protect plants against economic damage caused by infection with severe strains (Gonsalves, 2005).

1.4 Breeding

Papaya is generally regarded as a cross-pollinated species. Initially undomesticated papaya had few edible fruit. During domestication the species has undergone considerable changes in fruit size, fruit flesh colour, mating system and growth habit (Manshardt and Moore, 2003). Inbred papaya lines have been used to help fix useful genetic characteristics in both gynodioecious and dioecious lines (Aquilizan, 1987) with offspring inheriting the same genes from both parents (Chay-Prove et al., 2000). Disease resistance, increased yields and improved quality and storage traits are the most important breeding objectives (Nakasone and Paull, 1998).

1.4.1 Disease resistance

Diseases are the largest single variable cost facing growers. Conventional plant breeding for disease resistance facilitated by genetic markers selection, and transgenic modification with genes that confer resistance, have important roles to play in crop improvement programmes. Globalization of agriculture has meant crop plants, often with a narrow genetic base, are now grown far from their centres of origin and therefore far from the pathogens that have co-evolved with them. Resistant cultivars are the most cost-efficient and environmentally sustainable form of disease control. Disease resistance is an inherited characteristic governed by Mendel’s laws in the same manner as traits such as height and seed colour.

Plant disease resistance genes (\(R\) genes) have been used in resistance breeding programmes for decades, with varying degrees of success. \(R\) genes encode proteins that detect pathogens playing a crucial role in disease prevention (McDowell and Woffenden, 2003). In ‘gene-for-gene’ interactions between plants and their
pathogens, incompatibility (no disease) requires dominant or partially-dominant $R$ genes in the plant and requires specific dominant avirulence ($Avr$) genes in the pathogen for their function (Flor, 1946). This genetic interaction between plant and pathogen led to the current view that such $R$ genes encode receptors for $Avr$ gene-dependent pathogen molecules (Staskawicz et al., 1995). Upon recognition of these molecules, $R$ gene products activate plant defence mechanisms such as rapid production of an oxidative burst resulting in cell wall cross-linking, localized cell death, salicylic acid biosynthesis and induction of genes characteristic of systemic acquired resistance (Levine et al., 1994; Lamb, 1994).

$R$ genes fall into several distinct classes; namely, the tobacco $N$ gene, the Arabidopsis $RPS2$ and $RPM1$ genes and the flax $L6$ gene confer resistance, respectively, to viral, bacterial and fungal pathogens. They all encode proteins that are probably cytoplasmic, contain multiple leucine-rich repeats (LRRs) and a nucleotide-binding site (Whitham et al., 1994; Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995; Lawrence et al., 1995).

An important factor in controlling pathogens is the availability of resistant plants with favourable agronomic traits. For plant breeders, molecular biology offers invaluable tools which enable the products of genes controlling resistance to be identified, understood and controlled. With this information the gene pool of the plant and its relatives can be efficiently exploited and incorporated into breeding programmes. Kruijt (Netherlands Organization for Scientific Research, 2004) discovered two disease resistance genes that were already present in an ancestral tomato species prior to the evolution of the modern tomato species. These $Cf$ resistance genes provide resistance against a fungal disease in tomato plants caused by Cladosporium fulvum. As $C. fulvum$ was probably a pathogen of this ancestral species the resistance genes $Cf$-4 and $Cf$-9 have been retained in the various modern wild tomato species. Each of the $Cf$ resistance genes recognise a different product from the fungus and this recognition in the wild tomato leaf causes cells around the fungus to die ensuring that the fungus cannot infect the rest of the plant (Netherlands Organization for Scientific Research, 2004).
Kruijt also discovered that wild tomato plants in Peru contain not one but three resistance genes. All recognise the same fungal factor. These three genes were the result of a series of changes that have led to complete pieces of DNA being duplicated. He demonstrated that DNA exchange between the various *Cf* genes led to a new *Cf* resistance gene (Netherlands Organization for Scientific Research, 2004).

Several *Cf* genes have been identified in wild tomato species and bred into cultivated tomato to generate near isogenic lines (Dixon, et al., 1996). Both *Cf*-2 and *Cf*-9 were identified in *Lycopersicon pinnellifolium* species and *Cf*-5 was identified in the land race, *L. esculentum* var. *cerasiform*. Two *Avr* genes, *Avr*4 and *Avr*9, were cloned from *C. fulvum* and shown to encode small, secreted cysteine-rich peptides which are candidate ligands for the *Cf*-4 and *Cf*-9 gene products, respectively (Joosten et al., 1994; van Kan et al., 1991). *C. fulvum* races carrying *Avr*4 or *Avr*9 are avirulent on tomato lines that carry the corresponding *R* genes *Cf*-4 or *Cf*-9. The *Cf*-9 gene from the wild berry tomato, *Lycopersicon pimpinellifolium*, is frequently used in commercial varieties.

Vegetable and ornamental crops can be infected by tobamoviruses which include tobacco (TMV) and tomato mosaic virus (ToMV). Infections usually give rise to characteristic mosaic symptoms and lead to considerable fruit damage and yield losses. Both viruses consist of a characteristic proteinaceous rod, made up of 2140 coat protein (CP) copies which envelop the positive-stranded linear RNA genome. After infection of the plant cell the RNA genome is uncoated and the viral gene products, the RNA-dependent RNA polymerase (RdRP), the movement protein (MP) and the coat protein are produced. Infection of neighbouring cells commences with the movement of RNA-MP complexes through plasmodesmata with MP-induced altered size exclusion limits (Citovsky and Zambryski, 2000). Long-distance transport of the virus proceeds through the vascular tissue and depends both on MP and CP.
Cultivated tomatoes infected with tomato mosaic virus (ToMV) have been controlled by the introgressed *Tm-1*, *Tm-2* and *Tm-22* resistance (*R*) genes (Pelham, 1966; Hall, 1980). Research has been undertaken to identify, map and confirm the soybean gene for bud blight resistance (Fasoula et al., 2003). Bud blight caused by tobacco ringspot virus reduces the seed yield and quality of soybean (*Glycine max* (L.) Merr.). It moves into the roots and root nodules impairing symbiotic N fixation.

### 1.4.2 Biotechnology

Biotechnology applications are widely used in papaya breeding projects throughout the world (Manshardt and Drew, 1998). The development of plant tissue and cell culture technologies over the past four decades has provided opportunities to overcome the limitations of conventional papaya breeding methods. Micropropagation has been highly developed in sub-tropical regions to stabilize selected papaya dioecious genotypes (Manshardt and Drew, 1998). Micropropagation has been defined as ‘*in vitro* regeneration of plants from organs, tissues, cells or protoplasts’ (Beversdorf, 1990) and ‘the true-to-type propagation of selected genotype using *in vitro* culture techniques’ (Debergh and Read, 1991).

Clonal propagation has been used in papaya breeding programmes to multiply elite heterozygous papaya lines which may segregate for undesirable phenotypes when propagated by sexual means (Manshardt and Drew, 1998). *In vitro* systems, including shoot tip propagation, anther culture and somatic embryogenesis, and *in vitro* polyembryony in papaya ovules have been developed (Litz and Conover, 1982). Embryo and ovule rescue, embryogenesis, embryogenic regeneration, genetic transformation, intergeneric hybridization, genome mapping and marker-assisted selection have been incorporated into several papaya breeding programmes. Papaya has become a model for other fruit crops with respect to biotechnology applications (Manshardt and Drew, 1998). The Papaya Biotechnology Network of SE Asia was established to integrate these proven biotechnology applications into resource-poor farmers’ agricultural practices to increase their income generation, food production, nutrition and productivity (ISAAA, 1999).
1.4.3 Intergeneric hybridisation

Improvement of crops through sexual recombination is usually limited to the variation within the gene pool of the species under consideration. The ability of different species to exchange genes is restricted by reproductive barriers, such as pre-zygotic barriers that operate before gamete fusion or post-zygotic barriers that prevent normal development or fertility of the hybrid or its progeny (Manshardt and Drew, 1998). PRSV-P tolerance is found in some lines which have been used to introgress tolerance into cultivars such as ‘Cariflora’ (Conover et al., 1986). However, resistance rather than tolerance is desired.

The highland papayas, *Vasconcellea*, are regarded as the nearest relative to *C. papaya* though the relationship is not close (Aradhya et al., 1999). It has been suggested that there are two lineages within the Caricaceae family and that some members of *Vasconcellea* are more closely linked to *C. papaya* than others (van Droogenbroeck et al., 2004).

Hybridisation between wild *Vasconcellea* species and *C. papaya* can transfer useful genes from *Vasconcellea* into papaya. Both *C. papaya* and *Vasconcellea* behave genetically as diploids and share the same chromosome number, 2n = 18 (Storey, 1976; Purseglove, 1982). Intergeneric crosses between *C. papaya* and *Vasconcellea* species are problematic due to genome incompatibility (Magdalita et al., 1997a); however, embryo rescue techniques have been used to overcome these incompatibility barriers (Drew et al., 1998). Tissue culture provides a means to overcome pre-zygotic barriers where hybrid zygotes form and cannot develop due to failure of the nutritive endosperm tissue. By culturing excised hybrid embryos, or ovules containing them on artificial media, development into mature plants may occur, opening access to the genes of the wild species (Manshardt and Drew, 1998; Drew et al., 2006a, b). Post-zygotic barriers of hybrids between *Vasconcellea* and
papaya have been shown to be the main barriers, causing abortion of immature embryos (Jimenez and Horovitz, 1958; Manshardt and Wenslaff, 1989a; Drew et al., 1998). Also, the low pollen fertility in *C. papaya* x *Vasconcellea* spp. [F₁] hybrids (Drew et al., 1998) and poor hybrid vigour or hybrid breakdown due to incompatibility (Magdalita 1997a, b; Drew et al., 1998) has impeded the success of hybridisation.

Many of the wild *Vasconcellea* species are intercompatible and can be cross-pollinated to produce hybrids with various degrees of fertility (Aradhya et al., 1999). Intergeneric crosses were carried out to transfer PRSV-P resistance from several *Vasconcellea* species (*V. stipulata*, *V. pubescens*, *V. quercifolia* and *V. cauliflora*) to papaya in Venezuela (Jimenez and Horovitz, 1958; Horovitz and Jimenez, 1967; Vegas et al., 2003), India (Pandis et al., 1970), Hawaii (Manshardt and Wenslaff, 1989a, b), Brazil (Gama et al., 1985), Taiwan (Chen et al., 1991) and Australia (Drew et al., 1998). When these crosses were first performed it was thought that they were interspecific crosses. However the reclassification of the genus *Carica* has shown that these crosses are actually intergeneric not interspecific (van Droogenbroeck et al., 2002, 2004). This explains why it has been so difficult to develop these hybrids (Manshardt and Wenslaff, 1989b; Drew et al., 1998; Magdalita et al., 1997b).

Extensive research has been carried out on the development of hybrids between *V. cauliflora* and *C. papaya*; however, these hybrids lacked vigour, did not flower and often broke down at the F₁ stage (Chen et al., 1991; Horovitz and Jimenez, 1967; Khuspe et al., 1980; Litz and Conover 1983; Manshardt and Wenslaff 1989a, Magdalita, 1996). Genetic studies also show that *V. cauliflora* is the most distant wild relative from *C. papaya* (Jobin-Décor et al., 1997). Crossing between *C. papaya* and *V. stipulata* was also reported but again due to lack of vigour and fertility these crosses where unable to be developed further (Horovitz and Jimenez, 1967).
Studies in Australia have shown that hybrids between *C. papaya* and *Vasconcellea* species such as *V. pubescens*, *V. parviflora*, *V. quercifolia* and *V. goudotiana* are high in vigour both in the field and glasshouse (Drew et al., 1998). These studies have been successful in generating populations through the use of embryo rescue (Magdalita et al., 1997a) and micropropagation techniques (Drew, 1992). However, sex expression and fertility between these crosses has varied greatly. The F1 hybrids of *C. papaya* with *V. pubescens* were 100% female and infertile; crosses with *V. quercifolia* had a sex ratio of 2:49:49 male: hermaphrodite: female and only a small amount of pollen fertility (Drew et al., 1998). Crosses between *C. papaya* and both *V. goudotiana* and *V. parviflora* showed a sex ratio of 1:1 male: female and some pollen fertility (Drew et al., 1998). Heightened intergeneric compatibility between *C. papaya* and *V. parviflora* or *V. quercifolia* (Drew et al., 1998) may be exploited to bridge hybridisation between *C. papaya* and the PRSV-P resistant species, as interspecific barriers among *Vasconcellea* are less prominent.

Resistance to PRSV-P in F1 hybrids of *C. papaya* x *V. pubescens* and *C. papaya* x *V. quercifolia* was exhibited after manual inoculations of hybrids with PRSV-P (Drew et al., 1998). It has been suggested that the resistance expressed in *V. quercifolia* is controlled by more than one gene (Magdalita et al., 1997a; Gonsalves et al., 2006). This was demonstrated through a F1 population between *C. papaya* x *V. quercifolia* that segregated as 3:1 resistance: susceptibility (Drew et al., 1998). *V. pubescens* has demonstrated single gene resistance (R. Drew, personal communication). However, no F1 individuals of *C. papaya* x *V. pubescens* displayed any signs of susceptibility to PRSV-P (Drew et al., 1998). Crossing of resistant and fertile F1 individuals of *C. papaya* x *V. quercifolia* with *C. papaya* produced a BC1 population (Drew and O’Brien 2001; Drew et al., 2006a). However only small numbers of resistant and fertile BC1 plants were produced (Drew et al., 2006) to enable production of BC2 population. Resistant BC2 plants of *C. papaya* x *V. quercifolia* have been backcrossed again to *C. papaya* to produce BC3 and BC4 generations in the Philippines.
Some papaya genotypes are more compatible with the wild *Vasconcellea* species and more likely to produce embryos when crossed (Magdalita et al. 1998). Careful selection of papaya genotypes is critical in crossing programmes as most crosses do not yield embryos after pollination (Magdalita et al. 1997a). The use of embryo rescue before embryo abortion occurs and subsequent plantlet production *in vitro* is an essential step in hybridisation (Magdalita et al. 1997b). The genotype of the *C. papaya* parent is important in undertaking such crosses (Sajise et al., 2004).

### 1.4.4 Genetic transformation

Genetic transformation involves non-sexual transfer of genes from one kind of organism into virtually any other. Transformation increases the gene pool available to breeders and allows improvements within elite genotypes without the need for backcrossing generations to recover the original phenotype (Manshardt and Drew, 1998). Plant transformation methods depend on tissue culture procedures to regenerate complete transgenic plants from single cells that have incorporated foreign genes (Manshardt and Drew, 1998). The introduction of recombinant DNA techniques and the rapid developments in gene transfer and plant regeneration procedures have allowed the transfer of desired genes between plants and also into plants from other organisms. The concept of pathogen-derived resistance is to disrupt the normal pathogenic cycle through the host plant expressing a pathogen gene (Sanford and Johnston, 1985).

The greatest economic impact of biotechnology on the papaya industry was creating virus-resistant cultivars through genetic transformation with CP genes (Manshardt and Drew, 1998). The first demonstration of CP-mediated resistance in plants was for TMV where transgenic tobacco plants constitutively expressing the TMV CP gene were more resistant to infection by TMV than control, non-transgenic tobacco (Powell-Abel et al., 1986). Since then, the CP gene has been used to generate genetically engineered resistance (Gonsalves and Slightom, 1993) to potato virus Y (PVY) in potato (Shukla et al., 1994), to watermelon mosaic – 2 (WMV) and zucchini...
yellow mosaic (ZYMV) viruses in cucurbits (Namba et al., 1992) and PRSV-P in papaya (Fitch et al., 1992). CP-mediated resistance has been reported for at least 24 plant viruses representing at least 13 virus groups that differ in virion morphology and genome composition (Grumet and Lanina-Zlatkina, 1996).

The CP gene of PRSV-P was cloned and engineered by Quemada et al. (1990) and Ling et al. (1991) to constitutively express the gene when integrated into the genome of papaya cells. Transformation protocols involving both Agrobacterium tumefaciens and microprojectile bombardment were developed in the early 1990s (Fitch and Manshardt, 1990; Fitch et al., 1993) with the main pathway of regeneration via somatic embryogenesis and although embryogenic cultures have been induced from various explant types, the most successful explants are immature zygotic embryos (Fitch and Manshardt, 1990) and young seedling tissues (Fitch, 2005).

In Australia, microprojectile bombardment of somatic embryos (Mahon et al., 1996) derived from immature zygotic embryos (Drew et al., 1994) of an Australian male dioecious papaya cultivar resulted in an efficient transformation yielding 41% successful transformants (kanamycin resistance) and transformed plantlets within six months of bombardment. By using zygotic embryos as target tissue, it was impossible to apply the technology directly to an elite female or hermaphroditic cultivar. However, it was possible to use this technology on their progeny (Drew et al., 1994).

Two cultivars, ‘SunUp’ (red-fleshed) and ‘Rainbow’ (yellow-fleshed), that resist the virus by expressing PRSV-P CP were successfully developed in Hawaii (Gonsalves and Manshardt, 1996) in response to the devastating impacts of the virus in Hawaii and south-east Asia. They were approved for commercial production in the USA in 1996 (USDA/APHIS, 1996) and as a food source in USA in 1997 (FDA, 1997). The release of these cultivars increased papaya production in Hawaii. The transgenic ‘Rainbow’ is sold in Hawaii and exported to mainland U.S. and Canada (Gonsalves et al., 2006).
The technology to control PRSV-P via transgenic papaya is well established and workable; however, not all requirements of the different papaya growing regions are being met. To elicit transgenically induced silencing of viral genes, homology between the virus and transgene must be high (>98%) (Gonsalves, 1998). Because genetic divergence of the different PRSV-P strains correlate with their geographical distribution (Wang and Yeh, 1997), the development of unique transgenes for different papaya growing regions and locally adapted cultivars is required.

Controversy exists over the deployment of transgenic plants in many places outside of the United States. This controversy has had an important bearing on whether genetic transformation technology goes beyond academic curiosity (Gonsalves et al., 2006). The environmental risks, the impact on ecosystems and the interaction of genetically modified (GM) papaya with other organisms or viruses cannot be predicted with any certainty. Risks include creating new strains of viruses and the irreversible spread of the gene through the papaya population (Greenpeace, 2003). Research in Hawaii showed considerable GM contamination of neighbouring non-GM female papaya plants (43% of the seeds analysed) within 25 metres of a GM papaya field (Manshardt, 2002). There have been questions raised about the food safety of GM papaya, e.g. the use of antibiotic resistance marker genes and allergies to GM papaya (Greenpeace, 2003).

1.4.5 DNA analysis

There is great potential for the application of genetic markers for tropical, sub-tropical and perennial fruit crops (Drew, 1997). DNA markers provide a rapid method of analysis of genetic variation in a population. A number of marker types including morphological (Keller et al., 1996) and chromosomal markers (Buitendijk et al., 1995), protein or isozyme (Chaparoo et al., 1989) and DNA markers (McCoy and Echt, 1993) have been used to mark genetically true interspecific hybrid plants. There has been some application of isozyme markers to tropical and sub-tropical crops such as Musa spp. (Jarret and Litz, 1986), mango (Degani et al., 1990), papaya (Manshardt and Wenslaff, 1989 a, b) and pineapple (de Wald et al., 1988).
Conventional breeding and selection based on phenotype can be a difficult and slow process with perennial fruit species. Phenotypes are influenced by the environment, plant growing conditions, the developmental stage of the plants and other physiological features of growth (Moore and Litz, 1984) thus limiting their use for identifying interspecific hybrids (Tanksley et al., 1989). For *C. papaya* x *V. cauliflora* hybrids, intermediate morphological characters have been the traditional markers used (Manshardt and Wenslaff, 1989a, b).

A technique was developed for the analysis of DNA fragments amplified by the polymerase chain reaction (PCR) using arbitrary primers i.e., randomly amplified polymorphic DNA (RAPD, William et al., 1990). The markers produced through RAPD-PCR have been used for identifying interspecific hybrids of potato (*Solanum tuberosum* x *S. brevidens*, Baird et al., 1992) and rice (*Oryza sativa* x *O. punctata*, Farooq et. al, 1994) as well as tomato and maize (Drew, 1997).

Molecular markers can facilitate several aspects of plant breeding programmes (Drew, 1997), including gene introgression (Moore and Durham, 1992) and breeding for multigene resistance and resistance to a disease not yet in a region or country (Henry, 1996). Breeding of most fruit species is complicated by factors including self-incompatibility, apomixis, dioecy, seedlessness, embryo maturity, heterozygosity and long juvenile periods (Moore and Durham, 1992).

An effective and easy method to identify the sex type of a papaya in the seedling stage of development would be useful for papaya breeding and commercial production. Yet the genetic basis for the sex ratio of papaya is poorly understood (Villegas, 1997). A common hypothesis is sex expression in papaya is controlled by a single locus (M) with three alleles – M1 (male), M2 (hermaphrodite) and m (female). Male (M1m) and hermaphrodite plants (M2m) are heterozygous whereas female plants (mm) are homozygous recessive. Combinations of dominants, namely M1M1, M1M2, or M2M2 are lethal, leading to post-zygotic abortion of such ovules. This hypothesis predicts that viable males can only be M1m and viable
hermaphrodites can only be M2m (Somsri et al., 1998). Through PCR, markers were generated by DNA amplification fingerprinting (DAF) for male, female and hermaphrodite progenies of a cross between Khaeg Dum (Thai cultivar) and Richter Gold (Australian cultivar) (Somsri et al., 1998).

RAPD analysis was used to differentiate between the sexual forms of three commercial C. papaya Solo cultivars using the BC210 primer to detect hermaphrodites in the cultivars. The BC210438 molecular marker was better for papaya sex differentiation (Gertrudes et al., 2002).

Jobin-Décor et al. (1997) investigated C. papaya, V. cauliflora, V. parviflora, V. pubescens, V. goudotiana, V. stipulata, V. quercifolia and Jacaratia spinosa using isozyme and RAPD analysis. Data gave similar measures of genetic similarity with C. papaya about 70% dissimilar to the Vasconcellea species by both methods. J. spinosa was relatively distant from the other species with no common isozyme alleles and 84% dissimilarity in RAPD analysis. None of the Vasconcellea species were recommended as progenitor species; equally, none appeared close enough to be exploited as bridging species in the genetic improvement of papaya (Jobin-Décor et al., 1997). RAPD analysis revealed C. papaya and V. cauliflora to be the most distant species, suggesting it would be most difficult to cross with C. papaya. A more closely related species would be better for intergeneric hybridisation with papaya. As a result, RAPD markers proved to be reliable and highly useful in guiding intergeneric hybridisation efforts among Carica and Vasconcellea species.

Kim et al. (2002) utilized AFLP markers to establish the genetic relationships among C. papaya cultivars, breeding lines, unimproved germplasm and related species. There is considerable phenotypic variation within C. papaya; however, DNA fingerprinting of 63 accessions from different countries showed limited genetic variation and the level of genetic variation among dioecious cultivars was similar to that of the hermaphrodite cultivars (Kim et al., 2002).
Isozymes or nuclear markers for genetic diversity and mapping studies were used on *C. papaya* (isozymes, Morshidi, 1998; RAPD, Sondur et al., 1996; Stiles et al., 1993; AFLP, Kim et al., 2002). Van Droogenbroeck et al. (2002) used the AFLP technique to perform the most complete diversity study comprising 95 genotypes representing at least eight *Vasconcellea* species, *C. papaya* and two *Jacaratia* species. In agreement with the rehabilitation of *Vasconcellea* as a genus based on morphological evidence (Badillo, 2000); the study illustrated the large genetic distance between *C. papaya* and the *Vasconcellea* species (van Droogenbroeck et al., 2004).

Linkage maps built from DNA markers have been developed to assist breeding through mapping candidate genes, searching for quantitative trait loci of agronomic traits (Sondur et al., 1996) and developing marker-assisted selection. Most linkage maps have been concerned with markers for traits such as sex determination and fruit flesh colour (Fitch, 2005). Sex determination has been particularly studied because of its involvement in efficient commercial fruit production. A RAPD map of the papaya genome identified markers for sex-determination, flowering height and fruit carpelloidy (Manshardt and Drew, 1998). Liu et al. (2004) used chromosome walking and fine mapping to locate the sex determination locus in papaya and also found that there was suppression of recombination around the locus indicative of degeneration of the controlling Y chromosome. Devitt et al. (2006a, b) investigated transcript and carotenoid profiling and identification of genes expressed during papaya fruit.

Molecular mapping has proved to be an invaluable tool for pearl millet improvement. Genes that confer resistance to downy mildew have been mapped as well as over 600 molecular markers for different traits. By using molecular markers, the locations of genes that control much of the variation for heat and drought tolerance in pearl millet have been identified and expensive and unreliable field screening trials have been replaced by marker-assisted selection (New Agriculturist On-line http://www.new-ag.info/02-5/focuson/focuson7.html).
Brigneti et al. (1997) used bulked segregant analysis of a F\textsubscript{1} tetraploid population to identify three AFLP markers linked to and on either side of \textit{Ry\textsubscript{sto}}, a dominant gene which confers resistance to potato virus Y (PVY) in potato. The tomato homologue of one of these AFLP markers was assigned to linkage group XI by analysis of a F\textsubscript{2} mapping population of tomato. The map position confirmed that \textit{Ry\textsubscript{sto}} was linked to markers previously mapped on chromosome XI of the potato genome.

1.4.6 SCAR marker for PRSV-P resistance

AFLP is a powerful reproducible marker technique which can be used in generating linkage maps (Zhang et al., 2007a; Tanksley, 1992), screening markers around loci of interest (Zhang et al., 2007b; Zhang et al., 2007c), germplasm fingerprinting (Yoon et al., 2007; Yonemoto, 2007) and genetic diversity evaluation (Zhou et al., 2007). AFLP screens many different DNA regions distributed randomly throughout the genome and can generate many genome-wide polymorphic markers without prior sequence knowledge. However, it is difficult to identify homologous markers utilizing this technique in a marker-assisted selection programme. Converting AFLP markers into locus-specific markers such as sequence characterized amplified region (SCAR) or cleaved amplified polymorphism sequence (CAPS) is desirable. As a result, SCAR can be valuable for plant breeding programmes (Miao et al., 2009).

SCARs were developed as PCR-based genetic markers by Paran and Michelmore (1993). A SCAR is a DNA fragment at a single genetically defined locus that is amplified via PCR from genomic DNA using a pair of specific oligonucleotide primers. The primer sequences can be derived from individual RAPD or AFLP markers. The RAPD or AFLP fragments are sequenced to allow primer design for specific amplification of the desired fragment. Reproducible amplification of single loci is achieved when high annealing temperatures are used (Paran and Michelmore, 1993).
SCARs were developed from eight RAPD markers linked to disease resistance genes in lettuce (Paran and Michelmore, 1993). All pairs of SCAR primers resulted in the amplification of single major bands the same size as the RAPD fragment cloned and sequenced. Polymorphism was either retained as the presence or absence of amplification of the band or appeared as length differences that converted dominant RAPD loci into co-dominant markers.

Molecular markers have been developed that are tightly linked to Sex 1, the gene that determines plant sex in papaya (Deputy et al., 2002). Three RAPD products were cloned and sequenced. SCAR primers were developed from the sequences resulting in SCAR T12, SCAR W11 and SCAR T1. SCAR T12 and SCAR W11 produce products in hermaphrodite and male plants and only rarely in females. SCAR T1 produces a product in all papayas regardless of plant sex. The sexing technique using SCAR T12 with SCAR T1 as a positive control, was used to correctly predict hermaphrodite papaya plants (Deputy et al., 2002).

Research was carried out to characterise molecular markers for PRSV-P resistance in Vasconcellea species (Dillon et al., 2005a, b). Genetic maps were constructed of V. pubescens and V. parviflora using Random Amplified Fingerprinting (RAF) for the development of a map to detect valuable trait loci. Several DNA markers linked to a PRSV-P resistance gene locus were identified and mapped in V. pubescens. One of these markers, Opk4_1r, resulted from a Single Nucleotide Polymorphism (SNP) in close proximity to the PRSV-P resistance gene locus. A same-size SCAR marker was present in both parents and four Vasconcellea species but was absent in papaya. Sequence analysis revealed several polymorphisms, one resulting in the creation of a restriction site within the Opk4_1r region which was used to convert the SCAR into a co-dominant CAPS marker (Psi1k4) which can be applied as a diagnostic test for selection of homozygous PRSV-P resistant hybrids of V. pubescens (Dillon et al., 2005a, b). Both the SCAR marker Opk4_1r and the CAPS marker Psi1k4 were used in research described in this thesis. The marker identified a single gene
dominant trait associated with PRSV-P resistance in a segregating F$_2$ population of $V.\ pubescens \times V.\ parviflora$.

1.5 Research plan

Project objective

The objective of this research was to develop a resistant $V.\ parviflora$ population containing the PRSV-P allele from $V.\ pubescens$ and then transfer the resistance into $C.\ papaya$.

Project aims

- Produce a F$_3$ population of RR (homozygous resistant) from a sib cross of the $V.\ pubescens \times V.\ parviflora$ [F$_2$] population.
- Produce a cross between RR F$_2$ $V.\ pubescens \times V.\ parviflora$ and $C.\ papaya$.
- Study morphological characters of these populations and the inheritance of SCAR (Opk4_1r) and CAPS ($Psi1k4$) marker developed by Dillon et al. (2005b, 2006).
- Backcross $V.\ parviflora$ to selected homozygous resistant (RR) $V.\ pubescens \times V.\ parviflora$ [F$_2$] to produce a BC$_1$ population and screen for inheritance of the Opk4_1r and $Psi1k4$ marker.
- Develop a BC$_2$ generation to $V.\ parviflora$ from the BC$_1$ population and screen for inheritance of the Opk4_1r and $Psi1k4$ marker.
1) Use individuals of the *V. pubescens* x *V. parviflora* [F₂]

2) Produce a F₃ population of RR (homozygous resistant) *V. pubescens* x *V. parviflora* from the F₂ population.

3) Produce a cross between RR F₂ *V. pubescens* x *V. parviflora* and *C. papaya*.

4) Study morphological characters of these populations and the inheritance of SCAR marker developed by Dillon et al. (2005b, 2006).

5) Backcross *V. parviflora* to selected homozygous resistant (RR) *V. pubescens* x *V. parviflora* [F₂] to produce a BC₁ population.

6) Develop a BC₂ generation to *V. parviflora* from the BC₁ population and screen with the Opk4_1r marker and Psi1k4 marker.
Chapter 2*  Intrageneric and intergeneric hybridisation between *Vasconcellea* species and *Carica papaya* L.
2.1 Introduction

*Carica* and *Vasconcellea* are the two most important genera of the Caricaceae family with *C. papaya* being the only member of the genus *Carica* (Badillo, 2000) while *Vasconcellea* comprises 21 species. *Carica papaya* and *Vasconcellea* species are often treated as close relatives as they have similar morphologies. *C. papaya* has a hollow stem, leaves with more than seven primary veins, a one-celled ovary and floral types ranging from strictly pistillate to strictly staminate with different intermediate forms (Lassoudiere, 1969). *Vasconcellea* species have a medullose stem, leaves that are entire or lobed as well as heart-shaped (*V. candicans*) and compound palm-shaped (*V. palandensis*), a five-celled ovary and are dioecious possessing only female or male flowers having no bisexual flowers (Horovitz, 1954) although some are monoecious.

*Vasconcellea* species are native to dry coastal tropical lowlands, humid sub-tropical forests and temperate regions ranging from southern Mexico to Chile and Argentina whereas its well known tropical relative, *C. papaya*, originated from Central America (Manshardt and Drew, 1998; van Droogenbroeck et al., 2004). *Vasconcellea parviflora*, a semi-lignose, herbaceous plant, is native to the hot, dry coastal regions of southern Ecuador and has the same climatic preference as *C. papaya*. It can be dioecious or monoecious with both pistillate and staminate flowers (Badillo, 1993). Male flowers are multiple on long inflorescences while females are fewer on short inflorescences. *Vasconcellea pubescens*, also known as the highland papaya, is native to cool, moist habitats to 3000 metres in elevation in southern Ecuador (Badillo, 1993; Manshardt and Drew, 1998). It is dioecious although male trees can produce hermaphroditic flowers.

*C. papaya*, the fourth most important tropical fruit crop, globally produced 6,634,580 tonnes of fruit in 2005 (FAO, 2007). Worldwide, PRSV-P is the most significant problem to production affecting tree vigour, fruit set and quality (Gonsalves, 1998). The virus reduces fruit yield (Manshardt, 1992) and the plant’s productive life is reduced from three years to one or less (Manshardt, 1992).
Methods of controlling the virus have been limited. There has been some success in producing a virus-resistant papaya by introducing PRSV-P resistance genes via transformation (Manshardt and Drew, 1998). Although transgenic plants have been developed using coat protein mediated resistance to induce silencing of viral genes, homology between the virus and transgene must be high (>98%) (Gonsalves, 1998). Because genetic divergence of the different PRSV-P strains correlate with their geographical distribution (Wang and Yeh, 1997), the development of unique transgenes for different papaya growing regions is required. Controversy exists over the deployment of transgenic plants in places outside of the United States. Many governments are reluctant to allow commercial production of crops that are genetically modified by gene transfer; therefore, incorporating PRSV-P resistance genes via breeding is a viable alternative.

Intergeneric hybridization has been slow and relatively unsuccessful due to incompatibility between C. papaya and Vasconcellea species (Manshardt and Wenslaff, 1989a, b; Drew et al., 1998; van Droogenbroeck et al., 2002). However, Vasconcellea species (V. cauliflora, V. parviflora, V. pubescens and V. quercifolia) have been crossed with C. papaya (Manshardt and Wenslaff, 1989a, b; Magdalita et al., 1997a; Drew et al., 1998, 2006a, b).

In Australia hybrid populations of C. papaya x V. cauliflora, C. papaya x V. pubescens and C. papaya x V. parviflora were generated through the use of embryo rescue (Magdalita et al., 1997a; Drew et al., 1998) and micropropagation techniques (Drew, 1992). In research described in this thesis, V. pubescens x V. parviflora [F₁] and [F₂] hybrid populations from crosses among V. pubescens x V. parviflora [F₁] plants were evaluated. Morphological character investigations were carried out on the F₁ hybrid populations of C. papaya x V. pubescens and C. papaya x V. parviflora as well as V. pubescens x V. parviflora [F₁] and [F₂] hybrid populations.
Research was undertaken to develop a resistant *V. parviflora* population containing the PRSV-P resistance allele from *V. pubescens* and then transfer the resistance into *C. papaya*. Heightened intergeneric compatibility between *C. papaya* and *V. parviflora* (Drew et al., 1998) could be exploited to bridge hybridisation between papaya and the PRSV-P resistant species (*V. pubescens*), as interspecific barriers within *Vasconcellea* (i.e. between *V. paraviflora* and any of the resistant species) are less prominent.

The project aims are to:

- Produce a F3 population of RR (homozygous resistant) from a sib cross of the *V. pubescens x V. parviflora* [F2] population.
- Produce a cross between RR F2 *V. pubescens x V. parviflora* and *C. papaya*.
- Study morphological characters of these populations and the inheritance of SCAR (Opk4_1r) and CAPS (*Psi1k4*) marker developed by Dillon et al. (2005b, 2006).
- Backcross *V. parviflora* to selected homozygous resistant (RR) *V. pubescens x V. parviflora* [F2] to produce a BC1 population and screen for inheritance of the Opk4_1r and *Psi1k4* marker.
- Develop a BC2 generation to *V. parviflora* from the BC1 population and screen for inheritance of the Opk4_1r and *Psi1k4* marker.

Morphological characteristics (including PRSV-P resistance) of the following resultant intergeneric, intrageneric and backcross populations were examined and discussed in this chapter.

- *V. pubescens x V. parviflora* [F3] #RR (sib-crossed F2 #RR)
- *(V. pubescens x V. parviflora* F2 #RR) to *V. parviflora* [BC1]
- *(V. pubescens x V. parviflora* F2 #RR) to *V. parviflora* BC1 #Rr to *V. parviflora* [BC2]
- *C. papaya x (V. pubescens x V. parviflora* F2 #RR)
2.2 Materials and methods

2.2.1 Plant material

Controls

Plants and pollen of *V. pubescens*, *V. parviflora* and *C. papaya* were used as controls throughout the research. The *C. papaya* variety, *in vitro* clone 2.001 was chosen because of its high yield and crossibility with *Vasconcellea* species (Drew et al., 2005).

Intergeneric and intrageneric hybrid production

All crosses and resultant hybrids are represented as female x male.

*V. pubescens* x *V. parviflora* [F₁]
*V. pubescens* x *V. parviflora* [F₂] (sib-crosseed F₁)
*V. pubescens* x *V. parviflora* [F₃] #RR (sib-crossed F₂ #RR)
*(V. pubescens x V. parviflora F₂ #RR) to V. parviflora [BC₁]*
{(V. pubescens x V. parviflora F₂ #RR) to V. parviflora} BC₁ #Rr to V. parviflora
[BC₂]
*C. papaya x V. parviflora* [F₁]
*C. papaya x V. pubescens* [F₁]
*C. papaya x (V. pubescens x V. parviflora F₂ #RR)*

(*progeny produced by seed)
(#containing the RR or Rr genotype for PRSV-P resistance gene)
Seed of *V. pubescens* and *V. parviflora* were germinated and seedlings grown in a glasshouse with a mean daily ambient temperature of 30°C. Plants for all experiments were grown at 2 metre spacing in field plots of 72 metres x 1 metre aligned in a north-south direction and grown to maturity in the field in the Redlands District, in south-east Queensland in red krasnozem soil. Plants were fertilised every three months with 50 g Nitrophoska Blue (12N: 5P: 14K). Weeds were controlled by hand-chipping and herbicide application (20 ml/L of 360 g/L active ingredient glyphosate). Overhead irrigation was supplied once a week until field capacity of the soil was reached. Pests and diseases were monitored and treated for as needed.

### 2.2.2 Cross pollination

*V. pubescens* x *V. parviflora* [F1]; *V. pubescens* x *V. parviflora* [F2]; *C. papaya* x *V. parviflora* [F1]; *C. papaya* x *V. pubescens* [F1];

When parent plants had matured and were flowering, full-ballooned male flowers from each species were crossed with the respective unopened full ballooned female flowers which were bagged three days prior to crossing with white paper bags (6 cm long x 4 cm wide) to avoid pollen contamination. Stigmas were pollinated using whole anthers and the flowers were then re-bagged for a further two weeks until fruit set was achieved.

*V. pubescens* x *V. parviflora* [F3]; RR

Potential parent plants were screened for the RR allele for PRSV-P resistance with the Opk4_1r marker, and RR male and female flowers of F2 plants containing the RR allele were crossed and hermaphroditic flowers were bagged with white paper bags (6 cm long x 4 cm wide) three days prior to crossing to avoid pollen contamination. Stigmas were pollinated using whole anthers and the flowers were then re-bagged for a further two weeks until fruit set was achieved.
(V. pubescens x V. parviflora F₂ RR) to V. parviflora [BC₁]

Male flowers from V. parviflora were crossed to female F₂ plants containing the RR allele for PRSV-P resistance. Flowers were bagged with white paper bags (6 cm long x 4 cm wide) three days prior to crossing to avoid pollen contamination. Stigmas were pollinated using whole anthers and the flowers were then re-bagged for a further two weeks until fruit set was achieved.

{(V. pubescens x V. parviflora F₂ RR) to V. parviflora} BC₂ to V. parviflora [BC₂]

Male flowers from V. parviflora were crossed to female BC₁ plants containing the Rr allele for PRSV-P resistance. Flowers were bagged with white paper bags (6 cm long x 4 cm wide) three days prior to crossing to avoid pollen contamination. Stigmas were pollinated using whole anthers and the flowers were then re-bagged for a further two weeks until fruit set was achieved.

C. papaya x (V. pubescens x V. parviflora F₂ RR)

Male F₂ plants containing the RR allele for PRSV-P resistance were used to pollinate female C. papaya clone 2.001. Flowers were bagged with white paper bags (6 cm long x 4 cm wide) three days prior to crossing to avoid pollen contamination. Stigmas were pollinated using whole anthers and the flowers were then re-bagged for a further two weeks until fruit set was achieved.
2.2.3 Progeny produced by seed

Fruit from *V. pubescens* x *V. parviflora* [F₁]; *V. pubescens* x *V. parviflora* [F₂] and (*V. pubescens* x *V. parviflora* F₂ RR) to *V. parviflora* [BC₁] were harvested 120 days after pollination. The seed was extracted from the fruit and the sarcotesta removed by gently rubbing the seeds in a sieve under running water. After removing the sarcotesta the seeds were washed with distilled water and blotted dry with a paper towel prior to planting. Seeds were planted in polystyrene trays (60 cells) with steam-pasteurised peat: perlite: polystyrene balls at a ratio of (1:1:1) and grown under glasshouse conditions.

2.2.4 Progeny produced by embryo rescue

Ninety to one hundred days after cross-pollination, immature fruit from *V. pubescens* x *V. parviflora* [F₃] RR; {(*V. pubescens* x *V. parviflora* F₂ RR) to *V. parviflora*)} BC₁ to *V. parviflora* [BC₂]; *C. papaya* x *V. parviflora* [F₁]; *C. papaya* x *V. pubescens* [F₁]; and *C. papaya* x (*V. pubescens* x *V. parviflora* F₂ RR) were harvested. Fruit were washed with 7x® detergent (a non-toxic detergent for tissue culture purchased from MP Biomedicals Australasia Pty Limited, Australia) and rinsed with water, surface sterilised with 70% ethanol and bisected in a laminar flow cabinet. Immature embryos were removed from immature seeds (Figure 2.2.4.1) using the protocols described by Magdalita et al. (1996).
Single plants (Figure 2.2.4.2) grew from each embryo and plants were micropropagated *in vitro* following methods described by Drew (1992) on Drew and Smith (1986) medium (Appendix A). Plants from each clone were acclimatised following the procedures described by Drew (1988). Agar was washed from the roots with tap water and plants were grown in polystyrene trays (60 cells) which contained steam-pasteurised peat: perlite: polystyrene balls at a ratio of (1:1:1). Plants were established in a humidity cabinet (Figure 2.2.4.3) initially set at 90% humidity which was gradually decreased each day until ambient RH was reached.
Fertilizer (Aquasol 23N: 4P: 18K) was applied at ¼ strength initially and increased at weekly intervals to full strength after four weeks. A fungicide treatment of Alliette® (active ingredient aluminium tris) was applied at full strength (1g/L) during acclimatisation. Once plants were large enough, they were transferred into black forestry tubes measuring 25 mm square and 150 mm deep.

2.2.5 Morphological characters

Morphological characters of intergeneric and intrageneric hybrids as well as backcross populations were assessed and recorded at first flowering, i.e., sex, flower colour and shape, number and size of stigmas, number of anthers, leaf shape and venation, and number of main veins. Leaf length was measured from the base of the middle leaflet midrib up to the tip. Leaf width was measured at the maximum breadth using the same leaves. Petiole length was measured from the node to the base of the leaf in five mature leaves per plant. Height of plant at first flower was measured from the base of the plant to the apex. Stem diameter was measured 100 mm from the base of the plant using a vernier calliper. Petiole colour, stem colour, fruit size and fruit colour were recorded. Measurements of all characters were based on the descriptors for *C. papaya* provided by the International Board for Plant Genetic Resources (1988).

2.2.6 Pollen fertility

Pollen fertility of male and hermaphrodite flowers was tested with a 1% acetocarmine solution and germination on Brewbaker and Kwack (1963) medium (Appendix A). The acetocarmine solution was prepared by adding 45% glacial acid to 55% distilled water in a fumehood. The solution was boiled and 1% acetocarmine was added and stirred until dissolved. The solution was used when cooled to room temperature.

Flowers at full balloon stage were harvested in the morning and petals were removed. Anthers were placed in a disposable petri dish and allowed to dry for 3 hours under
ambient conditions. After drying time had elapsed, the anthers were dusted onto glass slides, stained with acetocarmine, covered with a clean cover slip and left for three minutes at room temperature (approximately 25°C). This procedure of dusting pollen onto slides was done by sterilizing the scalpel, forceps and fine needle with a glass bead sterilizer to avoid cross contamination of pollen grains.

Pollen grains were observed under a slide microscope and if grains had absorbed the stain they were deemed to be fertile. Pollen from plants that had shown evidence of fertility by staining was plated onto Brewbaker and Kwack germination medium to check for the growth of a pollen tube. Flowers were again harvested and treated as described previously. The pollen was then dusted onto the germination media by means of sterile forceps and a fine needle. These instruments were sterilized between each procedure to avoid cross-contamination of pollen grains. Growth of pollen tubes was assessed after 24 hours incubation on germination medium.

2.2.7 Screening for PRSV-P resistance

Resistance to PRSV-P was determined by screening in a glasshouse. PRSV-P infected *C. papaya* leaves (Figure 2.2.7.1) were crushed using mortars and pestles and a phosphate buffer (0.1 M, pH 7) was added to make an inoculum. Plants were infected by rubbing carborundum-dusted leaves with the inoculum. This process was repeated three times at two weekly intervals and plants were assessed one month after final inoculation for visual signs of the virus.

![Figure 2.2.7.1 PRSV-P infection on a *C. papaya* leaf.](image_url)
2.3 Results

2.3.1 *V. pubescens* x *V. parviflora* [F₁]

Seventeen plants of *V. pubescens* x *V. parviflora* [F₁] were produced. Plants grew vigorously from seed both in the glasshouse and in the field during winter but were slower during the warmer months, i.e., September to March. Plants were similar to *V. pubescens* in that they would defoliate during warmer months. Mean pollen fertility was 70%. Flowers were pink in colour with white streaks and were male and female; however, male plants did produce some hermaphroditic flowers (Figure 2.3.1.1). Male and hermaphroditic flowers were multiple and on long peduncles like *V. parviflora*. Female flowers were in clusters of 6-8 and close to the stem.

![Image of flowers](image)

**Figure 2.3.1.1** Flowers of *C. papaya*, *V. pubescens*, *V. parviflora*, *C. papaya* x (*V. pubescens* x *V. parviflora*), and *V. pubescens* x *V. parviflora* [F₁].
Leaf shape (Figure 2.3.1.2) was very similar to *V. pubescens*. Leaf venation was palmate-lobed like *V. pubescens*. The number of main veins was 7 for all the F₁ hybrids and *V. pubescens* whereas *V. parviflora* had 5. Leaf length in the F₁ hybrid (45 cm ± 0.47) was greater than that of both parents and width (35.2 cm ± 0.66) was similar to *V. pubescens*.

![Leaf shape of:](image)

**Figure 2.3.1.2** Leaf shape of:

A - *V. parviflora*

B - *V. pubescens*

C - *V. pubescens* x *V. parviflora* [F₁]

Petiole length (27.1 cm ± 0.47) was similar to *V. pubescens* and greater than *V. parviflora* (6.31 cm ± 0.29). Height of plant at first flower (45 cm ± 0.78) was similar to *V. pubescens* and greater than *V. parviflora* (10.25 cm ± 0.36). Fruit colour was orange-yellow and they were slightly larger than fruit of *V. parviflora* (Table 2.3.1.1). Sib crosses among F₁ plants were performed easily and embryos were rescued to produce an F₂ generation. The F₁ plants were not exposed to PRSV-P virus due to their importance as this cross had never been produced previously; however, all carried the Rr genotype of the PRSV-P resistant gene from *V. pubescens* as determined by the CAPS *Psilk*4 marker.
Table 2.3.1.1 A summary of morphological characters for *C. papaya*, *V. pubescens*, *V. parviflora* and their F₁ and F₂ hybrids. The data are the mean ± standard error determined from 10 plants of *C. papaya*, *V. pubescens*, *V. parviflora* and 30 hybrids.

<table>
<thead>
<tr>
<th>Character</th>
<th><em>C. papaya</em></th>
<th><em>V. pubescens</em></th>
<th><em>V. parviflora</em></th>
<th><em>C. papaya x V. pubescens [F₁]</em></th>
<th><em>C. papaya x V. parviflora [F₁]</em></th>
<th><em>V. pubescens x V. parviflora [F₁]</em></th>
<th><em>V. pubescens x V. parviflora [F₂]</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pollen fertility</td>
<td>90%</td>
<td>55%</td>
<td>70%</td>
<td>NA</td>
<td>45%</td>
<td>70%</td>
<td>75%</td>
</tr>
<tr>
<td>Flower colour</td>
<td>Cream</td>
<td>Green</td>
<td>Pink</td>
<td>Yellow-green</td>
<td>Pink</td>
<td>Pink with little white streaks</td>
<td>Shades of pink-white</td>
</tr>
<tr>
<td>Flower shape</td>
<td><em>C. papaya</em> large</td>
<td><em>V. pubescens</em> small</td>
<td><em>V. parviflora</em> small</td>
<td><em>V. pubescens</em> but larger</td>
<td><em>V. parviflora</em> but larger</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>No. of stigma lobes and size</td>
<td>5 thick</td>
<td>5 thin</td>
<td>5 thin</td>
<td>5 thin</td>
<td>5 thin</td>
<td>5 thin</td>
<td>5 thin</td>
</tr>
<tr>
<td>No. of anthers</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>All female</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Leaf shape</td>
<td><em>C. papaya</em></td>
<td><em>V. pubescens</em></td>
<td><em>V. parviflora</em></td>
<td>Intermediate</td>
<td>Intermediate</td>
<td><em>V. pubescens</em></td>
<td>Intermediate</td>
</tr>
<tr>
<td>Leaf venation</td>
<td>Palmate</td>
<td>Palmate</td>
<td>Palmate</td>
<td>Palmate</td>
<td>Palmate</td>
<td>Palmate</td>
<td>Palmate</td>
</tr>
<tr>
<td>No. of main veins</td>
<td>9±0.00</td>
<td>7±0.00</td>
<td>5±0.00</td>
<td>7±0.00</td>
<td>7±0.00</td>
<td>7±0.00</td>
<td>7±0.00</td>
</tr>
<tr>
<td>Leaf length (cm)</td>
<td>30.55±0.94</td>
<td>28±1.52</td>
<td>10.59±0.20</td>
<td>54.76±2.34</td>
<td>25.23±1.55</td>
<td>45±0.47</td>
<td>43.9±0.52</td>
</tr>
<tr>
<td>Leaf width (cm)</td>
<td>42.82±1.78</td>
<td>33±0.56</td>
<td>9.65±0.12</td>
<td>59.93±1.70</td>
<td>29.33±5.46</td>
<td>35.2±0.66</td>
<td>43.9±0.52</td>
</tr>
<tr>
<td>Petiole length (cm)</td>
<td>38.65±1.70</td>
<td>25±.68</td>
<td>6.31±0.29</td>
<td>43.26±2.06</td>
<td>21.24±1.82</td>
<td>27.1±0.47</td>
<td>25±.56</td>
</tr>
<tr>
<td>Petiole colour</td>
<td>Red-green</td>
<td>Green</td>
<td>Red-green</td>
<td>Red-green</td>
<td>Red-green</td>
<td>Red-green</td>
<td>Red-green</td>
</tr>
<tr>
<td>Height of plant at first flower (cm)</td>
<td>60.97±2.10</td>
<td>40±1.25</td>
<td>10.25±0.36</td>
<td>65.3±2.66</td>
<td>56.53±5.43</td>
<td>45±0.78</td>
<td>48±0.98</td>
</tr>
<tr>
<td>Stem diameter (cm)</td>
<td>3.55±0.18</td>
<td>1.9±0.15</td>
<td>2.1±0.08</td>
<td>6.60±0.28</td>
<td>2.89±0.28</td>
<td>2.34±0.11</td>
<td>2.16±0.05</td>
</tr>
<tr>
<td>Stem colour</td>
<td>Red-brown at base, green at apex</td>
<td>Brown at base, green at apex</td>
<td>Light brown at base, green at apex</td>
<td>Red-brown at base, green at apex</td>
<td>Red-brown at base, green at apex</td>
<td>Brown at base, green at apex</td>
<td>Brown at base, green at apex</td>
</tr>
<tr>
<td>Fruit size and colour</td>
<td>Large, yellow</td>
<td>Small, yellow</td>
<td>Extra small, yellow</td>
<td>Small, yellow</td>
<td>Small, yellow</td>
<td>Extra small, yellow</td>
<td>Extra small, yellow</td>
</tr>
</tbody>
</table>

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2.3.2 *V. pubescens* x *V. parviflora* [F₂]

Approximately 71% of the seed germinated to give a population of 268 plants of *V. pubescens* x *V. parviflora* [F₂]. Plants grew vigorously in the glasshouse and field. All plants of the F₂ generation did not defoliate like the F₁ or *V. pubescens* during the warmer months. Sex ratio was 1:1 of male and female flowers; however, male plants did produce some hermaphroditic flowers. Pollen fertility was 75%. Flowers ranged in colour from shades of pink to white (Figure 2.3.2.1 C).

![Variation observed in colour and morphology of flowers resulting from crosses of *V. pubescens* x *V. parviflora* [F₁], and [F₂] generations.](image)

**Figure 2.3.2.1** Variation observed in colour and morphology of flowers resulting from crosses of *V. pubescens* x *V. parviflora* [F₁], and [F₂] generations.

A – *V. parviflora*

B – *V. pubescens* x *V. parviflora* [F₁]

C – *V. pubescens* x *V. parviflora* [F₂]
Leaf shape was intermediate between that of *V. pubescens* and *V. parviflora*. Leaf venation was palmate-lobed or sub-orbicular. The number of main veins was 7 for all the F$_2$ hybrids, the same as F$_1$ hybrids and *V. pubescens* but more than *V. parviflora*. Leaf length (43.9 cm ± 0.52) and width (43.9 cm ± 0.52) in the F$_2$ hybrid was greater than that of both parents (Table 2.3.1.1). Petiole length (25 cm ± 0.56) was similar to *V. pubescens* and F$_1$ hybrids but greater than *V. parviflora* (6.31 cm ± 0.29). Height of the F$_2$ hybrid at first flower (48 cm± 0.98) was similar to *V. pubescens* and F$_1$ hybrids but greater than *V. parviflora* (10.25 cm ± 0.36). Fruit were orange-yellow in colour and slightly larger than *V. parviflora* and contained six seeds per fruit. Plants segregated in a 3:1 ratio for PRSV-P resistance: susceptibility.

### 2.3.3 *V. pubescens* x *V. parviflora* [F$_3$] RR

*V. pubescens* x *V. parviflora* [F$_2$] plants with the RR genotype for PRSV-P resistance were crossed to produce a F$_3$ generation. As seeds were difficult to germinate fifty embryos were rescued from 20 fruit and micropropagated plantlets grew vigorously *in vitro* (Table 2.3.3.1).

**Table 2.3.3.1** – Summary of hybridisation of *V. pubescens* and *V. parviflora* [F$_3$] RR

<table>
<thead>
<tr>
<th>Stage of Hybridisation</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flowers cross-pollinated</td>
<td>25</td>
</tr>
<tr>
<td>Successful cross-pollinations after 2 weeks</td>
<td>20</td>
</tr>
<tr>
<td>Fruit harvested</td>
<td>20</td>
</tr>
<tr>
<td>Hybrid seeds recovered</td>
<td>64</td>
</tr>
<tr>
<td>Embryos rescued</td>
<td>50</td>
</tr>
<tr>
<td>Number of plants in tissue culture</td>
<td>50</td>
</tr>
<tr>
<td>Number of micropropagation cycles to reach 10 clones of each plant</td>
<td>2</td>
</tr>
<tr>
<td>Plants acclimatized</td>
<td>3 of each clone totalling 150</td>
</tr>
<tr>
<td>Number of plants tested for PRSV-P in field</td>
<td>30</td>
</tr>
</tbody>
</table>

After three replicates of each clone were established in the field, plants were observed to be only vigorous during winter. During the hotter months plants showed signs of wilting and their leaves curled when heat-stressed. Sex ratio was 1:1 of male and female flowers; however, male plants did produce some hermaphroditic flowers. Pollen fertility was recorded and approximately 70% of pollen grains were stained by 1% acetocarmine solution. Pollen grains appeared normal and were well formed.
although grains which did not stain were irregular in shape. Flowers ranged in colour from shades of pink to white. Leaf shape was intermediate between that of the two parent species. Leaf venation was palmate-lobed or sub-orbicular (Figure 2.3.3.1). The number of main veins was 7 for all the F3 hybrids, the same as F1 hybrids, F2 hybrids and *V. pubescens* but more than *V. parviflora*. Leaf length (43.3 cm ± 0.49) in the F3 hybrid was greater than both parent species and width (33.5 cm ± 0.42) was similar to *V. pubescens* (Table 2.3.3.2). Thirty plants were field-tested for PRSV-P and plants remained symptom free.

![Variation observed in leaves of F1, F2 and F3 generations of V. pubescens x V. parviflora crosses.](image)

**Figure 2.3.3.1** Variation observed in leaves of F1, F2 and F3 generations of *V. pubescens* x *V. parviflora* crosses.

A – *V. parviflora*
B – *V. pubescens*
C – *V. pubescens* x *V. parviflora* [F1]
D – *V. pubescens* x *V. parviflora* [F2]
E – *V. pubescens* x *V. parviflora* [F3]
Table 2.3.3.2 A summary of morphological characters for *C. papaya*, *V. pubescens*, *V. parviflora* and their hybrids and backcross generations. The data are the mean ± standard error determined from 10 plants of *C. papaya*, *V. pubescens*, *V. parviflora* and 30 hybrids, *except BC1 cross as only two plants were produced.*

<table>
<thead>
<tr>
<th>Character</th>
<th><em>C. papaya</em></th>
<th><em>V. pubescens</em></th>
<th><em>V. parviflora</em></th>
<th><em>(V. pubescens x V. parviflora [F2]) x V. parviflora [BC2]</em></th>
<th><em>(V. pubescens x V. parviflora F2 RR) x V. parviflora [BC2]</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pollen fertility</td>
<td>90%</td>
<td>55%</td>
<td>70%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Flower colour</td>
<td>Cream</td>
<td>Green</td>
<td>Pink</td>
<td>Shades of pink-white, cream</td>
<td>Light pink</td>
</tr>
<tr>
<td>Flower shape</td>
<td><em>C. papaya</em> large</td>
<td><em>V. pubescens small</em></td>
<td><em>V. parviflora small</em></td>
<td>Intermediate 10% <em>C. papaya</em>; 20% <em>V. pubescens</em>; 70% <em>V. parviflora</em></td>
<td><em>V. parviflora</em></td>
</tr>
<tr>
<td>No. stigma &amp; size</td>
<td>5 thick</td>
<td>5 thin</td>
<td>5 thin</td>
<td>5 thin</td>
<td>5 thin</td>
</tr>
<tr>
<td>No. of anthers</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10 All female</td>
<td>NA both plants were female</td>
</tr>
<tr>
<td>Leaf shape</td>
<td><em>C. papaya</em></td>
<td><em>V. pubescens</em></td>
<td><em>V. parviflora</em></td>
<td>Intermediate 60% <em>C. papaya</em>; 30% <em>V. pubescens</em>; 10% <em>V. parviflora</em></td>
<td><em>V. parviflora</em></td>
</tr>
<tr>
<td>Leaf venation</td>
<td>Palmate</td>
<td>Palmate</td>
<td>Palmate</td>
<td>Palmate</td>
<td>Palmate</td>
</tr>
<tr>
<td>No. of main veins</td>
<td>9±0.00</td>
<td>7±0.00</td>
<td>7±0.00</td>
<td>7±0.00</td>
<td>7±0.00</td>
</tr>
<tr>
<td>Leaf length (cm)</td>
<td>30.55±0.94</td>
<td>28±1.52</td>
<td>10.59±0.20</td>
<td>43.3±0.49</td>
<td>49.3±0.61</td>
</tr>
<tr>
<td>Leaf width (cm)</td>
<td>42.82±1.78</td>
<td>33±0.56</td>
<td>9.65±0.12</td>
<td>33.5±0.42</td>
<td>37.3±0.42</td>
</tr>
<tr>
<td>Petiole length (cm)</td>
<td>38.65±1.70</td>
<td>25±0.68</td>
<td>6.31±0.29</td>
<td>23.4±0.36</td>
<td>35.78±1.32</td>
</tr>
<tr>
<td>Petiole colour</td>
<td>Red-green</td>
<td>Green</td>
<td>Red-green</td>
<td>Red-green</td>
<td>Red-green</td>
</tr>
<tr>
<td>Height of plant at first flower (cm)</td>
<td>60.97±2.10</td>
<td>40±1.25</td>
<td>10.25±0.36</td>
<td>47±0.57</td>
<td>45±2.26</td>
</tr>
<tr>
<td>Stem diameter (cm)</td>
<td>3.55±0.18</td>
<td>1.9±0.15</td>
<td>2.1±0.08</td>
<td>1.78±0.03</td>
<td>2.90±0.90</td>
</tr>
<tr>
<td>Stem colour</td>
<td>Red-brown at base, green at apex</td>
<td>Brown at base, green at apex</td>
<td>Light brown at base green at apex</td>
<td>Brown at base, green at apex</td>
<td>Brown at base, green at apex</td>
</tr>
<tr>
<td>Fruit size and colour</td>
<td>Large, yellow</td>
<td>Small, yellow</td>
<td>Extra Small, yellow</td>
<td>Extra Small, yellow</td>
<td>Extra Small, yellow</td>
</tr>
</tbody>
</table>
2.3.4 (*V. pubescens* x *V. parviflora* F₂ RR) to *V. parviflora* [BC₁]

Only two plants were produced from crossing as the 15 seeds were difficult to germinate. One plant showed abnormal (deformed at some stages of growth) leaf symptoms while the other appeared normal. The plants grew vigorously in the glasshouse. Flowers were light pink in colour (Figure 2.3.4.1) and petals were curled at thin terminal ends. The size of flowers on both hybrids was small like *V. parviflora*. Leaf shape was similar to *V. parviflora*. Leaf venation was palmate-lobed. The number of main veins was 7 for both hybrids and the same as F₁ hybrids, F₂ hybrids, F₃ hybrids and *V. pubescens*. Leaf length (30.9 cm ± 0.32) and width (24.5 cm ± 0.45) in the BC₁ hybrids was less than that of both parents (Table 2.3.3.2). Plants were not tested for PRSV-P as there were only two and they were not cloned.

![Figure 2.3.4.1](image)

**Figure 2.3.4.1** Variation observed in colour and morphology of flowers resulting from crosses of *V. pubescens* x *V. parviflora* [F₁], [F₂] and [BC₁] generations:

A – *V. parviflora*

B – *V. pubescens* x *V. parviflora* [F₁]

C – *V. pubescens* x *V. parviflora* [F₂]

D – (*V. pubescens* x *V. parviflora* F₂ RR) to *V. parviflora* [BC₁]
2.3.5 \{(V. pubescens \times V. parviflora F_2 \text{ RR}) \text{ to } V. \text{ parviflora}\} \text{ BC}_1 \text{ to } V. \text{ parviflora} [\text{BC}_2]

Thirteen embryos were rescued and plants were micropropagated. Plants grew vigorously \textit{in vitro} and in the glasshouse (Figure 2.3.5.1).

![Image of plants](image)

\textbf{Figure 2.3.5.1} Group of \text{BC}_2 \text{ plants} \{(V. \text{ pubescens} \times V. \text{ parviflora} \ F_2 \text{ RR}) \text{ to } V. \text{ parviflora} \text{ BC}_1\} \text{ to } V. \text{ parviflora}.

Plants were slow-growing like \textit{V. parviflora} in the field. Flower colour varied in shades of pink and petals were curled and thin at terminal ends (Figure 2.3.5.2 and Figure 2.3.5.3). The size of flowers on all hybrids was small in size like \textit{V. parviflora}. The number of main veins was 7 for both hybrids and the same as \text{F}_1 \text{ hybrids}, \text{F}_2 \text{ hybrids}, \text{F}_3 \text{ hybrids}, \text{BC}_1 \text{ hybrids}, and \text{V. pubescens}. Leaf shape was similar to that of \textit{V. parviflora}. Leaf venation was palmate-lobed. Leaf length (24.9 cm ± 0.45) and width (14.5 cm ± 0.85) in the \text{BC}_2 \text{ hybrids} was less than that of both parents (Table 2.3.3.2). Plants were not field-tested for PRSV-P resistance.
Figure 2.3.5.2 Female flowers of BC$_2$: \{(V. pubescens \times V. parviflora F$_2$ RR) to V. parviflora BC$_1$} to V. parviflora

Fig. 2.3.5.3 Male flowers of BC$_2$: \{(V. pubescens \times V. parviflora F$_2$ RR) to V. parviflora BC$_1$} to V. parviflora
2.3.6 *C. papaya* x *V. parviflora* [F₁]

Two hundred and fifty plants of *C. papaya* x *V. parviflora* [F₁] were produced. Fifty were micropropagated and the rest were planted out and grown in the glasshouse and field. All plants grew vigorously *in vitro* and in the glasshouse but when planted in the field, plants were slow growing similar to *V. parviflora*. Plants were deciduous in winter similar to *V. parviflora*. Pollen fertility of up to 45% was recorded. The sex ratio was 1:1 of male: female flowers. Flowers were pink in colour (Figure 2.3.6.1) and the stigma was pink with a white vein. Female flowers were in clusters of 4-6 and close to the stem while male flowers were multiple and on long peduncles.

![Flowers of C. papaya, V. parviflora and C. papaya x V. parviflora [F₁].](image)

**Figure 2.3.6.1** Flowers of *C. papaya*, *V. parviflora* and *C. papaya* x *V. parviflora* [F₁].
Leaves were yellow mottled with yellow and green sections and varied from light mottling to mostly chlorotic (Figure 2.3.6.2). Leaf shape was intermediate between parent species. Leaf venation was palmate-lobed (Figure 2.3.6.3). The number of main veins was 7 for all the F₁ hybrids compared to *C. papaya* which had 9. Leaf length (25.23 cm ± 1.55) and width (29.33 cm ± 5.46) in the F₁ hybrid was variable and intermediate between *V. parviflora* and *C. papaya*. Petiole length (21.24 cm ± 1.82) was less than *C. papaya*. Hybrid height at first flower (56.53 cm ± 5.43) was similar to *C. papaya* and greater than that of *V. parviflora*. Stem diameter (2.89 cm ± 0.28) was greater than that of *V. parviflora* and less than for *C. papaya* (Table 2.3.1.1). All plants of this cross were susceptible to PRSV-P according to field tests.

*Figure 2.3.6.2* *C. papaya* x *V. parviflora* [F₁] leaves showing varying degrees of mottling.
Figure 2.3.6.3 Leaves of *C. papaya*, *V. parviflora* and *C. papaya x V. parviflora* [F₁].

### 2.3.7 *C. papaya x V. pubescens* [F₁]

Three hundred plants of this cross were micropropagated and grown in the glasshouse. All plants grew vigorously *in vitro*, the glasshouse and field, although they were slower during summer and more cold tolerant than *C. papaya* in winter. There was no pollen fertility as all hybrids were female. Female flowers were green when immature similar to those of *V. pubescens* and yellow when fully mature like *C. papaya* (Figure 2.3.7.1). The shape of the flower was the same as the flowers of parent *V. pubescens*. Flowers occurred either singly on short peduncles similar to *C. papaya* female flowers or as multiple flowers on long peduncles similar to *C. papaya* male flowers.
Leaves had a palmate-lobed apex and obtuse base (Figure 2.3.7.2). The number of main veins was 7 for all the F1 hybrids, the same as *V. pubescens*, compared to leaves of *C. papaya* which had 9. Leaf length (54.76 cm ± 2.34) and width (59.93 cm ± 1.70) in the F1 hybrid was greater than both parents. Petiole length (43.26 cm ± 2.06) and height at the first flower (65.3 cm ± 2.66) in the F1 hybrid was similar to *C. papaya*. Stem diameter (6.60 cm ± 0.28) varied from both parents being nearly twice the size of *C. papaya* (3.55 cm ± 0.18) and three times the size of *V. parviflora* (2.1 cm ± 0.08) (Table 2.3.1.1). All plants were resistant to PRSV-P.

**Figure 2.3.7.1** Flowers of *C. papaya*, *V. pubescens* and *C. papaya x V. pubescens* [F1].
2.3.8 *C. papaya* x (*V. pubescens* x *V. parviflora* \(F_2\) RR)

One hundred and ten plants of *C. papaya* x (*V. pubescens* x *V. parviflora*) using an RR PRSV-P resistant seedling from the population described in 2.3.2) were produced and micropropagated. Plants grew vigorously *in vitro* and in the glasshouse. When plants were planted in the field some were slow growing like *V. parviflora* plants and others were vigorous. However, none were as vigorous as *C. papaya* plants (Table 2.3.8.1). Weak, slow growing plants died after 6-9 months in the field as they could not survive winter. All plants were infertile female.
Table 2.3.8.1 Summary of hybridisation of C. papaya x (V. pubescens x V. parviflora F2 RR)

<table>
<thead>
<tr>
<th>Stage of Hybridisation</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flowers cross-pollinated</td>
<td>35</td>
</tr>
<tr>
<td>Successful cross-pollinations after 2 weeks</td>
<td>19</td>
</tr>
<tr>
<td>Fruit harvested</td>
<td>19</td>
</tr>
<tr>
<td>Hybrid seeds recovered</td>
<td>452</td>
</tr>
<tr>
<td>Embryos rescued</td>
<td>110</td>
</tr>
<tr>
<td>Number of plants in tissue culture</td>
<td>110</td>
</tr>
<tr>
<td>Number of micropropagation cycles to reach 10 clones of each plant</td>
<td>2</td>
</tr>
<tr>
<td>Plants acclimatized</td>
<td>5 of each clone, totalling 550</td>
</tr>
<tr>
<td>Number of plants tested for PRSV-P in field</td>
<td>2 of each clone, totalling 220</td>
</tr>
</tbody>
</table>

Flower colour varied from shades of yellow to cream (Figure 2.3.8.1 D). Two flowering types occurred: flowers on short inflorescences were in clusters of 3-8, while multiple flowers which resembled C. papaya male flowers in appearance were on long inflorescences. The size of flowers on short inflorescences was larger than those of both V. pubescens and V. parviflora but smaller than those of C. papaya. Flowers that were on long inflorescences appeared to be like those of hermaphrodites of the F2 V. pubescens x V. parviflora (2.3.2) and F3 V. pubescens x V. parviflora (2.3.3) hybrids and were small in size. Variation for flower shape occurred, with 10% resembling those of C. papaya, 20% those of V. pubescens and 70% those of V. parviflora (Table 2.3.3.2).
Figure 2.3.8.1 Variations in flower colour on plants of *C. papaya* x (*V. pubescens* x *V. parviflora* F₂ RR) in comparison to flower colour of *V. parviflora* and *C. papaya* female and male plants.

A – *V. parviflora*
B – *C. papaya* female
C – *C. papaya* male
D – *C. papaya* x (*V. pubescens* x *V. parviflora* F₂ RR)
Leaf venation was palmate-lobed with varying degrees of lobing (Figure 2.3.8.2 D). Variation in leaf shape occurred: 60% C. papaya, 30% V. pubescens and 10% V. parviflora in appearance. The number of main veins in the leaves was 7 for all the C. papaya x (V. pubescens x V. parviflora F2 RR) hybrids, the same as F1 hybrids, F2 hybrids, F3 hybrids and V. pubescens but more than V. parviflora leaves which had 5 and less than C. papaya leaves which had 9. Leaf length and width in the C. papaya x (V. pubescens x V. parviflora F2 RR) hybrid varied with some being greater than both parents (Table 2.3.3.2) and others being smaller. None of the plants produced symptoms of PSRV-P.

Figure 2.3.8.2 Variations in leaf shape on plants of C. papaya x (V. pubescens x V. parviflora F2 RR) in comparison to leaves of parent species.

A – V. parviflora
B – V. pubescens
C – C. papaya
D – C. papaya x (V. pubescens x V. parviflora F2 RR)
2.4 Discussion

Interspecific crosses are often attempted to transfer desired traits such as disease resistance, stress tolerance or high yield from wild species to related crop species. Successful intergeneric crosses, for example, *Vasconcellea* spp. x *C. papaya*, are typically much more difficult to achieve and less common. Generation of these hybrids is usually possible only with significant human intervention, including use of techniques such as rescue of immature embryos and *in vitro* culture (Magdalita, 1996; Drew et al., 1998). Tissue culture provides a means to overcome pre-zygotic barriers where hybrid zygotes form but cannot develop due to failure of the nutritive endosperm tissue to develop. By culturing excised hybrid embryos or ovules containing them on artificial media, development into mature plants may occur, opening access to the genes of the wild species (Manshardt and Drew, 1998; Drew et al., 2006a, b). Other factors such as optimal time of year for cross-pollination (late spring in south-east Queensland), high pollen fertility of the male *Vasconcellea* species parent and the use of clone 2.001 as the female *C. papaya* parent, which produced a high yield of hybrid embryos, facilitated previous success in the production of *C. papaya x V. cauliflora*, *C. papaya x V. parviflora*, *C. papaya x V. pubescens* and *C. papaya x V. quercifolia* hybrids (Magdalita et al., 1998; Drew et al., 1998, 2006a).

Until recently there were limited reports of vigorous intergeneric hybrids within Caricaceae in field plantings. However, a large number of morphologically normal *C. papaya x V. parviflora* and *C. papaya x V. pubescens* hybrid plants (Drew et al., 1998) were produced and grew to maturity in the field, where many displayed good vigour. Similarly, high vigour was reported for field plantings of *C. papaya x V. quercifolia* hybrids (Drew et al., 2006a, b).

*Vasconcellea* species vary in many morphological characters including plant height, leaf shape, venation and size, fruit colour and shape. Flower colour and shape make each species distinct from each other and from *C. papaya* (Badillo, 2000). There was a range of morphological variability in *V. pubescens x V. parviflora* hybrids and BC₁
and BC2 populations observed in the present study. Plant vigour in the juvenile phase (as measured by plant height to first flower) of F1, F2 and F3 hybrids of V. pubescens x V. parviflora as well as C. papaya x (V. pubescens x V. parviflora F2 RR) [F1] was similar to V. pubescens unlike V. parviflora which is slow-growing (Badillo, 2000). However, when V. pubescens and V. parviflora were crossed directly with C. papaya, the F1 hybrids displayed the vigour of C. papaya. When V. pubescens x V. parviflora [F2] hybrids were backcrossed to V. parviflora plant vigour was intermediate between V. pubescens and V. parviflora. Yet {V. pubescens x V. parviflora [F2 RR] to V. parviflora} BC1 to V. parviflora [BC2] plants exhibited low plant vigour similar to growth of V. parviflora plants.

Leaf morphological characters are a useful visual guide for preliminary identification of hybrids. Magdalita et al. (1997a) used the number of main veins in the hybrids of C. papaya x V. cauliflora as a morphological marker of hybridity. The number of main veins seen in all hybrids and backcross generations was seven which is a dominant trait from V. pubescens, being more than V. parviflora which had five and less than C. papaya which had nine. Leaf shape for F1 hybrids of V. pubescens x V. parviflora was the same as V. pubescens while for F2 and F3 hybrids of V. pubescens x V. parviflora was intermediate between parent species. However, when the V. pubescens x V. parviflora [F2] was backcrossed to V. parviflora, leaf shape resembled that of V. parviflora. For the F1 populations of C. papaya x V. parviflora and C. papaya x V. pubescens the leaf shape resembled that of the respective wild parents and increased size could be attributed to hybrid vigour. When F2 hybrids of V. pubescens x V. parviflora were crossed to C. papaya, leaf shape resembled one of the parent species for each seedling but usually C. papaya (60%) rather than V. pubescens (30%) or V. parviflora (10%) which suggests multiple gene control or non-Mendelian ratios resulting from the incompatibility of the intergeneric cross.
The cross *C. papaya* x (*V. pubescens* x *V. parviflora* F₂ RR) [F₁] produced plants with infertile female flowers, similar to the *C. papaya* x *V. pubescens* [F₁]. No males were produced in either cross. A large degree of variability was observed in plants from the cross *C. papaya* x (*V. pubescens* x *V. parviflora* F₂ RR) [F₁], for example, some plants were weak and died several months after field planting while others exhibited high levels of vigour. This observation was consistent with variable vigour displayed by progeny of *C. papaya* x *V. cauliflora* (Magdalita et al., 1997a, b). The pink flower colour of *V. parviflora* was dominant in all hybrids of *V. pubescens* x *V. parviflora* over the green of *V. pubescens*. However when these hybrids were crossed to *C. papaya* cream was dominant over pink. By contrast, in the F₁ hybrids of *C. papaya* x *V. parviflora* and *C. papaya* x *V. pubescens* the colour of the flowers of the respective *Vasconcellea* parent species was dominant. This apparently aberrant result when *V. pubescens* x *V. parviflora* hybrids were crossed to *C. papaya* is consistent with the production of all female flowers on these plants, suggesting that abnormal pairing of chromosomes and possible preferential elimination of the *Vasconcellea* chromosomes is affecting the expression of these characteristics. Production of some hermaphroditic flowers in *V. pubescens* x *V. parviflora* [F₁], [F₂] and [F₃] generations is consistent with occurrence of hermaphroditic flowers on male trees of *V. pubescens* and *C. papaya* (Badillo, 2000).

Flower shape for intrageneric F₁, F₂ and F₃ hybrids between *V. pubescens* x *V. parviflora* was intermediate between both parents. When the *V. pubescens* x *V. parviflora* [F₂] was backcrossed to *V. parviflora*, flower shape was more like *V. parviflora*. As with flower colour, in the F₁s of *C. papaya* x *V. parviflora* and *C. papaya* x *V. pubescens* the flower shape resembled that of the respective wild parents with the only difference being in size. When F₂ hybrids of *V. pubescens* x *V. parviflora* were crossed to *C. papaya* there was the following segregation for flower shape: 10% *C. papaya*, 20% *V. pubescens*, 70% *V. parviflora*, again highlighting inconsistencies in gene expression in intergeneric hybrids.
Fruit size in all *V. pubescens* x *V. parviflora* [F₁], [F₂] and [F₃] hybrids was small and intermediate between *V. pubescens* and *V. parviflora*. Stem colour of *V. pubescens* x *V. parviflora* [F₁], [F₂] and [F₃] hybrids as well as backcross generations was brown at the base and green at the apex, the same as *V. pubescens*. Red-brown stem colour of *C. papaya* in *C. papaya* x *V. pubescens* and *C. papaya* x *V. parviflora* [F₁] hybrids and *C. papaya* x (*V. pubescens* x *V. parviflora* F₂ RR) [F₁] hybrids dominated over brown of *V. pubescens* which in turn was dominant over the light brown of *V. parviflora*.

Although crosses between *C. papaya* and *Vasconcellea* spp. have been of limited use due to incompatibility between papaya and the wild species (Sawant, 1958), there have been numerous attempts to transfer PRSV-P resistance from these wild relatives to *C. papaya*. The lack of success in these crosses can be attributed to the high degree of genetic divergence between *Vasconcellea* spp. and *C. papaya* (van Droogenbroeck et al., 2004). Although crosses between *C. papaya* and PRSV-P resistant species *V. cauliflora*, *V. stipulata*, *V. pubescens* and *V. quercifolia* have been reported (Manshardt and Wenslaff 1989a, b; Magdalita et al., 1997b; Drew et al., 1998), these plants have died in the F₁ hybrid stage or shown little fertility which is essential to continue backcrossing programmes. However, crosses reported in this thesis have demonstrated that PRSV-P resistance can be transferred to the F₁ plants. This resistance in *V. pubescens* and *V. cauliflora* is controlled by a single dominant gene (Drew et al., 1998; Magdalita et al., 1997b; Dillon et al., 2005b). A large *V. pubescens* x *V. parviflora* [F₂] population was exposed to PRSV-P and 26% of seedlings were susceptible to the virus and the rest were resistant (Dillon et al., 2005a). Of the F₂ plants that were susceptible to PRSV-P, variations in severity of the symptoms of the virus were observed (Dillon et al., 2005). Those results indicated multi-gene virus resistance in *V. pubescens* that was controlled by a single gene. The use of the CAPS marker which is diagnostic for the PRSV-P resistance gene in *V. pubescens* (Dillon et al., 2006) facilitated the identification of PRSV-P resistant homozygous (RR) or heterozygous resistant (Rr) plants in the current research and was used at the seedling or *in vitro* plantlet stage after embryo rescue.
The use of *V. parviflora* as a bridging species between *C. papaya* and PRSV-P resistant *V. pubescens* has been investigated in this research. The inability to produce a fertile male plant in *C. papaya x V. pubescens* [F₁] populations has been a barrier for backcrossing strategies (Drew et al., 1998). However, as reported previously, F₁ hybrids between papaya and *V. parviflora* have exhibited pollen fertility and hybrid vigour in a F₁ population (Drew et al., 1998). It has been shown in this research that morphologically normal F₂ populations of *V. pubescens x V. parviflora* containing the PRSV-P resistance genotype (RR) can be backcrossed to *V. parviflora* to produce BC₁ hybrids with high vigour and resistance. Fertility in the BC₁ population enabled further backcrossing to produce a BC₂ population. These BC₂ plants produced both fertile male and female plants some of which contained the Rr genotype. However, crossing BC₂ plants to *C. papaya* resulted in only female plants. As *V. parviflora* is one of the closest related *Vasconcellea* spp. to papaya (Jobin-Décor, 1997), it is expected further backcrossing to *V. parviflora* will eventually produce a PRSV-P plant that contains a predominant *V. parviflora* genome and will cross with *C. papaya* to produce fertile progeny. This should overcome the incompatibility between *V. pubescens* and *C. papaya* and enable PRSV-P resistance to be transferred from *V. pubescens* to *C. papaya*.

Future research could investigate crossing Rr BC₂ (87.5% *V. parviflora*) genome plants to *C. papaya* genotype 2.001, to produce a F₁ population which could inherit the R allele for PRSV-P resistance. If they were not fertile, backcrossing to *V. parviflora* should continue to produce a BC₃ population (93.75% *V. parviflora*) or BC₄ population (96.87% *V. parviflora*) with the resistance allele inherited and subsequently Rr backcross plants could be crossed to *C. papaya*. When fertility is achieved in crosses with *C. papaya*, sib-crossing could then produce *C. papaya* plants with the RR genotype for PRSV-P resistance.
Because of this research, genetic diversity of *Vasconcellea* species could be exploited in the future to provide breeders with options to develop new and more productive papaya varieties that are resistant to virulent pests and diseases and are adapted to changing environments. Plant genetic resources for food and agriculture are the basis of global food security (Strange, 2005). The world population is expected to reach eight billion by 2020 and to meet the need for more food, it will be necessary to make better use of a broader range of the world’s plant genetic diversity. Therefore, other *Vasconcellea* species could be incorporated into future intergeneric hybridisation programmes. For example, *V. goudotiana* has exhibited resistance to *Phytophthora*, tolerates some drought, bears heavily and its fruit have an apple-like taste. *V. pubescens* is cold tolerant, and *V. stipulata* is resistant to PRSV-P, has a strong pleasant aroma and is very rich in papain. Babaco (*V. x heilbornii*) tolerates cool, damp winters, has few seeds and useful fruit quality characteristics. New papaya varieties with different fruit shape, size, flavour and aroma could be explored. For example, creating a fruit that is small, with skin that is edible and seedless and has a pleasant aroma would increase papaya’s marketability. In addition, having papaya varieties that grow in a wider range of environments would allow more regions to grow the fruit thus giving resource-poor countries access to this valuable fruit crop.
Chapter 3* The use of a SCAR and CAPS marker to assist hybridisation between *C. papaya* L. and *Vasconcellea* species

* A summary of this chapter has been submitted for publication in Acta Horticulturae (Proceedings of International Symposium in Molecular Markers in Horticulture, Corvallis, Oregan, USA, July 29 – August 1, 2009).
3.1 Introduction

Marker-assisted selection assists breeders to identify useful traits with speed and precision. Conventional breeding and assessment based on morphological markers can be a difficult and slow process with perennial fruit species. Molecular markers can facilitate plant breeding programmes (Drew, 1997), and be useful for gene introgression (Moore and Durham, 1992), and breeding for multigene resistance and resistance to diseases not yet in a region or country (Henry, 1996).

Losses caused by diseases are the largest single variable cost facing growers. Conventional plant breeding for disease resistance facilitated by markers, and transgenic modification with genes that confer resistance, have important roles to play in crop improvement programmes. For plant breeders molecular biology offers useful tools which enable the products of genes controlling resistance to be identified, understood and controlled. With this information the gene pool of a plant and its relatives can be exploited and incorporated into breeding programmes (Strange, 2005).

*Vasconcellea* species contain genes that would be beneficial if transferred to *C. papaya.* Four *Vasconcellea* species (*V. pubescens, V. quercifolia, V. stipulata* and *V. cauliflora*) are resistant to PRSV-P. As previously discussed, several intergeneric and interspecific hybridisations between *C. papaya* and *Vasconcellea* species have been conducted despite limitations due to post-zygotic barriers. However, low fertility has prevented sustained hybridisation. *V. pubescens* is immune to PRSV-P and when crossed to papaya produced infertile hybrids (2.3.7). *V. parviflora* is susceptible to PRSV-P and when crossed to papaya, produced hybrids with some pollen fertility (2.3.6). As *V. parviflora* is compatible with *V. pubescens* (2.3.1-2.3.3) and PRSV-P resistance can be introgressed into hybrids while increasing the proportion of *V. parviflora* genetic background (2.3.4-2.3.5), *V. parviflora* could be used as a bridge species between *V. pubescens* and *C. papaya.*
A single dominant gene for PRSV-P resistance was mapped in *V. pubescens* (Dillon et al., 2005b). Genetic maps were constructed of *V. pubescens* and *V. parviflora* by use of dominant, polymorphic randomly amplified DNA fingerprint (RAF) markers (Peace et al., 2003) in a F2 interspecific population of *V. parviflora* and *V. pubescens*. Using a bulked segregant analysis strategy, several DNA markers linked to a PRSV-P resistance gene locus were identified and mapped in *V. pubescens*. One of these markers Opk4_1r resulted from a Single Nucleotide Polymorphism (SNP) (Dillon et al., 2005b) in close proximity to the PRSV-P resistance gene locus. The sequence characterized amplified region (SCAR) was present in both parent species and four *Vasconcellea* species but not in papaya (Dillon et al., 2005b). Sequence analysis revealed several polymorphisms, one resulting in the creation of a restriction site within the Opk4_1r region which was used to convert the SCAR into an easily detected co-dominant marker cleaved amplified polymorphism sequence (CAPS Psi1k4) which could be applied as a diagnostic test for selection of homozygous or heterozygous PRSV-P resistant hybrids of *V. pubescens* (Dillon et al., 2005a, b).

The SCAR marker Opk4_1r (Dillon 2005a, b) and the CAPS Psi1k4 marker (Dillon et al., 2006) were used in the research described in this chapter. The marker identified a single-gene dominant trait associated with PRSV-P resistance in a segregating F2 population generated from a *V. pubescens* x *V. parviflora* [F1] population. This chapter investigates the use of the Opk4_1r SCAR marker (Dillon et al., 2005a, b) for selection of plants which contain the R allele for PRSV-P resistance and the use of CAPS Psi1k4 for the selection of resistant genotypes within given intrageneric and intergeneric populations.
3.2 Materials and methods

3.2.1 Plant material

In all of the experiments *V. pubescens* and *V. parviflora* were used as controls. Production of plants used in these experiments was described in Chapter 2. Plants were maintained in tissue culture, the glasshouse and the field. Young leaf material was gathered from these plants for genotype analysis. Micropropagated plants of the following crosses were assessed for the PRSV-P resistance gene using the SCAR marker Opk4_1r and then for the allele using the CAPS *Psi1k4* marker.

- *V. pubescens* x *V. parviflora* [F₁]
- *V. pubescens* x *V. parviflora* [F₂]
- *V. pubescens* x *V. parviflora* [F₃]
- (*V. pubescens* x *V. parviflora* F₂ RR) to *V. parviflora* [BC₁]
- {(*V. pubescens* x *V. parviflora* F₂ RR) to *V. parviflora*} BC₁ to *V. parviflora* [BC₂]
- *C. papaya* x (*V. pubescens* x *V. parviflora* F₂ containing RR allele for PRSV-P resistance)

Six breeding lines of *C. papaya* from different growing regions were tested with both the Opk4_1r SCAR marker and CAPS *Psi1k4* marker.

- K1 (south-east Queensland)
- C3 (south-east Queensland)
- 35 (south-east Queensland)
- 70 (central Queensland)
- 97 (north Queensland)
- 5648 (Philippines)
Four *Vasconcellea* species and two resistant *C. papaya x V. quercifolia* [F₁] plants were tested with the Opk4_1r SCAR marker and CAPS *Psi1k4* marker:

- *V. quercifolia*
- *V. stipulata*
- *V. cauliflora*
- *V. goudotiana*
- 410 *C. papaya x V. quercifolia* [F₁]
- 468 *C. papaya x V. quercifolia* [F₁]

### 3.2.2 DNA extraction and marker analysis

A sample of 0.06 g of leaf material from each plant was labelled and weighed in small plastic weighing boats. Leaf material was ground into a fine powder in liquid nitrogen with a mortar and pestle. The fine powder was then transferred into a 1.5 ml eppendorf tube containing 600 μL of 2% CTAB buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.7 M NaCl). PVP (polyvinyl-polypyrrolidone) 10% (w/v) and sarkosyl (n-laursarcosine) were prepared fresh and 60 μL of each was added to eppendorf tubes. Tubes were vortexed lightly and left to incubate in a water bath for an hour. Every 15 minutes, tubes were carefully inverted once to mix the components. Following incubation tubes were left to cool at room temperature for 10 minutes before adding 600 μL of phenol: chloroform: isoamyl alcohol (25:24:1). Samples were then mixed gently on an orbital shaker for 5 minutes. After mixing, tubes were centrifuged at 4°C for 4 minutes.
Approximately 500 µL of the supernatant was transferred into a new eppendorf tube containing 500 µL of isopropanol and 80 µL of 3 M sodium acetate at a pH of 5.3. Tubes were inverted once to mix the components and placed in -20°C for overnight incubation. Tubes were centrifuged at 14000 rpm at 4°C for 15 minutes. The supernatant was removed from each tube aspirating as much as possible and leaving the pellet at the base of the tube. Five hundred µL of cold 70% (V/V) ethanol was added to each tube and tubes were centrifuged for a further 5 minutes. Ethanol was tipped off and a second rinse with 70% ethanol was performed. The ethanol was discarded to leave pellets at the base of the tube. The tubes were transferred to a laminar flow cabinet where they were left to air dry. Once dry, DNA pellets were resuspended in 50 µL of TE buffer (10 mM Tris, 1 mM EDTA) and incubated at 45°C overnight. DNA samples were stored at -20°C until they were used.

DNA concentrations of extracts were determined by using gel electrophoresis and standards of known lambda DNA. A 0.7% agarose gel was prepared with the number of lanes depending on the comb size used: 15, 20 or 40 lanes. To prepare the gel, 100 ml of 1 x TAE buffer (40 mM Tris-acetate, 1 mM NaEDTA pH 8.0) was dispensed in a 250 ml erlenmeyer flask and 0.7 g of agarose was added. Agarose was dissolved in a microwave for one minute and left to cool. Five µL of 10 mg/ml ethidium bromide was added to the 100 ml of liquid gel. Gel was mixed and poured into a mould, making sure all bubbles were removed. The comb was inserted and the gel was left to set in a fume hood.

Before the gel could be run, DNA had to be thawed on ice. This helped preserve the DNA extracts. 2 µL of DNA was combined with 8 µL of 1x gel loading dye (1% (w/v) xylene cyanol, 1% (w/v) bromophenol blue and 50% (v/v) glycerol) on parafilm®. After mixing, samples were loaded into lane on gels. Once samples were loaded, the standards of lambda, 5 µL (50 ng) and 10 µL (100 ng), were included as the last two lanes. Electrophoresis was carried out for 40 minutes at 100V. DNA was visualised by exposure to UV light and a gel photo was taken.
Sample concentrations were determined by comparing the 50 ng and 100 ng markers to genomic DNA bands. If DNA concentration was too low, a sodium acetate/ethanol precipitation was performed followed by resuspension. If the DNA concentration was too high, more TE buffer was added to samples and they were resuspended. After these adjustments, the DNA concentration was checked again on a 0.7% agarose gel.

Opk4_1r 10-mer primers were obtained from Sigma-Aldrich as dried desalted nucleotides. The appropriate amount of sterile milli-Q water was added to the primers to bring the concentration to 100 µM. Primers were made up to 10 µM by pipetting 50 µL of the 100 µM stock and adding 450 µL of sterile milli-Q water. Table 3.2.2.1 details the primers (Dillon et al., 2006) that were used.

**Table 3.2.2.1** Primers for the SCAR marker, OPK4_1r (from Dillon et al., 2006).

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opk4_1r (Forward)</td>
<td>CCGCCCAAACCTGCGGAACAC</td>
</tr>
<tr>
<td>Opk4_1r (Reverse)</td>
<td>CCGCCCAAACCCCCCAAACCTAG</td>
</tr>
</tbody>
</table>
DNA samples 10x buffer, MgCl₂, 10 mM dNTPs, Opk4_1r forward and reverse primers were thawed on ice. A master mix was prepared in a 1.5 ml eppendorf tube to provide each reaction with 19 µL of master mix. Table 3.2.2.2 lists master mix composition (from Dillon et al., 2006).

**Table 3.2.2.2** Master mix details (Dillon et al., 2006).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume(µl) per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile Milli-Q water</td>
<td>13.3</td>
</tr>
<tr>
<td>10x NH₄ buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>2.0</td>
</tr>
<tr>
<td>dNTP’s (10mM) from Sigma®</td>
<td>0.5</td>
</tr>
<tr>
<td>Primer Opk4_1r Forward from Sigma® Genosys</td>
<td>0.5</td>
</tr>
<tr>
<td>Primer Opk4_1r Reverse from Sigma® Genosys</td>
<td>0.5</td>
</tr>
<tr>
<td>Stoffel Fragment (10U/µL) Amplitaq® from Applied Biosystems</td>
<td>0.2</td>
</tr>
<tr>
<td>Total Volume (µL)</td>
<td>19.0</td>
</tr>
</tbody>
</table>
PCR tubes (0.2 ml) were labelled and put into a rack on ice, and 19.0 µL of master mix were added to each tube, followed by 1.0 µL of DNA. Tubes were mixed by flicking and a thermocycler (PC-960C) was set at 94°C for a hot start. The PCR reaction was run following the conditions outlined in Table 3.2.2.3 (Dillon et al., 2006).

**Table 3.2.2.3** PCR reaction protocols for SCAR marker Opk4_1r (from Dillon et al., 2006).

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>Repeat steps 2-4 25 times</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>72</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

After the PCR reaction was finished, a 1% agarose gel was prepared with the number of lanes depending on the comb size used: 15, 20 or 40 lanes. To prepare the gel, 100 mls of 1x TAE buffer (40 mM Tris-acetate, 1 mM NaEDTA pH 8.0) was dispensed in a 250 ml erlenmeyer flask and 1 g of agarose was added. Agarose was dissolved in a microwave for one minute and left to cool. Five µL of 10 mg/ml ethidium bromide was added to the 100 ml of liquid gel. The gel was mixed and poured into a mould, making sure all bubbles were removed. The comb was inserted and the gel was left to set in a fume hood.
Two and a half µL of the PCR template was mixed with 10.5 µL of 1x gel loading dye (1% (w/v) xylene cyanole, 1% (w/v) bromophenol blue and 50% (v/v) glycerol) on parafilm®. Once samples were loaded, the standard of a 1KB DNA ladder (10µL) was included as the last lane. Electrophoresis was carried out for 40 minutes at 100V. DNA was visualised by exposure to UV light and a gel photo was taken. If the PCR reaction had worked, a band would appear at 372 bp.

If the band was present, CAPS analysis was conducted. Samples were treated with the Psi1k4 restriction enzyme to determine genotype. A master mix was prepared in a 1.5 ml eppendorf tube to provide each reaction with 32.5 µL of master mix. Table 3.2.2.4 outlines master mix composition for CAPS analysis (Dillon et al., 2006).

**Table 3.2.2.4** Master mix details for the CAPS marker Psi1k4 (from Dillon et al., 2006).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume(µl) per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>27.4</td>
</tr>
<tr>
<td>10x Buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>Psi1 enzyme</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>32.5</strong></td>
</tr>
</tbody>
</table>

The PCR templates which revealed a band were brought to a total volume of 50 µL by adding 32.5 µL of master mix on ice. The tubes were incubated at 37°C for 60 minutes. A 2% agarose gel was prepared with the number of lanes depending on the comb size used: 15, 20 or 40 lanes. To prepare the gel, 150 ml of 1x TAE buffer (40 mM Tris-acetate, 1 mM NaEDTA pH 8.0) was dispensed in a 250 ml erlenmeyer flask and 3 g of agarose was added. Agarose was dissolved in a microwave for one minute and left to cool. Seven and a half µL of 10 mg/ml ethidium bromide was added to the 150 ml volume of liquid gel. The gel was mixed and poured into a mould, making sure all bubbles were removed. The comb was inserted and the gel was left to set in a fume hood.
After incubation, 25 µL of the PCR reaction solution was mixed with 10 µL of 1x gel loading dye (1% (w/v) xylene cyanole, 1% (w/v) bromophenol blue and 50% (v/v) glycerol) on parafilm®. Once samples were loaded, the standard of a 1KB DNA ladder (10µL) was included as the last lane. Electrophoresis was carried out for 30 minutes at 100V. DNA was visualised by exposure to UV light and a gel photo was taken. Genotype was determined. If there were two bands, it was RR (homozygous resistant); one band it was rr (homozygous susceptible); and all three bands present, it was Rr (heterozygous resistant).
3.3 Results

3.3.1 *V. pubescens x V. parviflora [F₁]*

The Opk4_1r SCAR marker was successfully amplified from genomic DNA of all plants in the *V. pubescens x V. parviflora [F₁]* population (Figure 3.3.1.1). When digested with the restriction endonuclease *Psi₁*, all F₁ plants produced a triple band pattern whereas *V. pubescens* samples only produced two bands (Figure 3.3.1.2).

![Amplified bands of the Opk4_1r SCAR marker in V. pubescens x V. parviflora [F₁]](image)

**Figure 3.3.1.1** Amplified bands of the Opk4_1r SCAR marker in *V. pubescens x V. parviflora [F₁]*

1. *V. pubescens*  
2. *V. pubescens*  
3. *V. parviflora*  
4. *V. parviflora*  
5. *V. pubescens x V. parviflora [F₁]*  
6. *V. pubescens x V. parviflora [F₁]*  
7. *V. pubescens x V. parviflora [F₁]*  
8. *V. pubescens x V. parviflora [F₁]*  
9. *V. pubescens x V. parviflora [F₁]*  
10. *V. pubescens x V. parviflora [F₁]*  
11. *V. pubescens x V. parviflora [F₁]*  
12. *V. pubescens x V. parviflora [F₁]*  
13. *V. pubescens x V. parviflora [F₁]*  
14. *V. pubescens x V. parviflora [F₁]*  
15. *V. pubescens x V. parviflora [F₁]*  
16. *V. pubescens x V. parviflora [F₁]*  
17. *V. pubescens x V. parviflora [F₁]*  
18. *V. pubescens x V. parviflora [F₁]*  
19. *V. pubescens x V. parviflora [F₁]*  
20. 1Kb DNA ladder

Note: Samples 1-19 are different individuals.
Figure 3.3.1.2 Application of CAPS Psi1k4 marker to *V. pubescens* x *V. parviflora* [F₁].

1 *V. pubescens* x *V. parviflora* [F₁] 8 *V. pubescens* x *V. parviflora* [F₁]
2 *V. pubescens* 9 *V. pubescens* x *V. parviflora* [F₁]
3 *V. pubescens* 10 *V. pubescens* x *V. parviflora* [F₁]
4 *V. pubescens* 11 *V. pubescens* x *V. parviflora* [F₁]
5 *V. pubescens* 12 *V. pubescens* x *V. parviflora* [F₁]
6 *V. pubescens* 13 1Kb DNA ladder
7 *V. pubescens*

Note: Samples 1-12 are different individuals.
3.3.2 *V. pubescens* x *V. parviflora* [F₃]

The Opk4_1r SCAR marker was successfully amplified from genomic DNA of all plants in the *V. pubescens* x *V. parviflora* F₃ population (Figure 3.3.2.1). When digested with the restriction endonuclease *Psi*₁, all F₃ plants produced two bands (Figure 3.3.2.2).

![Figure 3.3.2.1 Amplified bands of the Opk4_1r SCAR marker in V. pubescens x V. parviflora [F₃]](image)

1. *V. parviflora*
2. *V. parviflora*
3. *V. pubescens*
4. *V. pubescens*
5. *V. pubescens* x *V. parviflora* [F₃]
6. *V. pubescens* x *V. parviflora* [F₃]
7. *V. pubescens* x *V. parviflora* [F₃]
8. *V. pubescens* x *V. parviflora* [F₃]
9. *V. pubescens* x *V. parviflora* [F₃]
10. *V. pubescens* x *V. parviflora* [F₃]
11. *V. pubescens* x *V. parviflora* [F₃]
12. *V. pubescens* x *V. parviflora* [F₃]
13. *V. pubescens* x *V. parviflora* [F₃]
14. 1Kb DNA ladder

Note: Samples 1-13 are different individuals.
Figure 3.3.2.2 Application of CAPS Psi1k4 marker to *V. pubescens* x *V. parviflora* [F3].

1 *V. parviflora*  
2 *V. parviflora*  
3 *V. pubescens*  
4 *V. pubescens*  
5 *V. pubescens* x *V. parviflora* [F3]  
6 *V. pubescens* x *V. parviflora* [F3]  
7 *V. pubescens* x *V. parviflora* [F3]  
8 *V. pubescens* x *V. parviflora* [F3]  
9 *V. pubescens* x *V. parviflora* [F3]  
10 *V. pubescens* x *V. parviflora* [F3]  
11 *V. pubescens* x *V. parviflora* [F3]  
12 *V. pubescens* x *V. parviflora* [F3]  
13 *V. pubescens* x *V. parviflora* [F3]  
14 *V. pubescens* x *V. parviflora* [F3]  
15 *V. pubescens* x *V. parviflora* [F3]  
16 1Kb DNA ladder

Note: Samples 1-15 are different individuals.
3.3.3 (V. pubescens x V. parviflora F₂ RR) to V. parviflora [BC₁]

The Opk4_1r SCAR marker was successfully amplified from genomic DNA of both plants in the (V. pubescens x V. parviflora F₂ RR) to V. parviflora population (Figure 3.3.3.1). When digested with the restriction endonuclease Psi1, both BC₁ plants produced triple bands (Figure 3.3.3.2).

![Figure 3.3.3.1](image)

**Figure 3.3.3.1** Amplified bands of the Opk4_1r SCAR marker in backcross population (V. pubescens x V. parviflora F₂ RR) to V. parviflora [BC₁] and [BC₂] populations.

1 V. parviflora
2 V. parviflora
3 V. pubescens
4 V. pubescens
5 V. pubescens
6 V. pubescens
7 V. pubescens
8 (V. pubescens x V. parviflora F₂ RR) to V. parviflora [BC₁]
9 (V. pubescens x V. parviflora F₂ RR) to V. parviflora [BC₁]
10 V. pubescens
11 {(V. pubescens x V. parviflora F₂ RR) to V. parviflora BC₁} to V. parviflora [BC₂]
12 {(V. pubescens x V. parviflora F₂ RR) to V. parviflora BC₁} to V. parviflora [BC₂]
13 {(V. pubescens x V. parviflora F₂ RR) to V. parviflora BC₁} to V. parviflora [BC₂]
14 {(V. pubescens x V. parviflora F₂ RR) to V. parviflora BC₁} to V. parviflora [BC₂]
15 1Kb DNA ladder

Note: Samples 1-14 are different individuals.
Figure 3.3.3.2 Application of CAPS Psi1k4 marker to (*V. pubescens* x *V. parviflora* F$_2$ RR) to *V. parviflora* [BC$_1$]

1 *V. parviflora*
2 *V. parviflora*
3 *V. pubescens*
4 *V. pubescens*
5 *V. pubescens*
6 *V. pubescens*
7 *V. pubescens*
8 (*V. pubescens* x *V. parviflora* F$_2$ RR) to *V. parviflora* [BC$_1$]
9 (*V. pubescens* x *V. parviflora* F$_2$ RR) to *V. parviflora* [BC$_1$]
10 *V. pubescens*
11 {(V. pubescens x V. parviflora F$_2$ RR) to V. parviflora BC$_1$} to *V. parviflora* [BC$_2$]
12 {(V. pubescens x V. parviflora F$_2$ RR) to V. parviflora BC$_1$} to *V. parviflora* [BC$_2$]
13 {(V. pubescens x V. parviflora F$_2$ RR) to V. parviflora BC$_1$} to *V. parviflora* [BC$_2$]
14 {(V. pubescens x V. parviflora F$_2$ RR) to V. parviflora BC$_1$} to *V. parviflora* [BC$_2$]
15 1Kb DNA ladder

Note: Samples 1-14 are different individuals.
3.3.4 \{(V. pubescens \times V. parviflora F_2 RR) to V. parviflora BC_1\} to V. parviflora [BC_2]

The Opk4_1r SCAR marker was successfully amplified from genomic DNA of all plants in the \{(V. pubescens \times V. parviflora F_2 RR) to V. parviflora BC_1\} to V. parviflora [BC_2] (Figure 3.3.3.1). When digested with the restriction endonuclease \textit{Psi}1, four BC_2 plants produced three bands and nine had a single band (Figure 3.3.3.2).

3.3.5 \textit{Vasconcellea} species and hybrids (\textit{V. quercifolia}, \textit{V. stipulata}, \textit{V. cauliflora}, \textit{V. goudotiana} and \textit{C. papaya} \times \textit{V. quercifolia} [F_1])

The Opk4_1r SCAR marker was successfully amplified from genomic DNA of all \textit{Vasconcellea} species and the hybrid cross between \textit{C. papaya} \times \textit{V. quercifolia} F_1 (Figure 3.3.5.1). When digested with the restriction endonuclease \textit{Psi}1, all species and hybrid crosses produced a double except \textit{V. parviflora} which produced a single band (Figure 3.3.5.2). The different species showed bands of different sizes.
Figure 3.3.5.1 Amplified bands of the Opk4_1r SCAR marker in *Vasconcellea* spp. (*V. quercifolia*, *V. stipulata*, *V. cauliflora*, *V. goudotiana* and *C. papaya x V. quercifolia [F1]*)

1. *V. pubescens*
2. *V. pubescens*
3. *V. quercifolia*
4. *V. quercifolia*
5. *V. cauliflora*
6. *V. cauliflora*
7. *V. stipulata*
8. *V. stipulata*
9. *V. goudotiana*
10. *V. goudotiana*
11. *C. papaya x V. quercifolia [F1] (410)*
12. *C. papaya x V. quercifolia [F1] (410)*
13. *C. papaya x V. quercifolia [F1] (468)*
14. *C. papaya x V. quercifolia [F1] (468)*
15. *C. papaya x (V. pubescens x V. parviflora F2)*
16. *C. papaya x (V. pubescens x V. parviflora F2)*
17. *C. papaya x (V. pubescens x V. parviflora F2)*
18. *C. papaya x (V. pubescens x V. parviflora F2)*
19. *C. papaya x (V. pubescens x V. parviflora F2)*
20. 1Kb DNA ladder
21. *C. papaya x (V. pubescens x V. parviflora F2)*
22. *C. papaya x (V. pubescens x V. parviflora F2)*
23. *C. papaya x (V. pubescens x V. parviflora F2)*
24. *C. papaya x (V. pubescens x V. parviflora F2)*
25. *C. papaya x (V. pubescens x V. parviflora F2)*
26. *V. parviflora*
27. *V. parviflora*
28. *C. papaya x (V. pubescens x V. parviflora F2)*
29. *C. papaya x (V. pubescens x V. parviflora F2)*
30. *C. papaya x (V. pubescens x V. parviflora F2)*
31. *C. papaya x (V. pubescens x V. parviflora F2)*
32. *C. papaya x (V. pubescens x V. parviflora F2)*
33. *C. papaya x (V. pubescens x V. parviflora F2)*
34. *C. papaya x (V. pubescens x V. parviflora F2)*
35. *C. papaya x (V. pubescens x V. parviflora F2)*
36. *C. papaya x (V. pubescens x V. parviflora F2)*
37. *C. papaya x (V. pubescens x V. parviflora F2)*
38. *C. papaya x (V. pubescens x V. parviflora F2)*
39. *C. papaya x (V. pubescens x V. parviflora F2)*
40. 1Kb DNA ladder

Note: Samples 1-14 are different individuals.
Figure 3.3.5.2 Application of CAPS Psi1k4 marker to Vasconcellea spp. (V. quercifolia, V. stipulata, V. cauliflora, V. goudotiana and C. papaya x V. quercifolia [F₁]).

1 V. pubescens
2 V. pubescens
3 V. quercifolia
4 V. quercifolia
5 V. cauliflora
6 V. cauliflora
7 V. stipulata
8 V. stipulata
9 V. goudotiana
10 V. goudotiana
11 C. papaya x V. quercifolia [F₁] (410)
12 C. papaya x V. quercifolia [F₁] (410)
13 C. papaya x V. quercifolia [F₁] (468)
14 C. papaya x V. quercifolia [F₁] (468)
15 C. papaya x (V. pubescens x V. parviflora F₂)
16 C. papaya x (V. pubescens x V. parviflora F₂)
17 C. papaya x (V. pubescens x V. parviflora F₂)
18 C. papaya x (V. pubescens x V. parviflora F₂)
19 C. papaya x (V. pubescens x V. parviflora F₂)
20 1Kb DNA ladder
21 C. papaya x (V. pubescens x V. parviflora F₂)
22 C. papaya x (V. pubescens x V. parviflora F₂)
23 C. papaya x (V. pubescens x V. parviflora F₂)
24 C. papaya x (V. pubescens x V. parviflora F₂)
25 C. papaya x (V. pubescens x V. parviflora F₂)
26 V. parviflora
27 V. parviflora
28 C. papaya x (V. pubescens x V. parviflora F₂)
29 C. papaya x (V. pubescens x V. parviflora F₂)
30 C. papaya x (V. pubescens x V. parviflora F₂)
31 C. papaya x (V. pubescens x V. parviflora F₂)
32 C. papaya x (V. pubescens x V. parviflora F₂)
33 C. papaya x (V. pubescens x V. parviflora F₂)
34 C. papaya x (V. pubescens x V. parviflora F₂)
35 C. papaya x (V. pubescens x V. parviflora F₂)
36 C. papaya x (V. pubescens x V. parviflora F₂)
37 C. papaya x (V. pubescens x V. parviflora F₂)
38 C. papaya x (V. pubescens x V. parviflora F₂)
39 C. papaya x (V. pubescens x V. parviflora F₂)
40 1Kb DNA ladder
3.3.6 C. papaya genotypes tested with Opk4_1r marker

The Opk4_1r SCAR marker was successfully amplified from genomic DNA of six C. papaya genotypes (Figure 3.3.6.1). When digested with the restriction endonuclease *Psi1*, all genotypes produced a single band, just like *V. parviflora* (Figure 3.3.6.2).

![Figure 3.3.6.1 Amplified bands of the Opk4_1r SCAR marker in C. papaya](image)

1. *V. parviflora*
2. *V. parviflora*
3. *V. pubescens*
4. *V. pubescens*
5. C. papaya – K1
6. C. papaya – K1
7. C. papaya - 5648
8. C. papaya - 5648
9. C. papaya - 70
10. C. papaya - 70
11. C. papaya - 35
12. C. papaya - 35
13. C. papaya - 97
14. C. papaya - 97
15. C. papaya - C3
16. C. papaya - C3
17. 1Kb DNA ladder

372bp
**Figure 3.3.6.2** Application of CAPS Psil1k4 marker to *C. papaya*.

1 *V. parviflora*
2 *V. parviflora*
3 *C. papaya*
4 *C. papaya*
5 *V. pubescens*
6 *V. pubescens*
7 *V. pubescens*
8 *V. pubescens*
9 *V. pubescens*
10 *C. papaya* - 5648
11 *C. papaya* – K1
12 *C. papaya* - 97
14 *C. papaya* - 35
15 1Kb DNA ladder
3.3.7 *C. papaya* x *V. pubescens* [F₁] and *C. papaya* x *V. parviflora* [F₁]

The Opk4_1r SCAR marker was successfully amplified from genomic DNA of all plants in the *C. papaya* x *V. pubescens* [F₁] population (Figure 3.3.7.1). When digested with the restriction endonuclease *Psi*₁, all F₁ plants produced double bands (Figure 3.3.7.2).

**Figure 3.3.7.1** Amplified bands of the Opk4_1r SCAR marker in *C. papaya* x *V. pubescens* [F₁] and *C. papaya* x *V. parviflora* [F₁]

1 *V. pubescens* 11 *C. papaya* x *V. pubescens* [F₁]
2 *V. pubescens* 12 *C. papaya* x *V. pubescens* [F₁]
3 *V. parviflora* 13 *C. papaya* x *V. pubescens* [F₁]
4 *V. parviflora* 14 *C. papaya* x *V. parviflora* [F₁]
5 *C. papaya* x *V. pubescens* [F₁] 15 *C. papaya* x *V. parviflora* [F₁]
6 *C. papaya* x *V. pubescens* [F₁] 16 *C. papaya* x *V. parviflora* [F₁]
7 *C. papaya* x *V. pubescens* [F₁] 17 *C. papaya* x *V. parviflora* [F₁]
8 *C. papaya* x *V. pubescens* [F₁] 18 *C. papaya* x *V. parviflora* [F₁]
9 *C. papaya* x *V. pubescens* [F₁] 19 *C. papaya* x *V. parviflora* [F₁]
10 *C. papaya* x *V. pubescens* [F₁] 20 1Kb DNA ladder
Figure 3.3.7.2 Application of CAPS Psi1k4 marker to *C. papaya* x *V. pubescens* [F₁] and
*C. papaya* x *V. parviflora* [F₁].

1. *V. pubescens*
2. *V. pubescens*
3. *V. parviflora*
4. *V. parviflora*
5. *C. papaya* x *V. pubescens* [F₁]
6. *C. papaya* x *V. pubescens* [F₁]
7. *C. papaya* x *V. pubescens* [F₁]
8. *C. papaya* x *V. pubescens* [F₁]
9. *C. papaya* x *V. pubescens* [F₁]
10. *C. papaya* x *V. pubescens* [F₁]
11. *C. papaya* x *V. pubescens* [F₁]
12. *C. papaya* x *V. parviflora* [F₁]
13. *C. papaya* x *V. parviflora* [F₁]
14. *C. papaya* x *V. parviflora* [F₁]
15. *C. papaya* x *V. parviflora* [F₁]
16. *C. papaya* x *V. parviflora* [F₁]
17. *C. papaya* x *V. parviflora* [F₁]
18. 1Kb DNA ladder
3.3.8 *C. papaya* x *V. parviflora [F₁]*

The Opk4_1r SCAR marker was successfully amplified from genomic DNA of all plants in the *C. papaya* x *V. parviflora [F₁]* population (Figure 3.3.7.1). When digested with the restriction endonuclease * Psi*₁, all F₁ plants produced double bands (Figure 3.3.7.2).

3.3.9 *C. papaya* x (*V. pubescens* x *V. parviflora F₂ containing RR allele for PRSV-P resistance*)

The Opk4_1r SCAR marker was successfully amplified from genomic DNA of all plants in the *C. papaya* x (*V. pubescens* x *V. parviflora F₂ RR*) population (Figure 3.3.9.1). When digested with the restriction endonuclease * Psi*₁, 100 plants produced double bands and 10 plants produced single bands (Figures 3.3.5.2, 3.3.9.2 and 3.3.9.3).

The following gels (Figures 3.3.9.1, 3.3.9.2 and 3.3.9.3) are just a sample of the population of 110 plants.
Figure 3.3.9.1 Amplified bands of the Opk4_1r SCAR marker in C. papaya x (V. pubescens x V. parviflora F2).

1. V. pubescens  
2. V. pubescens  
3. C. papaya x (V. pubescens x V. parviflora F2)  
4. C. papaya x (V. pubescens x V. parviflora F2)  
5. V. parviflora  
6. V. parviflora  
7. C. papaya x (V. pubescens x V. parviflora F2)  
8. C. papaya x (V. pubescens x V. parviflora F2)  
9. C. papaya x (V. pubescens x V. parviflora F2)  
10. C. papaya x (V. pubescens x V. parviflora F2)  
11. C. papaya x (V. pubescens x V. parviflora F2)  
12. C. papaya x (V. pubescens x V. parviflora F2)  
13. C. papaya x (V. pubescens x V. parviflora F2)  
14. C. papaya x (V. pubescens x V. parviflora F2)  
15. C. papaya x (V. pubescens x V. parviflora F2)  
16. C. papaya x (V. pubescens x V. parviflora F2)  
17. C. papaya x (V. pubescens x V. parviflora F2)  
18. C. papaya x (V. pubescens x V. parviflora F2)  
19. C. papaya x (V. pubescens x V. parviflora F2)  

21. C. papaya x (V. pubescens x V. parviflora F2)  
22. C. papaya x (V. pubescens x V. parviflora F2)  
23. C. papaya x (V. pubescens x V. parviflora F2)  
24. C. papaya x (V. pubescens x V. parviflora F2)  
25. C. papaya x (V. pubescens x V. parviflora F2)  
26. C. papaya x (V. pubescens x V. parviflora F2)  
27. C. papaya x (V. pubescens x V. parviflora F2)  
28. C. papaya x (V. pubescens x V. parviflora F2)  
29. C. papaya x (V. pubescens x V. parviflora F2)  
30. C. papaya x (V. pubescens x V. parviflora F2)  
31. C. papaya x (V. pubescens x V. parviflora F2)  
32. C. papaya x (V. pubescens x V. parviflora F2)  
33. C. papaya x (V. pubescens x V. parviflora F2)  
34. C. papaya x (V. pubescens x V. parviflora F2)  
35. C. papaya x (V. pubescens x V. parviflora F2)  
36. C. papaya x (V. pubescens x V. parviflora F2)  
37. C. papaya x (V. pubescens x V. parviflora F2)  
38. C. papaya x (V. pubescens x V. parviflora F2)  
39. C. papaya x (V. pubescens x V. parviflora F2)  
40. 1Kb DNA ladder
Figure 3.3.9.2 Application of CAPS *Psi1k4* marker to *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$).

1 *V. pubescens*  
2 *V. pubescens*  
3 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
4 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
5 *V. parviflora*  
6 *V. parviflora*  
7 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
8 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
9 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
10 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
11 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
12 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
13 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
14 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
15 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
16 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
17 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
18 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
19 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
20 1Kb DNA ladder  
21 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
22 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
23 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
24 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
25 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
26 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
27 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
28 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
29 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
30 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
31 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
32 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
33 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
34 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
35 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
36 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
37 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
38 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
39 *C. papaya* x (*V. pubescens* x *V. parviflora* F$_2$)  
40 1Kb DNA ladder
**Figure 3.3.9.3** Application of CAPS Psi1k4 marker to *C. papaya* x (*V. pubescens* x *V. parviflora* F2)

1. *V. parviflora*
2. *V. parviflora*
3. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
4. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
5. *V. pubescens*
6. *V. pubescens*
7. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
8. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
9. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
10. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
11. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
12. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
13. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
14. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
15. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
16. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
17. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
18. *C. papaya* x (*V. pubescens* x *V. parviflora* F2)
19. 1Kb DNA ladder
Table 3.3.1 Results from application of the CAPS \( \text{Psi}1k4 \) marker to \textit{Vasconcellea} spp., \textit{C. papaya} and their hybrids.

<table>
<thead>
<tr>
<th>Plant ID</th>
<th>Marker Present/Absent</th>
<th>Homozygous Resistant (RR) as shown by \text{CAPS}</th>
<th>Homozygous Susceptible (rr) as shown by \text{CAPS}</th>
<th>Heterozygous resistant (Rr) as shown by \text{CAPS}</th>
<th>Expected Mendelian Segregation</th>
<th>Resistant or Susceptible PRSV ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V. \text{pubescens} \times V. \text{parviflora} ) [( \text{F}_1 )]</td>
<td>P</td>
<td>(17 plants)</td>
<td>1:1(Rr)</td>
<td>1:1 Resistant</td>
<td>Not field tested</td>
<td></td>
</tr>
<tr>
<td>( V. \text{pubescens} \times V. \text{parviflora} ) [( \text{F}_2 )]</td>
<td>P</td>
<td></td>
<td>1:1(RR):2(Rr)</td>
<td>3:1 Resistant : Susceptible*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V. \text{parviflora} ) [( \text{BC}_1 )] (Sib cross from F2 RR)</td>
<td>P</td>
<td>(50 plants)</td>
<td>1:1(RR)</td>
<td>1:1Resistant</td>
<td>Not field tested</td>
<td></td>
</tr>
<tr>
<td>( (V. \text{pubescens} \times V. \text{parviflora} ) [( \text{F}_2 ) RR to V. \text{parviflora}[( \text{BC}_1 )] ( ) )</td>
<td>P</td>
<td>(2 plants)</td>
<td>1:1(Rr)</td>
<td>1:1Resistant</td>
<td>Not field tested</td>
<td></td>
</tr>
<tr>
<td>( (V. \text{parviflora} ) [( \text{F}_2 ) RR] to V. \text{parviflora}[( \text{BC}_1 )] ( ) )</td>
<td>P</td>
<td>(9 plants)</td>
<td>1(rr):1(Rr)</td>
<td>1:1Resistant : Susceptible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V. \text{parviflora} )</td>
<td>P</td>
<td></td>
<td></td>
<td>Susceptible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V. \text{pubescens} )</td>
<td>P</td>
<td></td>
<td></td>
<td>Resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V. \text{cauliflora} )</td>
<td>P</td>
<td></td>
<td></td>
<td>Resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V. \text{goudotiana} )</td>
<td>P</td>
<td></td>
<td></td>
<td>All plants susceptible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V. \text{quercifolia} )</td>
<td>P</td>
<td></td>
<td></td>
<td>All plants resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V. \text{stipulata} )</td>
<td>P</td>
<td></td>
<td></td>
<td>Not field tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{C. papaya} ) (All 6 genotypes)</td>
<td>P</td>
<td></td>
<td></td>
<td>Susceptible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{C. papaya} \times V. \text{parviflora} ) [( \text{F}_1 )]</td>
<td>P</td>
<td>(10 plants)</td>
<td></td>
<td>Susceptible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{C. papaya} \times V. \text{quercifolia} ) [( \text{F}_1 )]</td>
<td>P</td>
<td>(2 plants)</td>
<td></td>
<td>3:1 Resistant : Susceptible#</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{C. papaya} \times V. \text{pubescens} ) [( \text{F}_1 )]</td>
<td>P</td>
<td>(10 plants)</td>
<td></td>
<td>Not field tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{C. papaya} \times (V. \text{pubescens} \times V. \text{parviflora} ) F2 RR)</td>
<td>P</td>
<td>(100 plants)</td>
<td>(10 plants)</td>
<td>Not field tested</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(*PRSV-P segregation as per Dillon et al., 2005a). □ Plant present in this category.
(# PRSV-P segregation as per Drew et al., 1998).
3.4 Discussion

Despite numerous attempts to transfer PRSV-P resistance from wild *Vasconcellea* species to *C. papaya* for more than 50 years, only limited success has been achieved to date and most researchers have not proceeded past the F1 intergeneric hybrid stage (Manshardt and Drew, 1998). However, two recent breakthroughs have increased chances that this transfer is possible. First, fertility has been achieved in F1 intergeneric hybrids between *C. papaya* and *V. quercifolia* (Drew et al., 2006a) and backcross generations developed from these F1 hybrids have expressed traits of the *V. quercifolia* parent including PRSV-P resistance (Drew et al., 2006b). Second, PRSV-P resistance in both *V. pubescens* and *V. cauliflora* has been shown to be regulated by a single gene, prsv-1 (Magdalita et al., 1997; Dillon et al., 2006). A SCAR marker, Opk4_1r, which was converted into a CAPS marker, Psilk4, was shown to be diagnostic for the three alleles of prsv-1 (Dillon et al., 2006).

The application of this SCAR and CAPS marker to marker-assisted breeding as recommended by Dillon et al. (2006) has been conducted in the current research for intrageneric populations of *V. pubescens x V. parviflora* [F2] and [F3] populations produced from the *V. pubescens* and *V. parviflora* [F1] and [BC1] and [BC2] generations resulting from *V. pubescens x V. parviflora* [F2] RR plants backcrossed to *V. parviflora*. Expected Mendelian segregation ratios were obtained for all hybrids and backcross generations. The markers facilitated the selection of resistant homozygous (RR) or heterozygous (Rr) plants without the labour-intensive PRSV-P screening in the glasshouse and the field. An added benefit was that the markers could be applied to small quantities of DNA extracted from leaves in the *in vitro* stage after embryo rescue. Thus plants with the required alleles of the resistance gene could be retained, micropropagated and acclimatised, while others could be discarded. As a result, plants containing the RR allele were selected from the F2 population and used to produce a F3 generation and to produce BC1 and BC2 populations. These results showed that it was possible to produce BC2 plants with the Rr genotype for PRSV-P resistance and that after
two or three more backcrosses, a PRSV-P resistant *V. parviflora* genotype could be produced.

The Opk4_1r SCAR marker was amplified in all *Vasconcellea* species that were tested, however, the CAPS marker was not consistent in identifying the genotype of *prsv-1* in crosses involving *Vasconcellea* species other than *V. pubescens* and *V. parviflora*. For example, the SCAR marker from *V. goudotiana* was digested by the *Psi*I restriction enzyme and produced two bands; however, *V. goudotiana* is susceptible to PRSV-P (rr). In addition, progeny of *C. papaya* (rr) and *V. parviflora* (rr), *V. quercifolia* (RR) and *V. pubescens* (RR) produced two bands when digested by the restriction enzyme which indicated the RR and not rr for *C. papaya* (rr) x *V. parviflora* (rr) [F1] and Rr for *C. papaya* (rr) x *V. quercifolia* (RR) [F1] and *C. papaya* (rr) x *V. pubescens* (RR) [F1] as would be expected. Similarly, when *C. papaya* (rr) was crossed to *V. pubescens* (RR) x *V. parviflora* (rr) [F2] RR, the CAPS marker identified 100 plants as being RR and 10 as being rr instead of all being Rr. It is likely that DNA sequences of the SCAR vary among the *Vasconcellea* species, thus other restriction sites and refined markers should be developed for crosses involving the other species. This is confirmed by the detection of similar band sizes in the SCAR marker for *V. pubescens* (RR), *V. cauliflora* and *V. goudotiana* (rr) and was consistent with the report of Dillon et al. (2006) who measured fragment sizes of 360, 360 and 361 bps, respectively, for these species and 372 and larger fragment lengths for *V. parviflora* (rr), *V. quercifolia* (RR) and *V. stipulata* (rr).

Previously, there have been conflicting reports on the Opk4_1r SCAR marker being absent (Dillon et al., 2005b) and present (Dillon et al., 2006) in *C. papaya*. In the current research the SCAR marker was present in six varieties of *C. papaya* (rr) that varied in their worldwide distribution; and was not digested by the restriction enzyme as expected for this R genotype.
The observed results confirm that a PRSV-P resistant *V. parviflora* (rr) could be produced by backcrossing the PRSV-P resistance allele from *V. pubescens* x *V. parviflora* Rr hybrids back to *V. parviflora* (rr). It has been reported previously that *V. parviflora* is more closely related to *C. papaya* than other *Vasconcellea* species (Jobin et al., 1997) and fertile progeny were produced when *C. papaya* was crossed to *V. parviflora* (Drew et al., 1998). Thus, it is proposed that PRSV-P resistance can be transferred from *V. pubescens* to *C. papaya* using *V. parviflora* as a bridging species and this transfer could be facilitated by SCAR and CAPS markers modified from those reported by Dillon et al. (2006).

Future research could be carried out to develop new markers (such as CAPS, SSR, or SNP) for *Vasconcellea* x *C. papaya* hybrids as *Psi1k4* is not a reliable marker for genotype selection in crosses with *C. papaya*.

Sequencing of the amplified SCAR marker fragments from each species would be a useful first step in identifying sequence differences on which to develop diagnostic markers able to trace the introgression of species-specific R alleles from *Vasconcellea* species into *C. papaya*. Such markers could then be applied to the populations developed in this described work and subsequent backcross (to *C. papaya*) populations to result in new PRSV-P resistant papaya cultivars with one or more R alleles from *Vasconcellea* species.
Appendix A

Drew and Smith Media (1986)

(100 X) Mineral #3 Stock Solution

<table>
<thead>
<tr>
<th>Mineral – Macroulements</th>
<th>(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_4$NO$_3$</td>
<td>160.100</td>
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<tr>
<td>KNO$_3$</td>
<td>101.110</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$.2H$_2$O</td>
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</tr>
<tr>
<td>CaCl$_2$</td>
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<tr>
<td>EDTAFeNa</td>
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<td>MgSO$_4$.7H$_2$O</td>
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<table>
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<th>Microelements</th>
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<tbody>
<tr>
<td>H$_3$BO$_3$</td>
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<tr>
<td>MnSO$_4$.4H$_2$O</td>
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<td>ZnSO$_4$.7H$_2$O</td>
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<td>CuSO$_4$.5H$_2$O</td>
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<td>CoCl$_2$.6H$_2$O</td>
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<td>KI</td>
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(10 X) Vitamin #6 Stock Solution

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<td>Nicotinic Acid</td>
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<tr>
<td>Pyridoxine HCL</td>
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<tr>
<td>Thiamine HCL</td>
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<tr>
<td>Biotin (heat)</td>
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<tr>
<td>Folic Acid (heat)</td>
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<td>Ca-Pantothenate</td>
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<tr>
<td>Riboflavin (heat)</td>
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<tr>
<td>Ascorbic Acid</td>
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<tr>
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<tr>
<td>Glycine</td>
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<tr>
<td>L. Cysteine HCL</td>
<td>0.1894</td>
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Drew and Smith Media (1986)

(100 X) Mineral #2 Stock Solution

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<td>CaCl$_2$</td>
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<td>EDTAFeNa</td>
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<td>MgSO$_4$.7H$_2$O</td>
<td>36.975</td>
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Microelements

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<td>(NH$_4$)$_6$Mo$<em>7$O$</em>{24}$.4H$_2$O</td>
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(10 X) Vitamin #5 Stock Solution

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<td>Folic Acid (heat)</td>
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Brewbaker and Kwack Medium (1963)

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<thead>
<tr>
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<th>Concentration</th>
</tr>
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<tbody>
<tr>
<td>H$_3$BO$_3$</td>
<td>1.6$\mu$M</td>
</tr>
<tr>
<td>Ca(NO$_3$)$_2$.4H$_2$O</td>
<td>1.3$\mu$M</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>8$\mu$M</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10g/100mls</td>
</tr>
<tr>
<td>Agar</td>
<td>1g/100mls</td>
</tr>
</tbody>
</table>
References


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