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Genetics of *Erwinia* resistance in *Zantedeschia*:
impact of plastome-genome incompatibility

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Voor mijn pa:
Wat je wat leuk is?
Pletten!

Wat je zoekt is niet te vinden
Wat je vindt niet wat je zocht
(Huub van der Lubbe)

Contents

1 General introduction

Ever since its introduction in 1731 (Bryan, 1989), the classic white calla lily has been appreciated world-wide as an ornamental crop. Scientifically known as *Zantedeschia aethiopica*, it had been the only cultivated representative of the genus *Zantedeschia* for a century. Since exploration of the Cape Peninsula, introduction of other *Zantedeschia* spp. followed around the middle of the nineteenth century, making available a wider range of flower colours. However, the types with coloured flowers are more sensitive on their growing conditions than *Z. aethiopica* and are (therefore) more susceptible to disease.

1.1 Zantedeschia

As a member of the *Araceae* family, the genus *Zantedeschia* is endemic to southern Africa and consists of two sections (Letty, 1973; Singh et al., 1996). The section *Zantedeschia* comprises of *Z. aethiopica* L. and *Z. odorata* Perry, while the section *Aestivae* consists of around six species: *Z. rehmannii* Engl., *Z. jucunda* Letty, *Z. elliotiana* (Watson) Engl., *Z. pentlandii* (Watson) Whittm., *Z. albomaculata* (Hook) Baill. and *Z. valida* (Letty) Y. Singh (Singh et al., 1996). All *Zantedeschia* spp. are diploid with 32 chromosomes (Yao et al., 1994b). Cultivars have been bred for ornamental value of either the flowers or the entire plants mainly from *Z. rehmannii*, *Z. albomaculata*, *Z. elliotiana* and *Z. pentlandii*. Most cultivars are propagated vegetatively, although some are F_1 hybrid cultivars, thus propagated by seeds.

Zantedeschia spp. (Figure 1.1) have a tuberous (section *Aestivae*) or rhizomatomous (section *Zantedeschia*) storage organ (Singh, 1996), are frost tender and, except for *Z. aethiopica*, require a period of dormancy (Funnell, 1993; Naor and Kigel, 2002). *Z. aethiopica* is an evergreen, but will become dormant if its environment limits growth. Therefore, storage organs of all *Zantedeschia* spp. are lifted after onset of dormancy and are stored in a growth-limiting environment.

Figure 1.1. a: Z. aethiopica*; b:* Z. rehmannii*; c:* Z. albomaculata *subsp.* albomaculata*; d:* Z. elliotiana

1.2 Erwinia carotovora

Soft rot caused by *Erwinia carotovora* subsp. *carotovora* (*Ecc*; syn. *Pectobacterium carotovorum* subsp. *carotovorum*) is an important disease of *Zantedeschia* spp. and the major disease in the section *Aestivae* (Funnell, 1993; Kuehny, 2000; Wright et al., 2002). This pathogen is a gram-negative necrotrophe, is widely distributed in both the temperate and tropical zones and has a wide range of host plants. It is a facultative anaerobic that causes maceration and rotting of parenchymatous tissue of the affected organ (Figure 1.2), resulting in loss of the whole plant (Pérombelon and Kelman, 1980; Pérombelon and Salmond, 1995). Much is known on pathogenesis and infection of soft rot on potato, which can be used as a basis for understanding soft rot on *Zantedeschia* spp.

Infections can be latent and bacteria have the ability to wait for optimal conditions, which makes them opportunistic pathogens (Toth et al., 2003). Bacteria are present in lumina, in lenticels and wounds and possibly, to a lesser extent, in vascular tissue (Pérombelon, 2002). Bacterial virulence determinants are stimulated by oxygen and nitrogen availability, temperature, osmolarity, iron deprivation, growth phase, catabolite repression, plant degeneration intermediates, DNA-damaging agents and presumably other yet unknown factors (Toth et al., 2003). An important factor for initiation of pathogenicity is 'quorum sensing' (Jones et al., 1993; Pirhonen et al., 1993; Fuqua et al., 1994). Bacteria constitutively express a signal molecule called N-(3-oxohexanoyl)-L-homoserine lactone (OHHL). The disease initiates at a threshold level (quorum) of OHHL that is believed to be around 10^6 bacteria per ml (Toth et al., 2003).

Figure 1.2. Plant that has collapsed after infection by Erwinia carotovora *subsp.* carotovora.

The most important pathogenicity factors are cell wall degrading enzymes, such as pectin lyase, pectin methyl esterase, pectate lyase, polygalacturonase, cellulase and protease (Pérombelon, 2002; Toth et al., 2003). These enzymes cleave structural pectic polymers and the middle lamella of the cell wall, enabling the penetration and colonisation of the tissue (Pérombelon and Salmond, 1995; Expert, 1999), but resulting in fully macerated plant tissue and leading to plant death.

The genus *Erwinia* was revised in a phylogenic study of the *Enterobacteriaceae* by Hauben et al. (1998), where *E. carotovora* and its subspecies were placed in the genus *Pectobacterium.* The most well-known and broadly used denomination, *E. carotovora*, will be used in this thesis and *P. carotovorum* will be regarded as a synonym.

1.2.1 Infection

Infection can take place in various ways. Tubers can become infected in storage or may be infected when planted in contaminated soil. Bacteria can overwinter in the soil on plant residues (Pérombelon and Kelman, 1980) and may spread via water (also aerosols) or via insects and handling. Furthermore, bacteria can be present in waterbodies such as lakes,

rivers and ponds (Pérombelon and Salmond, 1995; Norman et al., 2003). Bacteria present on or in the soil or already on the plant enter plant tissue via wounds, for example after flower harvest. Subsequent leaf tissue from diseased plants is an inoculum source for the crop.

During periods of stress, mainly plants of the section *Aestivae* are susceptible to soft rot. These stress periods arise during high temperature, high relative humidity, high nitrogen content of the soil or high moisture of the soil (low soil aeration) (Funnell and MacKay, 1999; Van Leeuwen et al., 2001). Infections of *Pythium* and *Rhizoctonia* have also been reported to be followed by *Erwinia* (Wright, 1994). Plants are vulnerable in particular under low oxygen conditions, as they are less efficient in their wound healing and pathogen defence responses, while the bacteria are not affected due to their facultative anaerobic nature (Pérombelon, 2002; Toth et al., 2003).

1.2.2 Soft rot control

A number of studies have been undertaken to assess the possibilities in restraining soft rot. These can be divided in studies on biological and chemical control, and on cultivation measures.

Biological control

Some measures involved in biological control could decrease soft rot incidence. These included steam treatment (Afek and Orenstein, 2002) or hot water treatment (McIntyre et al., 1978; Ranganna et al., 1998) before tuber storage (in potato). Additionally, some micro-organisms have been described to have an inhibitory effect to *E. carotovora* on potato tubers, namely *E. herbicola* (Vanneste et al., 1998) and *Pseudomonas fluorescens* (Cronin et al., 1997).

Chemical control

A number of chemicals have been described with an inhibitory effect on *E. carotovora*. These include mainly hazardous substances such as paracetic acid, sodium hypochlorite (Dosdall, 1955; Corr et al., 1993) and copper-based chemicals (Blom and Brown, 1999; Tzeng et al., 1999; Gracia-Garza et al., 2002). The antibiotic streptomycin (Farag et al.,

1982; Corr et al., 1993; Kuehny et al., 1998) could diminish disease incidence. All these chemicals are not practical due to their safety hazard. According to Funnell and MacKay (1999), there is no effective chemical control against soft rot.

Cultivation measures

Biological and chemical measures have been successfully applied in soft rot control, but only to a limited extent. Therefore, most promising are cultivation measures, since some methods did have success in decreasing disease incidences in *Zantedeschia* spp. These include mulching (Welsh and Clemens, 1992; Wright and Burge, 2000), early cessation of irrigation (Wright et al., 2002), application of rockwool (Mori et al., 1999), course river sand (Welsh and Clemens, 1992) or combined application of peatmoss, vermiculite and perlite as growing media (Chen and Lin, 2000), decreased nitrogen fertilisation (Van Leeuwen et al., 2001) or tuber curing (Wright, 1994) and fertilisation by gypsum (Funnell and MacKay, 1999). Unfortunately, none of these measures alone could fully control soft rot. Therefore, combining protective methods could result in better control of soft rot. Indeed, (Welsh and Clemens, 1992) claimed to have diminished losses down to 5% by a combination of protective methods.

The cultivation measures appear most promising, since they are durable. Sensitivity to chemicals could be overcome by the pathogen, and use of chemicals is being restricted by international legislation. Biological control measures also are promising. However, hot water treatment can become expensive due to its intensive energy use. More knowledge is needed on use of micro-organisms against *Ecc* before these can be applied.

Resistance of *Zantedeschia* spp. to soft rot has received only little attention. Conceivably, use of resistant cultivars would decrease the impact of soft rot and ease the cultivation of *Zantedeschia* spp. considerably. Combined use of resistant cultivars and protective cultivation measures could help in controlling soft rot even more.

1.3 Host resistance to soft rot

Plants have many different strategies to defend themselves against pathogens. Knowledge on resistance to soft rot in plants and resistance mechanisms in general is needed to define an approach to increasing resistance to soft rot in *Zantedeschia* spp.

1.3.1 Hypersensitivity response

The most common feature associated with active host resistance in general is the hypersensitive response (HR). This type of resistance is in bacteria associated with *hrp* genes (hypersensitive response and pathogenesis) that are required for production and secretion of avirulence gene (Avr) products by the pathogen. Plants with corresponding R genes (resistance) can recognise these products and initiate HR by strategies as local cell or tissue death (gene-for-gene action hypothesis; (Alfano and Collmer, 1996).

Ecc is not able to elicit HR (Chatterjee et al., 2002). Paradoxically, (Cui et al., 1996) found that a wild-type strain of *Ecc* did possess an analogue of *E. chrysanthemi-hrp* genes. The production of harpin by *Ecc* is activated by the quorum sensing signal, but is possibly occurring at a level not high enough to elicit HR (Mukherjee et al., 1997). However, harpin was required for symptom production on host *Arabidopsis thaliana*, but was not sufficient by itself to cause necrosis (Chatterjee et al., 2002). In short, *hrp* genes are involved in pathogenesis, but their precise role remains to be determined.

1.3.2 Induced resistance

Another resistance strategy against pathogens is the activation of protective mechanisms upon contact with invaders, which is called induced or acquired resistance. After inoculation with weakened micro-organisms, plants have been shown to be protected against infection after subsequent inoculation with the same or a related pathogen. Additionally, the activation of so-called systemic acquired resistance (SAR) was clearly proven by the findings that a localised infection could lead to resistance against all kinds of pathogens. The in planta signal for SAR is generally considered to be salicylic acid, but other signals can be translocated to upper leaves and induce resistance (Sticher et al., 1997).

Local application of isolated cell-wall degrading enzymes of *Ecc* triggered systemic resistance against the same pathogen in tobacco (Vidal et al., 1998; Norman-Setterblad et al., 2000) and *Arabidopsis thaliana* (Brader et al., 2001; Kariola et al., 2003). Treatment of tobacco with culture filtrate of *Ecc* induced local and systemic expression of a plant defence related glucanase gene. Somehow, pectinase and cellulases co-operate in this system (Vidal et al., 1998). Purified harpin could induce systemic resistance in *A. thaliana* (Kariola et al., 2003).

The precise role of salicylic acid in induced resistance to *Ecc* is unclear, since Vidal et al. (1998) found a central role in tobacco, whereas Norman-Setterblad et al. (2000) found a more central role for ethylene and jasmonic acid in *A. thaliana,* while Kariola et al. (2003) found that salicylic acid and jasmonic acid both act in induced resistance of *A. thaliana*. On the other hand, Vidal et al. (1997) found that a culture filtrate worked antagonistically towards salicylic acid, although both salicylic acid and jasmonic acid could induce systemic resistance.

Pérombelon and Salmond (1995) subscribed induced resistance in potato to in planta-formation of several compounds. They mentioned suberin and lignin to slow down enzymatic degradation of plant tissue, and phenolic compounds and phytoalexins to inhibit bacterial growth directly. Lulai and Corsini (1998) confirmed that phenolic compounds could confer potato tissue completely resistant. Glucosinolates, triggered by jasmonic acid, could also diminish an infection in *A. thaliana* (Brader et al., 2001)

1.3.3 Constitutive resistance

Another strategy of plants against invaders is being resistant constitutively. Constitutive resistance is caused by the physical nature of plant tissues. The common characteristic of constitutive resistance systems is that they confer resistance to enzymatic degradation of cell walls. For resistance to *Ecc*, this involves the diversity and extent of cell wall bonds (Pérombelon and Salmond, 1995), which coincide with the pathogen's wide array of cell wall degrading enzymes. Hence, the more complex the cell wall is, the higher the resistance to *Ecc*. Resistance to *Ecc* has been related to calcium concentration of plant tissue (Pagel

and Heitefuss, 1989; Tzeng et al., 1990; Pérombelon and Salmond, 1995; Flego et al., 1997) and to pectin methylation (Pérombelon, 2002).

Host plants can have high resistance to all isolates of a pathogen (horizontal resistance) or to only specific isolates of the pathogen (vertical resistance). Horizontal resistance is associated with many traits that influence plant resistance, therefore polygenic and quantitative or partial. Vertical resistance is qualitative and often associated with HR.

Complete resistance or immunity to bacterial soft rot is absent in any host species studied do far, but quantitative differences in resistance do exist. For example, only degrees of susceptibility have been identified in potato (Pérombelon & Salmond, 1995). Neither in *Brassica rapa* (Ren et al., 1996), nor in *Brassica oleracea* var. *italica* (Darling et al., 2000) any immunity or high resistance has been found.

1.4 Breeding for resistance to soft rot

There are several possibilities to breed for resistance to soft rot. The technique to introgress resistance into genotypes of the section *Aestivae* depends on the source of the resistance gene. The genetic variation of *Zantedeschia* spp. for resistance to soft rot has not been studied, but *Z. aethiopica* is presumed to be more resistant than cultivars of the section *Aestivae* (Funnell, 1993; Yao et al., 1995), although this difference has not been determined scientifically. The introgression of genes from the section *Zantedeschia* into genotypes of the section *Aestivae* is not possible due to severe breeding barriers (Wolff, 1919; Traub, 1948; Yao et al., 1994a). Therefore, if conventional breeding is to be successful, resistance sources must be located within the section *Aestivae*. If no resistance exists within the section *Aestivae*, it might be possible to introduce resistance genes from outside the *Zantedeschia* germplasm by genetic modification.

1.4.1 Genetic modification

Many studies have been undertaken to increase resistance to soft rot by introducing foreign genes by genetic modification. These are mainly genes coding for antimicrobial proteins such as magainin (Li et al., 2001), lysozymes (Düring et al., 1993; Serrano et al., 2000), tachyplesin (Allefs et al., 1996b), cecropin and attacin (Arce et al., 1999), but also genes associated with increasing cell wall complexity such as glucose oxidase (Wu et al., 1995) and peroxidase (Ray et al., 1998) and a gene associated with induced resistance, pectate lyase (Wegener et al., 1996; Wegener, 2002). All introduced genes except peroxidase resulted in some degree of elevated resistance in potato; magainin resulted in increased resistance in tobacco.

None of the mentioned introductions resulted in complete resistance of the plant, only some degrees of elevated resistance were obtained. Direct comparison of resistance levels obtained by introduction of the different genes is difficult since different genotypes were transformed and different disease testing methods were used. Above all, very little is mentioned on the stability of the expression levels of the introduced genes. Therefore, it is difficult to conclude which genes have best prospects. Most promising might be a synthetical chimerical peptide of melittin and cecropin which was introduced in tobacco and potato (Osusky et al., 2000). Transgenic plants appeared to be completely resistant. However, all transgenic plants of one genotype showed reduced vigour from the introduced gene. In summary, the measure of resistance obtained by introduction of the aforementioned foreign genes is still limited. Synthetic genes are promising, but their toxicity may harm plant growth.

1.4.2 Conventional breeding

Conventional breeding starts with the assessment of genetic variation in the cultivated species and its crossable species. The potential of resistance breeding is unknown, because variation for resistance to soft rot has not been determined in the genus *Zantedeschia*. Moreover, selection of resistant genotypes requires sensitive disease tests to determine resistance of plants. However, these have not been described yet.

Knowledge from other crops could be used as a basis for breeding *Zantedeschia* spp. for resistance to soft rot. So has it been possible to measure the level of resistance to soft rot in several crops, such as potato (Bain and Pérombelon, 1988; Tzeng et al., 1990; Lojkowska and Kelman, 1994; Allefs et al., 1996a), broccoli (Darling et al., 2000), chicory (Schober and Vermeulen, 1999), *Brassica rapa* (Ren et al., 2000), Chinese cabbage (Ren et al., 2001), dieffenbachia (Norman et al., 1997) and carrot (Michalik et al., 1992). Therefore, the perspectives to develop a resistance test for *Zantedeschia* spp. are very good. It was possible to determine genetic control for resistance or to improve resistance in potato (Wolters and Collins., 1995; Zimnoch Guzowska et al., 2000), Chinese cabbage (Ren et al., 2001) and *Brassica rapa* (Ren et al., 2000). So it should be possible to breed for resistance to soft rot in *Zantedeschia* spp. as well.

1.4.3 Breeding barriers

The occurrence of a major breeding barrier prevents the introgression of genes from the section *Zantedeschia* into cultivars of the section *Aestivae* (Wolff, 1919; Traub, 1948; Yao et al., 1994a). This barrier results in albino plants that can only survive heterotrophically and is caused by reciprocal incompatibility between plastomes and genomes (Yao et al., 1994a). Plastid development is probably blocked in an early stage in these albino plants (Yao and Cohen, 2000).

Plastome-genome incompatibility (PGI) exists between species of different sections of the genus *Zantedeschia*, as well as between species within the section *Zantedeschia* (Yao et al., 1994a; 1995). The plastome of *Z. odorata* is incompatible to the genome of *Z. aethiopica*, resulting in albino plants. The plastome of *Z. aethiopica* is partially incompatible to the genome of *Z. odorata*, resulting in virescence (a condition where plants initially are pale-green or yellow, but turn green as they age) (Yao et al., 1994a).

Although functional interspecific hybrids can be obtained after artificial hybridisation among species of the section *Aestivae* (Wolff, 1919; Shibuya, 1956; Horn, 1962), it is possible that PGI exists. An indication for PGI was delivered by New and Paris (1967), who mentioned hybrid variegation and a non-Mendelian segregation of virescence in interspecific hybrids. If PGI exists among the section *Aestivae*, then it could have a major

impact on genetic studies and breeding. Knowledge of breeding barriers and PGI within the section *Aestivae* is a prerequisite of genetic analyses and breeding. Directions of PGI between species of the section *Aestivae* must therefore be determined to identify the most promising parents and crossing directions.

1.5 Scope of the thesis

The research described in this thesis aims to explore possibilities for breeding *Zantedeschia* spp. for resistance to soft rot caused by *Ecc*. This first chapter starts with a review on the genus *Zantedeschia* and the causal agent of soft rot, *Ecc*. The infection process and possibilities how to control the pathogen are described and general resistance mechanisms in plants are reviewed with emphasis on resistance to soft rot caused by *Ecc*.

Since breeding relies on genetic variation and the selection of the best parents, methods to determine levels of resistance are described in Chapter 2. The variation for resistance needed for selection of the best parents is described and discussed in Chapter 3. Key factors for successful breeding are knowledge of the inheritance of the traits and the efficiency in selecting superior offspring. The development of the offspring was hampered by PGI barriers and by biparental plastid inheritance. The effect of PGI and biparental plastid inheritance on genetic analyses is discussed in Chapter 4 before a genetic analysis of resistance is described in Chapter 5, so that conclusions could be drawn on genetic control of resistance to soft rot. Relevance and opportunities of the presented results for biological science in general and breeding of *Zantedeschia* in particular are discussed in Chapter 6

2 Evaluation of tests to determine resistance in *Zantedeschia* spp. (*Araceae*) to soft rot caused by *Erwinia carotovora* subsp. *carotovora*

Ronald C. Snijder and Jaap M. van Tuyl

Abstract

Bacterial soft rot caused by *Erwinia carotovora* subsp. *carotovora* is a major disease in *Zantedeschia* spp., particularly in cultivars from the section *Aestivae*. The disease can be partly controlled by cultural measures, but by combining cultural methods with resistant plant material a promising strategy for control of soft rot can be developed. No tests are available for resistance testing in breeding *Zantedeschia* spp. Therefore, three tests developed for use in potato breeding were adapted for use on eight cultivars of *Zantedeschia* spp. Variation was found in all three tests. Resistant control cultivar *Zantedeschia aethiopica* 'Crowborough' scored most resistant in all three tests. Within the section *Aestivae*, degrees of susceptibility were identified that were in agreement with each other and with field observations, indicating reliability of two of the methods in which tubers were used. The correlation coefficient of these two tests was high. A new non-destructive test method was developed for use on seedlings which involved immersion of leaf disks in a bacterial suspension. The percentage of decayed leaf area was a measure of resistance and results were in general agreement with the other tests. These methods will be useful for breeding for soft rot resistance and performing genetic analyses.

2.1 Introduction

Zantedeschia Sprengel (*Araceae*), also called 'calla lily' or 'arum lily', is a genus of about eight species in two sections, all from southern Africa (Letty, 1973; Singh et al., 1996). *Z. aethiopica* bears a rhizome and belongs to section *Zantedeschia*; hybrids with coloured flowers are developed from crosses of species from section *Aestivae*, mainly *Z. rehmannii, Z. elliotiana* and *Z. albomaculata* (Funnell, 1993; Singh et al., 1996). Species from this section all produce a root tuber as a storage organ (Robinson et al., 2000) and in contrast to *Z. aethiopica* require a dormancy period (Funnell, 1993; Singh et al., 1996).

Bacterial soft rot caused by *Erwinia carotovora* subsp. *carotovora* is a major disease in section *Aestivae* of *Zantedeschia* and occurs world-wide (Corr et al., 1993; Funnell and MacKay, 1999; Kuehny, 2000; Wright and Burge, 2000). This soil borne facultative anaerobic bacterium causes maceration and rotting of parenchymatous tissue of all plant organs, resulting in loss of the entire plant (Pérombelon and Kelman, 1980; Wright, 1998).

Plants become infected during storage or in the field, but infection does not necessarily result in soft rot, since the bacterium can be present latently (Blom and Brown, 1999). Infected plants turn yellow, emit a foul smell and can be completely macerated resulting in death within a few days (Wright, 1998). Spread of bacteria takes place mainly by watering or by handling during tuber lifting, storage and planting. Soft rot symptoms can develop at any time during the growing cycle when conditions favourable for soft rot, such as high relative humidity occur, or when plants are under stress due to low soil aeration or high temperature (Funnell and MacKay, 1999; Wright and Burge, 2000).

Preventive soft rot control measures include irrigation, mulching and soil ventilation (Funnell, 1993; Funnell and MacKay, 1999; Wright and Burge, 2000). The bacteria are endemic in soil, making the disease difficult to control even with clean seed tubers (Pérombelon and Salmond, 1995; Funnell and MacKay, 1999). Increased calcium levels have been correlated positively with resistance to soft rot in calla lily, but no method gives full control of disease (Funnell and MacKay, 1999). Therefore, combining different cultural measures, including the use of resistant cultivars, is a promising approach in overcoming problems caused by the soft rot pathogen.

However, all *Aestivae* cultivars are susceptible to bacterial soft rot and no research is being conducted to determine variation in resistance within *Zantedeschia* spp. According to Funnell (1993) and Yao et al. (1995), *Z. aethiopica* is more tolerant to soft rot than *Aestivae* genotypes, but details were not provided. Incorporating resistance from *Z. aethiopica* into *Aestivae* genotypes is not possible due to major compatibility barriers (Yao et al., 1994a; 1995). Therefore, sources of resistance must be located within the *Aestivae* gene pool and testing methods are required that can assess resistance levels in leaves and tubers. If possible sources of resistance are to be used in breeding for resistance, more knowledge is required on variation in and genetics of resistance. Therefore, methods for testing the resistance of clones must be developed, along with those individual seedlings. The latter requires special attention, since seedlings that are to be used in further crossing and evaluation must be tested by non-destructive methods.

Variation in resistance to *E. carotovora* has been studied in many crops (Allefs et al., 1995; Carputo et al., 1996; Darling et al., 2000; Ren et al., 2001). The potato–*Erwinia* complex, in particular, can be used as a basis for understanding the calla lily–*Erwinia* complex. Both crops are comparable in propagation and cultural methods. Both are mainly vegetatively propagated and the storage organ is used for over-wintering. In potato, this is a stolon-borne tuber, whereas *Z. aethiopica* bears a rhizome and plants from section *Aestivae* carry a root tuber (Robinson et al., 2000). Since *Aestivae* genotypes also carry tubers, it can be expected that methods developed for potato tubers could be used on *Zantedeschia* spp.

This paper describes the evaluation of four methods for determining resistance levels in tubers and leaves of *Zantedeschia* spp. The objectives were to evaluate methods previously used with potato to develop a new test method for *Aestivae* seedlings and to explore variation of resistance in *Zantedeschia* spp. to soft rot.

2.2 Materials and methods

2.2.1 Plant material

Plant material was obtained from commercial sources in The Netherlands. These included *Z. aethiopica* 'Crowborough' (section *Zantedeschia*), 'Best Gold', 'Black Magic', 'Galaxy', 'Pink Persuasion', 'Sensation', 'Treasure' and 'Florex Gold' (section *Aestivae*). For the tuber test, tubers from one-year-old plants were produced from in vitro propagated plants (T_1-tubers). Tubers used in the tuber slice test were from T_2-plants (two years from in vitro propagated plants). All tubers were stored at 9°C at 70 % relative humidity (RH) and treated for 10 min in 100 mg/l gibberellic acid (GA_3, Berelex, Bayer) before planting as is common practice to promote flowering (Funnell, 1993). Plants were grown from T_1-tubers in a greenhouse with day temperature of 20–30°C and night temperatures of 15°C.

2.2.2 Bacterial strain

E. carotovora subsp. *carotovora*, isolate PD 1784, isolated from an unknown *Zantedeschia* accession, was obtained from the Dutch Plant Protection Service and stored at -80ºC using 'Protect' beads (Technical Service Consultancy). Inocula were prepared from 48-h-old cultures in 'Lab-Lemco' Broth (Oxoid) and 86 mM NaCl (shaken at 100 rpm). Bacterial cultures were centrifuged for 10 min at 1800 g and the pellets resuspended in sterile tap water. The bacterial concentration was estimated using a haemocytometer.

2.2.3 Petiole test

The oldest leaves of plants were cut around flowering time with a knife disinfected in 80 % ethanol. After discarding leaf blades, petioles were cut 20 cm from the top, washed three times in sterile water and surface dried. Petioles were placed into 5ml inoculum ($1*10^5$ cfu/ml) in plastic tubes (Ø = 2.0 cm) and incubated in an environmental chamber at 100 % RH for five days. The length of healthy tissue (LH) was measured to the nearest 0.5 cm

(modified from Bisht et al. (1993)). The experiment was done in two replicates using eight petioles per cultivar.

2.2.4 Tuber test
Whole T_1-tubers (8 per cultivar, not treated with GA_3) were disinfected by washing in tap water, immersing in 1 % hypochlorite for 20 min and then again washing in tap water. Tubers were wounded by pushing a 200-µl pipette tip 3–5 mm into the base of the tuber (modified from Allefs et al. (1993) and Lojkowska and Kelman (1994)) and 20 µl inoculum ($1*10^7$ cfu/ml) was pipetted into the wound. Subsequently, tubers were incubated with the apical meristem pointed downwards in 100 % RH, 20ºC until observation after six days. To measure the degree of resistance, the tubers were weighed before (W1) and after (W2) washing away infected tissue.

2.2.5 Tuber slice test
T_2-tubers were disinfected as described above. Ten slices, 7–9 mm thick, were cut longitudinally from three tubers using a clean knife. They were inoculated by placing a piece of conventional lab paper (Ø = 5 mm) soaked with $1*10^5$ cfu/ml onto the middle of the cut surface. The slices were placed in a layer of water (approx. 1–2 mm deep), with the inoculated side up, to prevent drying of the cut surface. Incubation and analysis were done as in the tuber test, but the observations were done after two days.

2.2.6 Leaf disk test
Two young leaves of a newly sprouting plant were harvested just after folding of their leaf blades. Twelve disks (Ø = 22 mm) per leaf blade were made using a cork-borer and transferred to a 12-well plate in 5ml inoculum ($1*10^7$ cfu/ml). Disks were kept immersed by constant pressure of a 1.5-ml eppendorf tube. Incubation was done in an environmental chamber (20ºC; 100 % RH). Observations were made after three, four or six days of incubation, depending on level of symptoms. The percentage of decayed surface area (P)

was visually estimated on a light-box. Every replicate included two leaves with 12 disks each.

The petiole and the tuber tests were carried out in duplicate during one season, the tuber slice test was done in duplicate in the second season, the leaf disk test was replicated at least three times in the first season, but most cultivars were measured in six replicates.

2.2.7 Statistical analyses

For the petiole and the leaf disk tests, differences between cultivars of healthy tissue (LH) and the percentage of macerated disk area (P), respectively, were estimated according to the iterative reweighted residual maximum likelihood algorithm (IRREML, assuming a binomial distribution using a logit link). This is a technique that can fit data sets to a Generalised Linear Mixed Model (GLMM). A GLMM is able to fit unbalanced data sets with random components of variance that are not normally distributed (Engel and Keen, 1994; Keen and Engel, 1998). In the tuber and tuber slice test, differences between cultivars for response variable $\sqrt{\{(W1-W2)/W1\}}$ were estimated by ANOVA according to Haynes et al. (1997). Spearman's rank correlation coefficients of the estimates of means of the cultivars in all test methods were calculated. Hereby, two calculations were made, one by inclusion and the second by exclusion of 'Crowborough'. This was done to assess whether 'Crowborough', which is the resistant control, biases the results. Moreover, the main interests were resistance differences within section *Aestivae* and use of the test methods in this group. All analyses were done using the statistical analysis software package Genstat 5, release 4.2 (GenStat, 2000).

2.3 Results

Four test methods were compared for measuring resistance levels to *Erwinia* in *Zantedeschia* spp. (Table 2.1).

Table 2.1. Comparison of four test methods for estimating resistance of several Zantedeschia *cultivars to* Erwinia carotovora *subsp.* carotovora *PD 1784.*

Cultivar	Petiole test[1]		Tuber test[2]	Tuber slice test[2]	Leaf disk test[3]	
		LH				P
Crowborough	2.90 a	18.5	0.00 a	0.00 a	-3.55 a	6
Pink Persuasion	0.82 ab	13.6	0.11 b	0.16 b	0.58 bc	55
Black Magic	1.27 ab	15.3	0.25 b	0.17 b	0.33 bc	54
Best Gold	1.33 ab	15.6	0.60 c	0.18 b	-0.28 b	40
Galaxy	1.68 ab	15.9	4	0.20 b	0.91 bc	74
Treasure	-0.81 b	7.1	0.82 c	0.24 bc	0.92 bc	68
Sensation	-0.78 ab	6.9	0.73 c	0.31 c	-0.08 b	47
Florex Gold	0.31 ab	10.9	0.75 c	0.41 d	1.61 c	80
lsd[5]	3.00		0.11	0.07	1.55	

[1]Length of unmacerated petiole tissue on logit scale and in cm (LH).
[2]Relative weight of decayed tuber tissue: $\sqrt{\{(W1-W2)/W1\}}$, where W1 represents the weight in grams of the tuber slice before and W2 after washing away macerated tissue.
[3]Percentage of decayed leaf disk area on logit scale and in % (P).
[4]Missing data.
[5]Lsd-values ($\alpha = 0.05$) are not applicable for LH and P, since these are not normally distributed.

2.3.1 Petiole test

'Crowborough' had the greatest length of healthy petiole tissue at 18.5 cm (see Table 2.1) which suggested a resistant phenotype. 'Best Gold', 'Black Magic', 'Galaxy' and 'Pink Persuasion' had less healthy tissue ranging from 13.6 to 15.9 cm. 'Sensation', 'Treasure' and 'Florex Gold' had little healthy tissue (susceptible phenotype) ranging from 6.9 to 10.9 cm which suggested that they were susceptible. However, due to high within-cultivar variance only 'Treasure' was significantly different (Table 2.1).

2.3.2 Tuber test and tuber slice test

Using the tuber and the tuber slice test, respectively three and four groups could be distinguished (Table 2.1). As with the petiole test, 'Crowborough' had a resistant phenotype with no infected tuber tissue and all *Aestivae* cultivars were susceptible with a range of 0.11 to as much as 0.82 relative amount of infected tuber tissue. The most susceptible phenotypes in the petiole test, 'Sensation', 'Treasure' and 'Florex Gold' also were the more susceptible in these two tests (Table 2.1).

2.3.3 Leaf disk test

Decayed areas of the leaf disks were fully macerated. These were more transparent and visible as light green sectors in the disks when viewed on a light-box and disintegrated when touched.

Table 2.2. Means of percentage decayed leaf disk tissue (on logit scale) after three, four and six days after inoculation of seven Zantedeschia *cultivars with* Erwinia carotovora *subsp.* carotovora *PD 1784*

Cultivar	3 days	4 days	6 days
Crowborough	-3.82 a	-3.55 a	-3.32 a
Best Gold	[1]	-0.28 b	1.63 b
Black Magic	-1.23 b	0.33 bc	1.13 b
Galaxy	-0.41 bc	0.91 bc	1.63 b
Pink Persuasion	-0.14 bc	0.58 bc	1.62 b
Sensation	-0.58 bc	-0.08 b	0.72 b
Treasure	-1.09 b	0.92 bc	2.34 b
Florex Gold	1.00 c	1.61 c	2.57 b
lsd (α=0,05)	1.60	1.55	2.75

[1] missing data

Discrimination of cultivars after four days was slightly better than after three days (Table 2.2). After six days, only the resistant 'Crowborough' could be discriminated from all *Aestivae* cultivars. After both three and four-day incubation, three *Aestivae* cultivars could be assigned to two levels of susceptibility. 'Treasure' was known to be susceptible

(Geerlings, pers. comm.), but after three days, 'Treasure' did not score as such. Therefore, a four-day incubation time was chosen as the best observation time for screening cultivars. The four methods resulted in largely similar groups (Table 2.1). 'Crowborough' was least susceptible in all four test methods. Both 'Florex Gold' and 'Treasure' were most susceptible by all methods, while 'Pink Persuasion' and 'Black Magic' showed a lower susceptibility. 'Best Gold', 'Sensation' and 'Galaxy' did not respond consistently. In the petiole and both tuber tests, 'Sensation' had a relatively susceptible phenotype, whereas in the leaf disk test, 'Sensation' was relatively resistant. 'Galaxy' was classed in the high susceptible group in the leaf disk test and in the low susceptible group using the petiole and the tuber slice tests. 'Best Gold' scored relatively resistant using the leaf disk test, but scored moderately susceptible using the tuber and petiole tests. Including 'Crowborough' in the statistical evaluation resulted in higher correlation coefficients (Table 2.3).

Table 2.3. Spearman's rank correlation coefficients of estimates of means from four test methods to measure resistance of seven Zantedeschia *cultivars to* Erwinia carotovora *subsp.* carotovora*; including and excluding the results from resistant cultivar* Z. aethiopica *'Crowborough' for determining correlation coefficients of results from six cultivars from section* Aestivae.

Test	Including 'Crowborough'			Excluding 'Crowborough'		
	petiole	tuber	tuber slice	petiole	tuber	tuber slice
Petiole	-	-		-	-	-
			-			
Tuber	0.82[1]	-	-	0.71	-	-
Tuber slice	0.67[1]	0.89[2]	-	0.50	0.83[1]	-
Leaf disk	0.55	0.64	0.62	0.32	0.43	0.43

[1], [2] Significant at the 0.05 and 0.01 probability levels, respectively

Only the correlation coefficient of the tuber and the tuber slice test was still significant after exclusion of 'Crowborough'. This indicated that the tests using tuber tissue are highly reproducible. The correlation between the leaf disk test and all other test results was not statistically significant.

2.4 Discussion

Four tests were compared for measuring resistance levels to soft rot. In general, all four test methods resulted in similar groups (Table 2.1), but there were considerable differences in variation and reproducibility. The tests using tubers gave the highest discrimination among the cultivars by revealing three and four significantly different groups in the tuber and tuber slice test, respectively (Table 2.1).

The high correlation (r = 0.83) between the results of *Aestivae* types in both tests using tuber tissue indicates high reproducibility (Table 2.3). However, only T_1-tubers were infected successfully after inoculation, i.e., resulting in a measurable amount of decay. T_2-tubers with a more irregular surface developed fewer symptoms (data not shown) and could have been latently infected. Latent infection of *Zantedeschia* spp. with *Erwinia* is known to occur in the field (Funnell, 1993). Hélias et al. (2000) also found latent infections after inoculation of potato plants.

In order to circumvent the establishment of latent infections, the tuber slice test was evaluated. All slices had a measurable amount of infected tissue after inoculating the cut surface of the tuber slice. Successful infection of irregularly shaped tubers apparently is dependent on the site of inoculation on the tuber. Bain and Pérombelon (1988) and Lojkowska and Kelman (1994) also found different results when inoculating different sites on the potato tuber. This could be related to an altered calcium concentration or cell wall content at the inoculation site (Pagel and Heitefuss, 1989; Pérombelon and Salmond, 1995).

The cultivar rankings by the tuber and the tuber slice tests were very similar (Table 2.3), so these results were not dependent on the site of inoculation. Although Allefs et al. (1995) found similar results in potato, Bain and Pérombelon (1988) and Lojkowska and Kelman (1994) found that cultivars were ranked differently by inoculating different sites on potato tubers.

The reasons for the high within-cultivar variances and the low correlation coefficients of the petiole and the leaf disk test (r = 0.32) are not clear. Other studies on *Erwinia* resistance also noted low reproducibility (Lojkowska and Kelman, 1994; Schober and Vermeulen, 1999), but was not explained. It could be related to the complex machinery

involved in initiation of pathogenesis by *E. carotovora*, as modelled by Mukherjee et al., (2000), but this has not been studied in relation to infection in the field.

The low correlation coefficient of *Aestivae* cultivar rankings in the petiole and the leaf disk test (r = 0.32) compared with the tuber and the tuber slice test (r = 0.83) indicates a low reproducibility of results using leaf material. However, the composition of susceptibility groups by all the tests is similar (Table 2.1). The correlation coefficients of the two tests using tubers and the tests using leaf material are also low (Table 2.3). Allefs et al. (1996a) also found low correlation coefficients between results of a tuber and a stem resistance test in potato after inoculation with *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi*. They interpreted this as being due to different components of resistance.

Neither the petiole nor tuber tests are applicable for screening seedlings, as they use too much tissue or are destructive, respectively. The leaf disk method is the only non-destructive method available for testing *Zantedeschia* seedlings, since each plant develops only one tuber and 4–6 small leaves in the first year and 2–10 bigger leaves the second. A large number of leaf disks were used in this study. In seven replicates, up to 180 leaf disks were tested for some cultivars. Such large amounts of tissue are not available from single seedlings, but were necessary to find significant differences. It can be expected that less leaf material is needed when genotypes to be analysed have different levels of resistance. Hence, the most resistant and the most susceptible seedlings can be selected. The leaf disk test could be optimised by including more than two leaves per genotype per replicate and using parts of leaves instead of whole leaves. This is supported by preliminary results from testing wild accessions and seedlings of section *Aestivae* (data not shown).

Observations of Shibuya (1956), (Brown, 1988) and Geerlings (pers. comm.) support the composition of groups as found in this study. They stated that *Z. elliotiana* was the most susceptible from section *Aestivae*, followed by *Z. rehmannii* and *Z. albomaculata*. Similarly, *Z. elliotiana*-resembling 'Florex Gold' was found most susceptible and *Z. rehmannii*-resembling 'Pink Persuasion', the least susceptible of the *Aestivae* cultivars in this paper. This indicates that there is agreement between field experiences and results obtained in our disease tests.

For selecting soft rot resistance during breeding of *Aestivae* genotypes, it is recommended to pre-screen seedlings using the leaf disk test and to screen subsequent

clones at a later stage of the breeding programme using the tuber slice test. The pre-screen test can differentiate most susceptible and most resistant genotypes. The tuber slice test can be applied to estimate the level of resistance in selected clones more accurately. Hence, the way to breeding and genetic analyses has been opened. Variation in resistance within the genus *Zantedeschia* and genetics of resistance within section *Aestivae* are now being investigated.

3 Genetic variation in *Zantedeschia* spp. (*Araceae*) for resistance to soft rot caused by *Erwinia carotovora* subsp. *carotovora*

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Abstract

Bacterial soft rot caused by *Erwinia carotovora* subsp. *carotovora* is a major disease in *Zantedeschia* spp., particularly in cultivars from the section *Aestivae*. The disease can partially be controlled by cultivation measures, so a combination with resistant cultivars could effectively protect the crop. However, resistant commercial *Aestivae* cultivars are not available yet. By means of a recently developed non-destructive resistance test, variation in aggressiveness was observed among five isolates of *Erwinia carotovora* subsp. *carotovora* without interactions between the isolates and three *Zantedeschia* accessions. Within eleven accessions of *Z. aethiopica*, variation was observed from almost complete to moderate resistance, while the *Z. odorata* accession was susceptible. All 21 *Aestivae* cultivars were susceptible. Within the *Aestivae* species, *Z. elliotiana* and *Z. pentlandii* were also susceptible, but within twelve accessions of *Z. albomaculata*, as well as in six accessions of *Z. rehmannii*, variation was found from susceptible to moderately resistant. Hence, new sources of resistance were identified that show good potentials for resistance breeding.

3.1 Introduction

The genus *Zantedeschia* (*Araceae*), consists of eight species in two sections, all from southern Africa. Section *Zantedeschia* is characterised by the presence of a rhizomatous tuber and consists of the species *Z. aethiopica* and *Z. odorata* (Singh et al., 1996). Species of section *Aestivae* are characterised by the presence of a discoid tuber (Singh et al., 1996), referred to as a 'root tuber' by Wright and Burge (2000). *Z. odorata* and species from the section *Aestivae* require a dormancy period*,* in contrast to *Z. aethiopica* (Funnell, 1993). Most cultivars have been developed from crosses between species of section *Aestivae*, mainly *Z. albomaculata*, *Z. elliotiana, Z. rehmannii* and *Z. pentlandii* (Funnell, 1993).

Bacterial soft rot caused by *Erwinia carotovora* subsp. *carotovora* occurs world-wide in many crops. This soil borne facultative anaerobic pathogen causes maceration and rotting of parenchymatous tissue of all plant organs, eventually resulting in plant death (Pérombelon and Kelman, 1980; Wright, 1998). It is an important disease in *Zantedeschia* spp. and the major disease in cultivars of section *Aestivae* (Corr et al., 1993; Funnell and MacKay, 1999; Kuehny, 2000; Wright and Burge, 2000).

Bacteria are spread mechanically during cultivation. Plants can become infected during storage or in the field, but infection does not necessarily result in soft rot, since the infection can be latent (Funnell, 1993; Pérombelon and Salmond, 1995; Blom and Brown, 1999). When the infection progresses, plants turn yellow, produce a foul smell and can become completely macerated resulting in plant death within a few days (Wright, 1998). Soft rot symptoms can become visible during all stages of plant growth and development. Conditions are favourable for soft rot when plants are under stress during low soil aeration, high temperature or high relative humidity (Funnell and MacKay, 1999; Wright and Burge, 2000).

Cultural measures like drainage, mulching and soil ventilation can reduce disease development (Funnell, 1993; Funnell and MacKay, 1999; Wright and Burge, 2000), but better control of the disease could be achieved by use of resistant cultivars. However, neither resistant *Aestivae* cultivars nor resistant *Zantedeschia* spp. have been documented yet. Only some practical experiences about presumed resistance are known. For example,

according to Funnell (1993) and Yao et al. (1995), *Z. aethiopica* is more resistant to soft rot than *Aestivae* genotypes, but they did not prove it. Recently developed methods enable to screen for resistance in *Zantedeschia* spp. Moreover, even genotypes of which only few plants exist, can be evaluated since non-destructive methods were developed (Snijder and van Tuyl, 2002).

The objective of this research was to identify genetic variation in resistance within the genus *Zantedeschia*. We hypothesised that *Z. aethiopica* is relatively resistant, that cultivars of section *Aestivae* are susceptible and that resistant genotypes exist among *Aestivae* accessions from the wild. Therefore, we selected the most informative isolate of *E. carotovora* to screen 66 accessions of *Zantedeschia* for variation in resistance by using a non-destructive leaf disk test.

3.2 Materials and Methods

3.2.1 Plant Material

Accessions and cultivars representing the entire genus *Zantedeschia* were collected from breeding companies, nurseries and botanical gardens, and screened in three experiments in three years for their resistance to soft rot caused by *E. carotovora* subsp. *carotovora* (Table 3.1). Within the section *Aestivae*, emphasis was put on plant material that has not been used in breeding, since cultivars were expected to be susceptible. To determine variation for resistance among *Zantedeschia* spp., accessions of both species of the section *Zantedeschia*, and of four species and 21 cultivars of the section *Aestivae* were collected from breeding companies, nurseries and botanical gardens. Two cultivars were grouped among accessions from the wild: 'Helen O'Connor' is an old cultivar that was introduced from wild species without breeding (Toogood and Mattin, 1998; Stoltz, 2001), and 'Solfatare' is an old cultivar and a hybrid of wild accessions (Magnus, 1901). Unfortunately, accessions of the rare *Z. jucunda* and *Z. valida* were not available and had to be excluded.

Table 3.1. Accessions of the Zantedeschia *collection that were evaluated for their resistance to soft rot caused by* Erwinia carotovora *subsp.* carotovora *isolate PD 1784. (a) section* Zantedeschia*, (b) section* Aestivae

a.

PRI-no	Species/cultivar	Origin[1]	Experiment
00005	*Z. aethiopica*	N1, France	1,4
00030	*Z. aethiopica*	B1, South Africa	1,4
00054	*Z. aethiopica* 'Pink Mist'	N2, The Netherlands	1,4
00901	*Z. aethiopica* 'Highwood'	N3, The Netherlands	1,4
99001	*Z. aethiopica* 'Calla white'	N4, The Netherlands	1
99005	*Z. aethiopica* 'Green Goddess'	B1, The Netherlands	1
99006	*Z. aethiopica* dwarf	B1, The Netherlands	4
99007	*Z. aethiopica*	B1, The Netherlands	4
99008	*Z. aethiopica* 'Crowborough'	N2, The Netherlands	0,1,4
99009	*Z. odorata*	N5, USA	1,4
99027	*Z. aethiopica* 'Giant Vanetti'	N1, France	1,4
99144	*Z. aethiopica* 'Green Goddess'	PRI, The Netherlands	4

b.

PRI-no	Species/cultivar	Origin[1]	Prop[2]	Duplicate of[3]	Experiment
99003	*Z.* 'Majestic Red'	N4, The Netherlands	Clone		2
99004	*Z. elliotiana*	N4, The Netherlands	Clone		3
99010	*Z.* 'Best Gold'	B3, The Netherlands	Clone	00043	2,5
99011	*Z.* 'Black Magic'	B3, The Netherlands	Clone	00048	2,5
99012	*Z.* 'Galaxy'	B3, The Netherlands	Clone	00034	2,5
99014	*Z.* 'Pink Persuasion'	B3, The Netherlands	Clone	00036	2,5
99015	*Z.* 'Sensation'	B3, The Netherlands	Clone	00042	2,5
99016	*Z.* 'Treasure'	B3, The Netherlands	Clone	00052	2,5
99017	*Z.* 'Florex Gold'	B3, The Netherlands	Clone	00046	0-7
99022	*Z. rehmannii*	B1, New Zealand	Clone		0-7
00001	*Z.* 'Shadow'	N3, The Netherlands	Clone		5
00006-1	*Z. albomaculata*[4]	N1, France	Clone		3,6
00031	*Z. albomaculata* subsp. *albomaculata*	B1, South Africa	Seed		6
00033	*Z.* 'Chianti'	B1, The Netherlands	Clone		5
00034	*Z.* 'Galaxy'	B1, The Netherlands	Clone	99012	2,5
00035	*Z.* 'Fandango'	B1, The Netherlands	Clone		5
00036	*Z.* 'Pink Persuasion'	B1, The Netherlands	Clone	99014	2,5
00038	*Z.* 'Black Eyed Beauty'	B1, The Netherlands	Clone		5
00039	*Z.* 'Cameo'	B1, The Netherlands	Clone		5
00040	*Z.* 'Coral Sunset'	B1, The Netherlands	Clone		5
00041	*Z.* 'Mango'	B1, The Netherlands	Clone		5
00042	*Z.* 'Sensation'	B1, The Netherlands	Clone	99015	2, 5
00043	*Z.* 'Best Gold'	B1, The Netherlands	Clone	99010	2,5
00045	*Z.* 'Hot Shot'	B1, The Netherlands	Clone		5
00046	*Z.* 'Florex Gold'	B1, The Netherlands	Clone	99017	5
00047	*Z.* 'Ruby'	B1, The Netherlands	Clone		5
00048	*Z.* 'Black Magic'	B1, The Netherlands	Clone	99011	2,5
00049	*Z.* 'Neroli'	B1, The Netherlands	Clone		5
00050	*Z.* 'Celeste'	B1, The Netherlands	Clone		5

Table 3.1 (*continued*)

00051	*Z.* 'Hazel Marie'	B1, The Netherlands	Clone		5
00052	*Z.* 'Treasure'	B1, The Netherlands	Clone	99016	5
00056	*Z. albomaculata* subsp. *albomaculata*	N1, South Africa	Seed		6,7
00057	*Z. rehmannii*	N1, South Africa	Seed		3,6,7
00058	*Z. albomaculata* hybrid	N5, The Netherlands	Clone		3,6
00060	*Z. albomaculata* subsp. *albomaculata*	B4, The Netherlands	Clone		3
00061	*Z. albomaculata* subsp. *albomaculata*	B4, The Netherlands	Clone		3
00062-1	*Z. elliotiana*	B4, The Netherlands	Clone		3
00063	*Z. rehmannii*	B4, The Netherlands	Clone		3
00069-2	*Z. pentlandii*	B4, The Netherlands	Clone		3
00073	*Z. elliotiana*	N1, South Africa	Seed		6,7
00074	*Z. rehmannii*	N1, South Africa	Seed		6,7
00075	*Z. albomaculata* subsp. *albomaculata*	N1, South Africa	Seed		7
00076	*Z. albomaculata* subsp. *albomaculata*	N1, South Africa	Seed		6,7
008047	99022 × 99014	PRI, The Netherlands	Seed		7
018001	*Z. albomaculata* 'Helen O'Connor'	N3, South Africa	Seed		6,7
018002	*Z. albomaculata* subsp. *macrocarpa*	*N3, South Africa*	*Seed*		7
018004	*Z. albomaculata* subsp. *albomaculata*	N3, South Africa	Seed		7
018005	*Z. albomaculata* subsp. *albomaculata*	N3, South Africa	Seed		6,7
018006	*Z. albomaculata* subsp. *macrocarpa*	N3, South Africa	Seed		7
018007	*Z. rehmannii*	N3, South Africa	Seed		7
018008	*Z. rehmannii*	G, Germany	Seed		7
018009	*Z. albomaculata* subsp. *albomaculata*	G, Germany	Seed		7
018010	*Z.* 'Solfatare'	N5, The Netherlands	Clone		6

[1] B = breeding company, N = nursery, G = botanical garden; numbers indicate identical origin per country

[2] Mode of propagation, 'seed'= raised from seed, 'clone'= raised from vegetatively propagated plants

[3] Duplicates are accessions from the same cultivar, but from another source

[4] Subspecies unclear, accession 00006-1 has characteristics of both subsp. *albomaculata* and subsp. *macrocarpa*, and is therefore possibly of hybrid origin

3.2.2 Cultivation of plants

Plants were grown in a heated greenhouse, with day temperatures ranging from 15 to 30°C and night temperatures from 10 to 18°C. All tubers were treated with 100 ppm gibberellic acid (GA_3, Berelex, Bayer) for 10 min before planting to promote flowering, as is common practice in commercial production (Funnell, 1993; Brooking and Cohen, 2002). Plants in the greenhouse were planted in poor soil, all plants were watered until five months after planting and liquid nutrients were applied weekly (NPK ratio=777) until four months after planting. Tubers of *Aestivae* genotypes were lifted when leaves had wilted, and air-dried thoroughly (20°C, 30 % relative humidity (RH)) before storage (9°C, 70 % RH).

3.2.3 Bacterial isolates

Bacterial isolates of *E. carotovora* were obtained from the Dutch Plant Protection Service (Table 3.2). All isolates were stored at –80°C using 'Protect' beads (Technical Service Consultants Limited, United Kingdom) after being cultured on solid medium (0.8 % 'Lab-Lemco' Broth (Oxoid B.V., Netherlands), 86 mM $NaCl_2$, 1.5 % bacteriological agar No.1 (Oxoid B.V., Netherlands).

Table 3.2. Isolates of Erwinia carotovora *that were evaluated for variation in aggressiveness, obtained from the Dutch Plant Protection Service.*

No	Subspecies	Isolated from	Year of isolation
PD 755	*atroseptica*	*Solanum tuberosum*	1986
PD 909	*carotovora*	*Zantedeschia* sp.	1987
PD 1784	*carotovora*	*Zantedeschia* sp.	1990
PD 3884	*carotovora*	*Z.* 'Golden Star'	2000
PD 3885	*carotovora*	*Z. aethiopica* 'Childsiana'	2000
PD 3886	*carotovora*	*Z.* 'Dominique'	2000

3.2.4 Resistance test

A leaf disk test was used for determining resistance levels of *Zantedeschia* plants ((Snijder and van Tuyl, 2002). Six disks of at least two young leaves of a newly sprouting plant were harvested just after unfolding of the leaf blade. Each disk was submerged in a 5 ml bacterial suspension (1^*10^7 bacteria/ml in sterilised, demineralised water) and incubated in a moist chamber (100 % relative humidity, 20°C). Observations were done visually on a slide view-box after two, three or four days of incubation for the section *Aestivae*, depending on level of symptoms of reference genotypes *Z. rehmannii* (99022) and 'Florex Gold' (99017), and after six days for the section *Zantedeschia*. Plants of the section *Zantedeschia* were tested and analysed apart from the section *Aestivae*, because of a different growing season and because observations were done at a later stage. Table 3.3 shows the experimental procedure for all resistance tests.

Table 3.3. Experimental procedure for screening resistance levels to Erwinia carotovora *subsp*. carotovora *PD 1784 in the genus* Zantedeschia

Goal	2000	2001	2002
Variation *Erwinia* isolates	Experiment 0		
Variation section *Zantedeschia*	Experiment 1	Experiment 4	
Variation *Aestivae* cultivars	Experiment 2	Experiment 5	
Variation *Aestivae* species	Experiment 3	Experiment 6	Experiment 7

3.2.5 Statistical analysis

Resistance levels were calculated per leaf. The unit leaf was considered as the experimental unit, because the between-leaf variance was different from the within leaf variance (Table 3.4).

Table 3.4. Analysis of variance of pooled leaf disks per leaf of control genotypes 'Florex Gold' (99017) and Z. rehmannii *(99022) for determining between-leaf and within-leaf variances.*

Leaf	df	Mean Square	
	63	21.95	
Residual	320	1.90	
		F-value	F-ratio
Between leaf variance[1]		3.34	1.76 (p=0.001)
Within leaf variance		1.90	

[1](Mean Square$_{Leaf}$)= (Within leaf variance) + 6*(Between-leaf variance);
(Within leaf variance) = Mean Square$_{Residual}$; (6 = number of disks per leaf)

Differences between cultivars for the percentage of macerated disk area were estimated according to the iterative reweighted residual maximum likelihood algorithm (IRREML, assuming a binomial distribution using a logit link). This is a technique that can fit data sets to a Generalised Linear Mixed Model (GLMM). A GLMM is able to fit unbalanced data sets with random components of variance that are not normally distributed (Engel and Keen, 1994; Keen and Engel, 1998). Predicted percentages of macerated leaf tissue were retrieved after backtransformation of predicted means from logit-scale that were obtained after analysis by IRREML. All analyses were done using the statistical analysis software package Genstat 5, release 4.2 (GenStat, 2000). All genotypes were arbitrarily assigned to a resistance class, relative to the very susceptible control 'Florex Gold', which was set at 100,

resulting in an index for macerated leaf disk area (M); 0-5: highly resistant; 6-30: moderately resistant; 31-90: susceptible; 91-100: very susceptible.

3.3 Results

To determine variation for resistance to *E. carotovora* subsp. *carotovora* in *Zantedeschia* spp., a representative collection of plants and a representative isolate of *E. carotovora* subsp. *carotovora* were identified. For the plant collection, representatives of *Zantedeschia* species and a representative group of cultivars of both sections was selected. In this way, a comparison could be made between resistance of genotypes from wild and cultivated accessions. All *E. carotovora* subsp. *carotovora* isolated from the genus *Zantedeschia* that were available at the Dutch Plant Protection Service were included, as well as an isolate of subsp. *atroseptica* from potato.

3.3.1 Variation in aggressiveness of Erwinia *isolates (Experiment 0)*

To identify a representative isolate of *E. carotovora*, six isolates were explored for aggressiveness. Hereto, levels of resistance of three genotypes of *Zantedeschia* to six isolates (Table 3.2) were determined by the leaf disk test. Isolate PD 755, which is of subsp. *atroseptica*, was least aggressive. All isolates could significantly discriminate the three species tested, where *Z. aethiopica* 'Crowborough' was most resistant to all isolates (Figure 3.1) and 'Florex Gold' most susceptible, while *Z. rehmannii* was more resistant than 'Florex Gold' to isolates of subsp. *carotovora* (PD 1784-3886). The ranking of the three species was similar for all isolates, which indicates that interactions are absent between the isolates of subsp. *carotovora* and the three *Zantedeschia* genotypes.

Although isolates PD 3884-3886 were more aggressive, we chose isolate PD 1784 for screening variation in resistance, because interactions were absent and because this isolate was also used in the development of the leaf disk test (Snijder and van Tuyl, 2002), enabling the comparison to previous research.
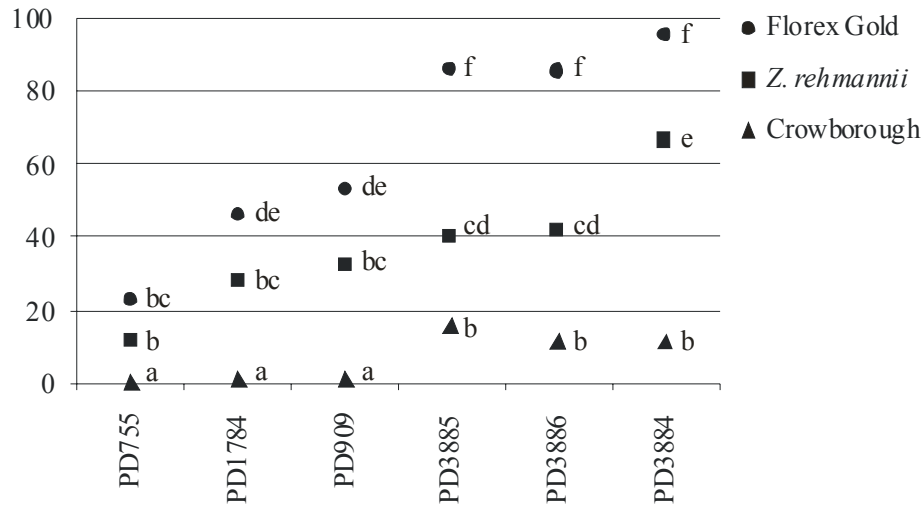
Figure 3.1. Percentage of macerated leaf disk area of three Zantedeschia *genotypes four days after inoculation by six isolates of* Erwinia carotovora. *Letters indicate significant differences (α=0.05). triangle:* Z. aethiopica *'Crowborough' (99008), square:* Z. rehmannii *(99022), circle: 'Florex Gold' (99017).*

Table 3.5. Index of macerated leaf disk area (M) macerated by Erwinia carotovora *subsp.* carotovora *PD 1784 among twelve genotypes of* Zantedeschia, *section* Zantedeschia.

PRI-no	Genotype	M[1,2]		
		2001	2000	Class[3]
99005	*Z. aethiopica* 'Green Goddess'		1a	R
99001	*Z. aethiopica* 'Calla White'		2ab	R
00030	*Z. aethiopica*	3a	8bc	R/MR
99008	*Z. aethiopica* 'Crowborough'	7ab	4ab	R
00901	*Z. aethiopica* 'Highwood'	3a	6abc	R
00054	*Z. aethiopica* 'Pink Mist'	6ab	12c	MR
99027	*Z. aethiopica* 'Giant Vanetti'	7ab	11c	MR
99006	*Z. aethiopica*	10ab		MR
99007	*Z. aethiopica*	10ab		MR
00005	*Z. aethiopica*	20ab	1a	MR
99144	*Z. aethiopica* 'Green Goddess'	28b		MR
99009	*Z. odorata*	50b	15c	S
99017	'Florex Gold'	100c	100d	VS

[1]'Florex Gold' was set at 100, scoring 100% in both years
[2]letters indicate significant differences within the year by Student's t-test of pairwise differences (α=0.05)
[3]assigned resistance levels, where 'R' stands for highly resistant, 'MR' for moderately resistant, 'S' for susceptible and 'VS' for very susceptible

3.3.2 Variation in resistance between accessions of the section Zantedeschia (Experiments 1 and 4)

To evaluate the section *Zantedeschia* for variation in resistance to *E. carotovora* subsp. *carotovora,* accessions were screened using the leaf disk test in two experiments in two successive years (Table 3.5). All accessions showed a similar level of resistance in both experiments and were more resistant than 'Florex Gold'. The accessions could be classified in three levels of resistance from almost completely resistant for accession 99005 to susceptible for *Z. odorata* in 2000 and 2001 (Table 3.5).

3.3.3 Variation in resistance between accessions of the section Aestivae (Experiments 2, 3, 5, 6, and 7)

To verify whether cultivars of the section *Aestivae* are susceptible, 21 cultivars were tested for resistance to *E. carotovora* subsp. *carotovora* using the leaf disk test. The duplicate five cultivars (Table 3.1b) that were included during two seasons did not score significantly different (Table 3.6a), indicating that the results of both experiments were reproducible and that the levels of resistance among cultivars from different sources are similar. Only 'Black Magic' (99011) and *Z. rehmannii* (99022) scored differently over both trials. 'Black Magic' (99011) was very susceptible (103) in 2001 and susceptible (37) in 2000, while *Z. rehmannii* (99022) was moderately resistant (8) in 2001 and susceptible (62) in 2000, which was not observed in other experiments (Table 3.6b). Cultivars 'Florex Gold', 'Treasure' and 'Galaxy' were most susceptible, while 'Neroli', 'Hazel Marie' and 'Coral Sunset' were least susceptible (Table 3.6a). In summary, some cultivars were as resistant as *Z. rehmannii* (99022), but most cultivars of the section *Aestivae* were as susceptible as the very susceptible control 'Florex Gold' and all were classified as susceptible or very susceptible (Table 3.6a).

Apparently, little resistance to *E. carotovora* subsp. *carotovora* is present among *Aestivae* cultivars. Wild germplasm was therefore screened for resistance. Most of these wild accessions were more resistant than the susceptible control 'Florex Gold' (Table 3.6b), while *Z. elliotiana* (99004), *Z. pentlandii* (00069-2) and *Z. elliotiana* (00073) were as susceptible as 'Florex Gold'. Some of the wild accessions showed a similar resistance level as *Z. rehmannii* (99022), such as *Z. rehmannii* (00074) and *Z. albomaculata* (00031, 00056,

018004, 018006). Other wild accessions appeared more resistant than *Z. rehmannii* (99022), such as *Z. rehmannii* (00057) and *Z. albomaculata* (00058, 018002).

Table 3.6. Index of macerated leaf disk area (M) of Zantedeschia *spp. of the section* Aestivae *cultivars (a) and wild accessions (b) after inoculation by* Erwinia carotovora *subsp.* carotovora *PD1784.*

a.

PRI-no	Cultivar/genotype	$M^{1,2}$		
		2001	2000	Class[3]
99022	*Z. rehmannii*	8a	62ab	S
00049	Neroli	30ab		S
00040	Coral Sunset	38abc		S
00051	Hazel Marie	41abc		S
00001	Shadow	52bc		S
00038	Black Eyed Beauty	53bc		S
00033	Chianti	61bc		S
00047	Ruby	66bcd		S
00035	Fandango	69bcd		S
00036	Pink Persuasion	60bcd	43ab	S
99014	Pink Persuasion	74bcd	93bc	S
00043	Best Gold	69bcd	43ab	S
99010	Best Gold	84bcd	72abc	S
00045	Hot Shot	76cd		S
00050	Celeste	77cd		S
99003	Majestic Red		74ab	S
00042	Sensation	82cd	32ab	S
99015	Sensation	94cd	86b	S
00039	Cameo	87cd		S
00048	Black Magic	66bcd	72b	S
99011	Black Magic	103d	37ab	S
00034	Galaxy	94cd	103bc	VS
99012	Galaxy	95d	99bc	VS
00041	Mango	100d		VS
99016	Treasure	102cd	99bc	VS
00052	Treasure		91bc	VS
99017	Florex Gold	100d	100bc	VS
00046	Florex Gold		100bc	VS

b.

PRI-no	Cultivar/Genotype	M[1,2]			Class[3]
		2002	2001	2000	
99022	*Z. rehmannii*	48ab	44a	62ab	S
00057	*Z. rehmannii*	24a	29a	57ab	MR
00058	*Z. albomaculata* hybrid		27a	26a	MR
018002	*Z. albomaculata* subsp. *macrocarpa*	10a			MR
00063	*Z. rehmannii*			39a	S
00074	*Z. rehmannii*	39ab	34a		S
018007	*Z. rehmannii*	59b			S
018008	*Z. rehmannii*	65b			S
018006	*Z. albomaculata* subsp. *macrocarpa*	48ab			S
00031	*Z. albomaculata* subsp. *albomaculata*	55ab	45a		S
00056	*Z. albomaculata* subsp. *albomaculata*	53ab	26a		S
00060	*Z. albomaculata* subsp. *albomaculata*			56ab	S
018004	*Z. albomaculata* subsp. *albomaculata*	53ab			S
00075	*Z. albomaculata* subsp. *albomaculata*	63b			S
00076	*Z. albomaculata* subsp. *albomaculata*	75bc	56a		S
00061	*Z. albomaculata* subsp. *albomaculata*			81b	S
00006-1	*Z. albomaculata*	46ab	119b		S
018001	*Z. albomaculata* 'Helen O Connor'	63b	103b		S
018005	*Z. albomaculata* subsp. *albomaculata*	68b	134b		S
018009	*Z. albomaculata* subsp. *albomaculata*	69b	127b		S
00073	*Z. elliotiana*	40ab	68ab		S
00062-1	*Z. elliotiana*			84b	S
018010	Solfatare		94b		VS
99004	*Z. elliotiana*			141bc	VS
00069-2	*Z. pentlandii*			122c	VS
99017	Florex Gold	100c	100b	100c	VS

[1]'Florex Gold' (99017) was set at 100, scoring 69% in 2000, 62% in 2001 and 80% in 2002
[2]letters indicate significant differences within the year by Student's t-test of pairwise differences (α=0.05)
[3]assigned resistance levels, where 'MR' stands for moderately resistant, 'S' for susceptible and 'VS' for very susceptible

Much variation for resistance was observed within *Z. rehmannii* and *Z. albomaculata*. *Z. rehmannii* (00057) was significantly more resistant than other *Z. rehmannii* (018007 and 018008), while some *Z. albomaculata* (018002, 00058) were more resistant than other *Z. albomaculata* (00075, 018005 and 018009). In conclusion, accessions from the wild germplasm showed a high variation for resistance and some may be useful genitors for resistance breeding programmes.

3.3.4 Genetic variation within accessions of the section Aestivae

Variation for resistance might not only be present between, but also within accessions. Since many of the wild accessions were seed-propagated (Table 3.1b), it can be expected that there was genetic variation among plants within these accessions. To determine the genetic variation of a seed-propagated accession, the variances within seed-propagated accessions were compared to the variances of reference clones (Table 3.7), which are genetically homogenous and hence show only environmental variation.

The variances of reference clones 'Galaxy' and 'Florex Gold' were larger than the variance of *Z. rehmannii* (99022) (Table 3.7). Apparently, the environmental variation is not constant and may be dependent on genotype. Seed-propagated accessions were therefore only compared to the reference clones that were most related. Accessions of *Z. rehmannii* were compared to clone *Z. rehmannii* (99022), *Z. albomaculata* to 'Galaxy' and *Z. elliotiana* to 'Florex Gold'.

The variance of *Z. rehmannii* (00074) was larger than the variance of *Z. rehmannii* (99022), so it can be concluded that there was genetic variation within *Z. rehmannii* (00074). Similarly, there was genetic variation within *Z. albomaculata* (00075) and in the population (008047), since their variances were larger than the variances of the susceptible references 'Galaxy' and 'Florex Gold'. It is however not evident if there was genetic variation within any of the other accessions, since their variances were not different from 'Galaxy'.

Table 3.7. Within-accession variance of percentage of leaf disk tissue macerated by Erwinia carotovora subsp. carotovora *PD 1784 (logit scale).*

PRI-no	Genotype	Variance[1]	Degrees of freedom	Prop[2]	Descent[3]
99017	'Florex Gold'	1.41a	20	Clone	E
00073	*Z. elliotiana*	1.08a	16	Seed	E
99012	'Galaxy'	0.62a	7	Clone	R×A
00075	*Z. albomaculata*	7.52b	13	Seed	A
018009	*Z. albomaculata*	1.36a	5	Seed	A
018006	*Z. albomaculata* subsp. *macrocarpa*	0.88a	8	Seed	A
008047	*Z. rehmannii* × 'Pink Persuasion'	9.02b	10	Seed	{R×(R×A)}
99022	*Z. rehmannii*	0.06a	6	Clone	R
00074	*Z. rehmannii*	0.63b	13	Seed	R
018007	*Z. rehmannii*	0.39b	8	Seed	R
018008	*Z. rehmannii*	0.13a	11	Seed	R

[1] letters indicate significant differences within descent group by F-test at α=0.05
[2] mode of propagation, 'seed'= raised from seed, 'clone'= raised from vegetatively propagated plants
[3] descent as determined from morphology, 'R'=*Z. rehmannii*, 'A'=*Z. albomaculata*, 'E'=*Z. elliotiana*, 'R×A'=hybrid descent of *Z. rehmannii* and *Z. albomaculata*

In conclusion, some accessions of the section *Aestivae* were identified that could be used as genitors in breeding for resistance to soft rot caused by *E. carotovora* subsp. *carotovora*. These include moderately resistant accessions *Z. albomaculata* (00058), *Z. albomaculata* subsp. *macrocarpa* (018002) and *Z. rehmannii* (00057). Also accession *Z. rehmannii* (00074) is a promising source of resistance, for it showed a low level of susceptibility and proved to have a high within-accession genetic variation.

3.4 Discussion

To our knowledge, no scientific study on *Erwinia* resistance in *Zantedeschia* spp. has been undertaken before. To identify the most informative isolate for this study, six *E. carotovora* isolates were compared. Isolate *E. carotovora* subsp. *atroseptica* PD 755 appeared the least aggressive and variation in aggressiveness was found between five isolates of *E. carotovora* subsp. *carotovora* (Figure 3.1). *E. carotovora* subsp. *atroseptica* was not expected to be aggressive on *Zantedeschia* spp, because it is primarily a potato pathogen (Pérombelon and

Salmond, 1995). Differences in aggressiveness between isolates of *E. carotovora* subsp. *carotovora* were also found by many other workers (McIntyre et al., 1978; Smith and Bartz, 1990; Darling et al., 2000; Ren et al., 2001) and reflect the large genetic variation in this pathogen (Avrova et al., 2002; Seo et al., 2002). Although PD 1784 was selected, any aggressive and discriminating isolate can be used in a resistance screening test, since interactions between isolates and host genotypes have not been proven before (Ren et al., 2001) and was neither proven in this study.

The results were reproducible and in concordance with Brown (1988) and Shibuya (1956), who both stated but did not prove that *Z. elliotiana* is more susceptible than *Z. rehmannii* and *Z. albomaculata*. Furthermore, the high level of susceptibility of 'Galaxy' was also observed by Funnell and MacKay (1999).

No complete resistance was observed in any of the tested *Zantedeschia* accessions. Within the section *Zantedeschia*, variation was observed among accessions of *Z. aethiopica,* while *Z. odorata* was as susceptible as the most susceptible *Z. aethiopica*. *Z. aethiopica* is a variable species with a great area of distribution (Letty, 1973) and this variability can be the cause for the high variation in resistance level of this species. In contrast, it is not likely that there is much variation within *Z. odorata*, since it is known from only one location (Perry, 1989).

Z. aethiopica is more resistant than cultivars from the section *Aestivae*. All accessions of *Z. aethiopica* showed some degree of susceptibility, but none was as susceptible as any of the cultivated *Aestivae* accessions. The resistance level of the most susceptible *Z. aethiopica* accession was comparable to that of the most resistant wild *Aestivae* accession. Possibly, two different distributions of resistance occur in *Z. aethiopica* and the section *Aestivae*, which could reflect different but still unknown mechanisms of resistance in both groups.

In conclusion, sources of resistance were found that have not yet been used in breeding for resistance to soft rot caused by *E. carotovora* subsp. *carotovora*. These include some accessions of *Z. rehmannii,* some accessions of *Z. albomaculata* subsp. *albomaculata,* and *Z. albomaculata* subsp. *macrocarpa*. The genetic variation in *Z. rehmannii* (00074) reflects potentials for resistance breeding in *Zantedeschia*.

4 Plastome-genome incompatibility and biparental plastid inheritance affect vigour of interspecific hybrids of *Zantedeschia* spp. (*Araceae*), section *Aestivae*

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Abstract

The combination of alien plastomes and genomes does not result in functional green interspecific hybrids in all plant genera; hybrids in some genera suffer from incompatibility between the parental plastomes and genomes. The aim was to determine plastid inheritance and plastome-genome incompatibility (PGI) among species of *Zantedeschia* from the section *Aestivae*. To this end, plastomes and genomes of four species (*Z. albomaculata*, *Z. elliotiana*, *Z. pentlandii* and *Z. rehmannii*) were combined by interspecific hybridisation. Plastomes were differentiated using plastome specific CAPS-markers. Degrees of PGI existed between the hybrid genomes of *Z. rehmannii* and *Z. albomaculata*, *Z. rehmannii* and *Z. elliotiana*, *Z. rehmannii* and *Z. pentlandii* and the plastomes of *Z. albomaculata*, *Z. elliotiana* and *Z. pentlandii*, respectively. The plastome of *Z. rehmannii* appeared compatible to these hybrid genomes. The plastomes of *Z. albomaculata* and *Z. elliotiana* appeared compatible to the hybrid genome of *Z. albomaculata* and *Z. elliotiana*. Plants that suffered from PGI exhibited a decreased vigour as judged from tuber weight, plant length, leaf number and chlorophyll content. Three plastomes were differentiated among the section *Aestivae*. Biparental plastid inheritance (3 to 90 %) was observed among all crossing combinations, which produced more than 1600 interspecific offspring. Biparental inheritance of plastids thus appeared to be a general phenomenon among interspecific hybrids within the section *Aestivae*. A literature review on plastid inheritance in interspecific hybrids among angiosperms shows that only *Oenothera* spp. exhibit such a wide range of degrees of biparental plastid inheritance. Possible evolutionary significance of the observed phenomena is discussed.   (Submitted to Theoretical and Applied Genetics in modified form)

4.1 Introduction

Since the plastid's and mitochondrion's bacterial endosymbiotic origin (Mereschowsky, 1905; Goksoyr, 1967), the genetic material of the symbionts has re-organised enormously. Hundreds of genes have migrated from the plastidial genome (further: plastome) and have integrated in the nuclear genome (Martin et al., 2002; Huang et al., 2003) (further: genome). The mitochondrion, plastid and nucleus have thus co-evolved a close metabolic interaction where many mitochondrial and plastid proteins are encoded in the nucleus (Leister, 2003).

A taxonomically distant plastid transfer results in functional green plants in most genera (Zubko et al., 2001). In some species, however, plastomes and genomes have co-evolved to such a degree that they cannot be exchanged interspecifically. This plastome-genome incompatibility (PGI) has been reported in *Oenothera* (Hupfer et al., 2000), *Rhododendron* (Michishita et al., 2002) and *Zantedeschia* (Yao et al., 1994a). Plants that suffer from PGI show leaves with varying degrees of chlorophyll deficiency, reflecting different levels of incompatibility (Kirk and Tilney-Bassett, 1978). Mechanisms and the evolutionary significance of PGI remain unclear, but PGI favours speciation in *Oenothera* spp. (Hupfer et al., 2000).

Although plastids inherit mainly maternally in flowering plants (Birky, 2001), the mode of plastid inheritance is not strict; it has shown reversals and parallel changes (Birky, 1995). Many mechanisms governing uniparental inheritance of organelles exist (Birky, 2001), the molecular basis is however poorly understood. In *Chlamydomonas reinhardtii,* methylation of the inherited plastome plays a central role in the preferential digestion of the not-inherited plastome (Umen and Goodenough, 2001), but the precise mechanism is unclear. The molecular background of mechanisms that differentiate the parental organelles in flowering plants is not known. It is possible that mechanisms that preferentially exclude or divide the parental organelles do not work as efficiently in interspecific crosses, as was demonstrated for mitochondria in interspecific hybrids of mice and as probably also exists in interspecific hybrids of cows (Birky, 2001). It can therefore not be concluded a priori that the modes of plastid inheritance in intraspecific hybrids and interspecific hybrids are similar.

Considerable numbers of offspring are needed for determining the mode of plastid inheritance. Over 600 offspring are needed to conclude with a power of 95% that the frequency of uniparental inheritance is > 99.5% (Milligan, 1992). Many studies have concluded a maternal inheritance in interspecific hybrids, but only one (*Epilobium* spp.) had sufficient number of offspring to conclude it reliably (Table 4.1). It can therefore not be ruled out that some degree of biparental plastid inheritance occurs in any interspecific hybrid, since plastids can inherit biparentally in interspecific hybrids among many flowering plant genera (Table 4.1).

Table 4.1. Plastid inheritance in interspecific F[1] hybrids among flowering plants; percentage of plants showing maternal (M) and biparental (B) or paternal inheritance (P), determined with genetic evidence; and the power of the test that plastids inherit >99.5 % uniparental (ß)

Mother	Father	M	B	P	N	ß[2]	Reference
Actinidia 4 spp.	*A.* 5 spp.			100	119	0.45	Cipriani et al., 1995; Testolin and Cipriani, 1997
Brassica 3 spp.	*B.* 3 spp.	100			124[1]	0.46	Song et al., 1993; Oszmin-kowski and Jourdan, 1994
Brassica rapa	*B. oleracea*	90	10		10		Song et al., 1993
Bromus arvensis	*B.* 2 spp.	100			28	0.13	Pillay and Armstrong, 2001
Chlorophytum elatum	*C. comosum*	97	2.7	0.3	600[1]		Kirk and Tilney-Bassett, 1978
Coffea arabica	*C. canephora*	100			?[1]		Lashermes et al., 1996
Daucus carota subsp. *sativus*	*D. muricatus*	100			?[1]		Steinborn et al., 1995
Epilobium 5 spp.	*E.* 5 spp.	100			>2000	~1	Schmitz and Kowallik, 1986
Epilobium watsonii	*E. montanum*	Frequ ently	Rar ely		2000		Schmitz and Kowallik, 1986
Eucalyptus globulus	*E. nitens*	100			231	0.69	McKinnon et al., 2001
Festuca pratensis	*Lolium perenne*	100			14	0.07	Kiang et al., 1994
Festuca pratensis × *Lolium perenne*	*Lolium perenne*	50	50		2		Kiang et al., 1994
Fraxinus excelsior	*F. angustifolia*	100			17	0.08	Morand-Prieur et al., 2002
Geranium bohemicum	*G. lanuginosum*		+		?[1]		Dahlgren, 1923; 1925
Glycine tabacina	*G. canescens*	100			?		Harris and Ingram, 1991
Gossypium 4 spp.	*G.* 4 spp.	100			?		Wendel, 1988; Galau and Wilkins, 1989
Helianthus annuus	*H.* 2 spp.	100			108	0.42	Rieseberg et al., 1994
Hypericum tetrapterum	*H. montanum*	+	+	+	>500		Noack, 1932
Hordeum 2 spp.	*Secale* 3 spp.	+	+	+	7		Soliman et al., 1987
Iris fulva	*I. hexagona*	97	2	1	100		Cruzan et al., 1993
Larrea 2 spp.	*L.* 2 spp.			100	20	0.10	Yang et al., 2000
Lens lamottei	*L. nigricans*	50	50		8		Van Oss et al., 1997
Liriodendron tulipifera	*L. chinense*	97		3	34		Sewell et al., 1993

Table 4.1 (continued)

Lotus corniculatus	*L. alpinus*	100			~200[1]	0.63	Gauthier et al., 1997
Magnolia tripetala	*M. fraseri*	89		11	9		Sewell et al., 1993
Nicotiana plumbaginifolia	*N. tabacum*	99.93	0.07		1500		Medgyesy et al., 1986
Oenothera 2 spp.	*O. hookeri*	85	15		455		Chiu and Sears, 1993
Oenothera atrovirens	*O. hookeri*	14.3	85.7		196		Chiu and Sears, 1993
Oenothera hookeri	*O. ammophila*	94.1	5.9		438		Chiu and Sears, 1993
Oenothera parviflora	*O. hookeri*	23.9	77.1		218		Chiu and Sears, 1993
Oryza 2 spp.	*O.* 2 spp.	100			?		Dally and Second, 1990
Phalaenopsis spp.	*Doritis pulcherrima*	100			?		Chang et al., 2000
Phlox drummondii	*P. cuspidata*	100			62[1]	0.27	Ferguson et al., 1999
Poncirus trifoliata	*Citrus reticulata × C. paradisi*	100			26	0.12	Moreira et al., 2002
Populus deltoides	*P.* 2 spp.	100			50	0.22	Rajora and Mahon, 1995
Rhododendron japonicum	*R.* 11 spp. (section *Tsutsusi*)	>95	<5		?		Smith, 1988
Rhododendron kiusianum (section *Tsutsusi*)	*R. japonicum* f. *flavum*	73		27	45		Ureshino and Miyajima, 2002
Rhododendron serpyllifolium (section *Tsutsusi*)	*R.* spp. (series *Kaempferia*)	79	5	16	199[1]		Ureshino and Miyajima, 2002
Stellaria longifolia	*S. porsildii*	100			24	0.11	Chong et al., 1994
Stellaria porsildii	*S. longifolia*	67	17	17	12		Chong et al., 1994
Triticum 3 spp.	*Secale cereale*	100			?		Vedel et al., 1981
Z. odorata	*Z. aethiopica*	8	23	69	26		Yao et al., 1994a; Yao and Cohen, 2000
Zantedeschia aethiopica	*Z. odorata*	100			60	0.26	Yao et al., 1994a; Yao and Cohen, 2000
Zantedeschia aethiopica	Cultivars (section *Aestivae*)	69	31		13		Yao et al., 1994a
Zantedeschia spp. (section *Aestivae*)	*Z. aethiopica*	25	75		4		Yao et al., 1994a
Zea mays	*Z. perennis*	100			?[1]		Conde, 1979

[1] including reciprocals, no reciprocal difference

[2] Power of the test that plastid inheritance is >99.5% uniparental, assuming a binomial distribution so $\beta = 1 - (1 - 0.995)^n$

Biparental plastid inheritance and PGI were recently demonstrated in interspecific hybrids of the monocotyledonous genus *Zantedeschia* as well (Yao et al., 1994a; 1995). The range of biparental plastid inheritance appears to be very broad in *Zantedeschia* interspecific hybrids (0 - 90 %; Table 4.1). Only in *Oenothera* spp. such a wide range of biparental plastid inheritance patterns (5 - 95 %) has been demonstrated in interspecific hybrids (Chiu and Sears, 1993). *Zantedeschia* spp. may therefore be as appropriate as *Oenothera* spp. for studying biparental inheritance, plastome evolution and PGI.

The genus *Zantedeschia* consists of eight species in two sections. The section *Zantedeschia* consists of *Z. aethiopica* and *Z. odorata*, while the section *Aestivae* consists

of *Z. albomaculata*, *Z. elliotiana*, *Z. jucunda*, *Z. pentlandii*, *Z. rehmannii* and *Z. valida* (Singh et al., 1996). The plastomes and genomes of *Z. aethiopica* and cultivars of the section *Aestivae* were demonstrated to be incompatible to such a high degree that hybrids were chlorophyll deficient (albino) and could only survive heterotrophically. Some albinos had inherited their plastids biparentally (Yao et al., 1995). Partial PGI exists between the plastome of *Z. aethiopica* and the hybrid genome of *Z. aethiopica* and *Z. odorata*. Some hybrids of *Z. aethiopica* and *Z. odorata* inherited their plastids biparentally (Yao and Cohen, 2000). It is likely that PGI and biparental plastid inheritance exists among the section *Aestivae* as well, since non-Mendelian inheritance of leaf colour and hybrid variegation were documented (New and Paris, 1967).

In order to study the co-evolution of plastomes and genomes in the section *Aestivae*, PGI and plastid inheritance were determined in reciprocal interspecific hybrids of four species (*Z. albomaculata*, *Z. elliotiana*, *Z. rehmannii* and *Z. pentlandii*). Plastome-specific CAPS markers were used to verify PGI. Plastid inheritance was determined in about 1600 interspecific hybrids. The consequence of PGI for plant vigour was determined by assessing various growth characteristics.

4.2 Materials and Methods

4.2.1 Plant material and cultivation

Accessions of *Aestivae* species were obtained from nurseries and a botanical garden (Table 4.2).

Table 4.2. Plant material used for producing interspecific hybrids within section Aestivae *of* Zantedeschia.

PRInr	Code	Genotype	Source[1]
00060	Aa1	*Z. albomaculata* subsp. *albomaculata*	N, The Netherlands
00061	Aa2	*Z. albomaculata* subsp. *albomaculata*	N, The Netherlands
00031	Aa3	*Z. albomaculata* subsp. *albomaculata*	G, South Africa
00056	Aa4	*Z. albomaculata* subsp. *albomaculata*	N, South Africa
00062-1	E1	*Z. elliotiana*	N, The Netherlands
99004	E2	*Z. elliotiana*	N, The Netherlands
00069-2	P	*Z. pentlandii*	N, The Netherlands
00063	R1	*Z. rehmannii*	N, The Netherlands
00078	R2	*Z. rehmannii*	N, The Netherlands
99022	R3	*Z. rehmannii*	N, New Zealand
00057	R4	*Z. rehmannii*	N, South Africa

[1]N=nursery, G=botanical garden

All plants were raised in poor soil in a greenhouse with temperatures ranging from 20-30ºC during the day and 15-18ºC during the night. Seedlings were raised in standard potting soil in the first growing season and in poor soil in the second growing season. Tubers were lifted after leaves had withered completely and the tubers were surface-dried in a ventilated climate room (18ºC, 30 % relative humidity (RH)) before storage (9ºC, 70 % RH). Before planting, tubers were pre-treated at 18ºC for four weeks. Parental plants were promoted to flower by incubating tubers 10 min in 100 ppm GA_3 (Berelex, Bayer) just before planting.

4.2.2 Interspecific hybridisation

Flowers were pollinated at opening of the spathe after emasculation. Hybridisations were carried out in the greenhouse (with day temperatures ranging from 20-30ºC and night temperatures from 15-18ºC) with fresh pollen using a 70 %-ethanol sterilised brush. Fruits were harvested after the plants had wilted, seeds were collected from ripe fruits and directly sown in standard potting soil. The hybrid character was judged from plant morphology and the origin of the plastomes was verified using plastome specific CAPS markers.

4.2.3 Species specific plastid CAPS markers

DNA extraction

Total DNA was extracted from ca. 0.3 g lyophilised young leaf material that was ground using the RETCH method (Qiagen). DNA was extracted from each sample by thoroughly mixing with 400 μl extraction mix (0.13 M Tris.HCl, 0.02 M EDTA, 0.9 M NaCl, 0.9 % CTAB, 0.15 M sorbitol, 5 % $Na_2S_2O_5$, 0.6 % sarkosyl) and incubating for one hour at 65ºC. After applying one volume chloroform/isoamylalcohol (24:1 v/v), the resulting suspension was centrifuged for 20 min at 5500 g. DNA was precipitated from the supernatant by one volume ice-cold (-20ºC) isopropanol and collected by centrifuging for five min at $21^{.}10^3$ g After discarding the supernatant, the DNA was washed in 70 % ethanol and suspended in TE (1M Tris, 0.1 M EDTA).

To develop plastome specific CAPS (cleaved amplified polymorphic sequence), two plastid intergenic regions were screened for restriction site polymorphisms.

Primers

The intergenic region of *psb*C and *trn*S, (about 1.6 kb, further called CS) was amplified by PCR using primer pair CS as described by Demesure et al. (1995). The intergenic region of *trn*D and *trn*C (about 3.1 kb, further called DC) was amplified by PCR using primer pair DC_{ron} (DC_{ron-F}: 5'-AGAGCACCGCCCTGTCAAG-3' and DC_{ron-R}: 5'-GCATGGCCRAGYGGTAAGG-3'). DC_{ron} was designed after alignment of flowering plant sequences of *trn*C and *trn*D using Primerselect (DNASTAR). The sequences were obtained from GenBank (NCBI).

PCR amplification

The polymerase chain reaction was carried out in 25 µl volume (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 3.5 mM $MgCl_2$, 0.1 % Triton X-100, 1 mM dNTP (Invitrogen), 5 pM CS primers for CS, 5 pM DC_{ron-F} for DC, 10 pM DC_{ron-R} for DC, 0.5 U SuperTaq (HT Biotechnology), 0.05 U *Pfu* polymerase (Promega; only for DC) and 100-500 ng template DNA). *Pfu* polymerase was added for amplification of the 3.1 kb DC-amplicon, for *Pfu* polymerase was demonstrated to improve the amplification of products up to 35 kb (Barnes, 1994). The MJ Research PTC-200 thermal cycler was programmed to carry out a temperature profile of three min at 94ºC, 40 cycles of one min 94ºC, one min at 55ºC (for CS) or 64.5ºC (for DC), and one min 72ºC (for CS) or 3.5 min (for DC), with a final fifteen min at 72ºC.

Restriction digestion of PCR products

The intergenic regions were assessed for restriction site polymorphisms by digesting 10 µl PCR product overnight by 10U of the restriction enzyme. CS was digested by *Alu*I, *Dra*I, *Hae*III, *Hpa*II, *Mse*I, *Pst*I, *Pvu*II, *Rsa*I and *Taq*I (Invitrogen). DC was digested by *Alu*I and *Hae*III. Digested PCR products were separated on agarose gels (Invitrogen, Ultra Pure, electrophoresis grade).

4.2.4 Growth characteristics

Growth characteristics (plant length, number of leaves and chlorophyll content) were observed at two months after planting. The tuber weight was determined during planting. Chlorophyll content was determined of the youngest leaf by a SPAD-502 instrument (Minolta Camera Ltd.). This instrument estimates leaf chlorophyll content in situ, by transmitting light through a leaf at wavelengths of 650 and 940 nm. The signal of 650 nm is absorbed by chlorophyll, while the signal of 940 nm is not. The ratio of transmittance of both signals through the leaf is a measure for the amount of chlorophyll, resulting in a unitless SPAD (Soil Plant Analysis Development)-value. Three measurements were made on the abaxial side of the upper half of the oldest leaf, two months after planting and averaged to reduce variability (Adamsen et al., 1999).

4.2.5 Statistical analysis

Growth characteristics were analysed by generalised linear models (GLMs). The chlorophyll content (measured in SPAD units) and plant length were estimated assuming a normal distribution, tuber weight by assuming a lognormal distribution and the number of leaves assuming a Poisson distribution. All analyses were done with the statistical software package Genstat 6, release 6.1 (GenStat, 2002).

4.2.6 Determination of the plastome of interspecific F_1 hybrids

The plastome types of interspecific F_1 hybrids were determined using plastome-specific CAPS-markers and by judging from the leaf morphology. The plastome types in a part of each progeny of all hybrid combinations were verified by CAPS markers and associated with leaf morphology and chlorophyll content. Leaf morphology and chlorophyll content appeared to be distinctive for the plastome in all hybrid combinations, so the plastome types of individual interspecific hybrids were determined by judging from the leaf morphology and chlorophyll content as listed in Table 4.5.

4.3 Results

Reciprocal interspecific F_1 hybrids of four species were generated to determine plastid inheritance and directions of PGI within the section *Aestivae*. Seeds germinated irregularly. Germination was poor (0-1%) in some crossing combinations (E1×Aa1 and Aa2×E), while seeds germinated very well (up to 100%) in other combinations (E2×R3 and R3×Aa4) (Table 4.3). Seeds from the selfing of Aa2 germinated very well (100%), while germination percentages were low in the selfings of R1 (31%) and P (3%). Therefore, low germination percentages of interspecific hybrids cannot a priori be attributed to incompatibility of crossing combinations.

Table 4.3. Germination percentages of F_1 hybrid families of four species of section Aestivae.

Mother	Father					
	R1	R2	Aa1	Aa2	E1	P
Z. rehmannii (R1)	31		56	77	47	42
Z. albomaculata (Aa1)	42		79	97	62	29
Z. albomaculata (Aa2)	20	93		100	1	
Z. elliotiana (E1)	36	35	0	79	84	75
Z. pentlandii (P)			88			3

Mother	Father				
	R3	R4	Aa3	Aa4	E2
Z. rehmannii (R3)			91	98	100
Z. rehmannii (R4)			83	98	
Z. albomaculata (Aa3)	79				
Z. albomaculata (Aa4)		90			
Z. elliotiana (E2)	99				

4.3.1 Plastome-genome incompatibility between Aestivae species

To determine the plastome composition of hybrid plant tissue, species specific CAPS markers were developed from two plastidial intergenic regions. Sequence variations between the species were demonstrated on the intergenic region of *trn*D and *trn*C (DC) by polymorphic restriction patterns of *Alu*I and *Hae*III, while no polymorphism could be demonstrated on the intergenic region of *trn*S and *psb*C (Table 4.4).

Table 4.4. CAPS: Alu*I- and* Hae*III-restriction patterns of the plastid* trn*D-*trn*C intergenic region from* Zantedeschia albomaculata subsp. albomaculata, Z. elliotiana, Z. rehmannii *and* Z. pentlandii

Species	Accessions	Restriction pattern (in kb)	
		*Alu*I	*Hae*III
Z. rehmannii	R1, R3	1.6, 1.25, 0.21	2.1, 0.37, 0.37
Z. albomaculata subsp. *albomaculata*	Aa1, Aa2	1.55, 1.25, 0.21, 0.05	2.1, 0.37, 0.37
Z. elliotiana and *Z. pentlandii*	E1, P	1.7, 1.35	1.1, 0.97, 0.41, 0.36

Z. rehmannii (R1, R3) and *Z. albomaculata* subsp. *albomaculata* (Aa1, Aa2) demonstrated similar DC-*Hae*III restriction patterns and could be differentiated by the *Alu*I polymorphism of the largest and smallest fragment (Table 4.4 and Figure 4.1). Both *Z. elliotiana* and *Z. pentlandii*, which did not demonstrate any polymorphisms and could therefore not be differentiated, showed a DC-*Alu*I and a DC-*Hae*III restriction pattern that was different from *Z. rehmannii* and *Z. albomaculata* subsp. *albomaculata* (Table 4.4).
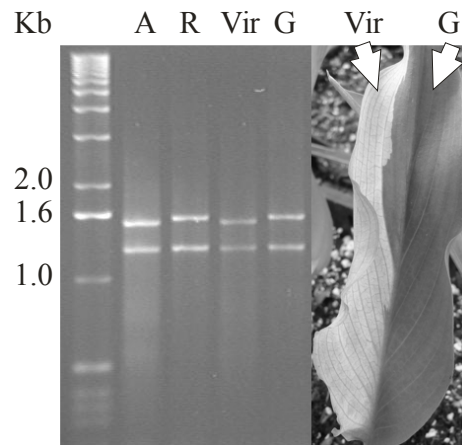

Figure 4.1. CAPS-markers of Z. rehmannii *(R) and* Z. albomaculata *subsp.* albomaculata *(A) and their chimerical (virescent (Vir)-green (G)) hybrid, retrieved after PCR of the plastidial intergenic spacer of* trn*D and* trn*C and digestion by* Alu*I*

Progeny originating from self-pollinations did not segregate for leaf colour, indicating that plastome-genome interactions within species were compatible. All interspecific progenies, however, segregated in three classes of leaf colour: green, variegated, and virescent or pale

green. Virescence occurs when a leaf is initially pale or yellow, but turns green while ageing and can result from incompatible plastome-genome combinations (Kirk and Tilney-Bassett, 1978).

The species specific CAPS markers were used to determine whether the different leaf morphologies corresponded to different plastome types (Table 4.5). Green tissue in hybrids with R1 as one of the parents showed the CAPS marker of R1 (the 1.6 kb DC-*Alu*I fragment, Table 4.4 and Figure 4.1). Tissue from totally virescent plants and from virescent sectors of chimerical plants showed the restriction pattern of the other parent (Figure 4.1), indicating that indeed PGI causes the virescence. The level of virescence depended on the genomic background: yellow leaf tissue turned green fast in R1Aa1 progeny, while yellow tissue in hybrids of R1E1 progeny turned green slowly.

Table 4.5. Association between plastome-genome combination as determined by CAPS-markers and leaf morphology in interspecific hybrids of Zantedeschia rehmannii *(R1),* Z. albomaculata *subsp.* albomaculata *(Aa1, Aa2),* Z. elliotiana *(E) and* Z. pentlandii *(P).*

Cross	Leaf morphology	Plastome	# plants
R1 × Aa1	Green	R1	20
Aa1 × R1	Virescent	Aa1	9
	Variegated (green and virescent sectors)	R1 (green sector) + Aa1 (virescent sector)[1]	2
R1 × Aa2	Green	R1	4
R1 × E1	Green	R1	2
	Variegated (green and virescent sectors)	R1 (green sector) + E1 (virescent sector)[1]	4
R1 × P	Green	R1	10
	Variegated (green and virescent sectors)	R1 (green sector) + P (virescent sector)[1]	2
Aa1×E1	Green	Aa1	13
	Variegated (green + pale green sectors)	Aa1 (green) + E1 (pale green)[2]	5
Aa2 × E1	Green	Aa2	1
E1 × Aa2	Green	Aa2	1
	Variegated (green + pale green sectors)	Aa2 (green) + E1 (pale green)[2]	13

[1]Green sectors contained plastids of *Z. rehmannii*, virescent sectors contained plastids of the other parent (Figure 4.1)
[2]Green sectors contained plastids of *Z. albomaculata* subsp. *albomaculata*, pale green sectors contained plastids of *Z. elliotiana*

Aa2E1 progeny were not virescent, since lighter coloured tissue did not change intensity while it aged. Leaf tissue of Aa2E1 progeny demonstrated two leaf colours: green and pale green, and plants were green, pale-green, or variegated with green and pale green sectors. Green tissue contained the plastome of Aa2 and the pale green tissue contained the plastome of E1 (Table 4.5). Although pale green plants on average contained less chlorophyll than green plants (Table 4.6), the SPAD values of individual plants overlapped, so plastomes of individual plants could not unambiguously be determined using chlorophyll content.

4.3.2 Effects of plastome-genome incompatibility to vigour

To verify whether PGI affected plant vigour, F_1 hybrids were assessed for tuber weight, plant height, number of leaves and chlorophyll content (Table 4.6). Green plants in the progeny of R1 and Aa1, which contained the plastome of R1, had more chlorophyll (SPAD = 42 ± 6) than virescent plants (SPAD = 17 ± 6), which contained the plastome of Aa1 (Table 4.6). Green R1Aa1 progeny carried larger tubers, were taller and had fewer leaves than virescent plants (Table 4.6). The plastome of R1 thus did not affect vigour as compared to the plastome of Aa1, which affected vigour negatively in R1Aa1 progeny.

Table 4.6. Effect of plastome type to vigour as measured by tuber weight during planting, plant length, number of leaves and chlorophyll content in F₁ hybrids of Z. rehmannii *(R1, R2),* Z. albomaculata *subsp.* albomaculata *(Aa1, Aa2) and* Z. elliotiana *(E)*

Genome	Leaf	Inferred Plastome[3]	Number of plants	Tuber Weight[1] (g)	Plant Length[1] (cm)	Number of leaves[1]	Chlorophyll (SPAD)[1][2]
R1Aa1	Green	R1	43	8.1 a	31.8 b	4.1 a	42 ± 6 b
R1Aa1	Virescent	Aa1	38	7.4 a	25.5 a	3.7 a	17 ± 6 a
R1E1	Green	R1	14	7.2 b	28.4 b	2.6 a	39 ± 5 b
R2E1	Virescent	E1	8	2.6 a	20.5 a	1.9 a	16 ± 4 a
Aa2E1	Green	Aa2	10	8.3 b	38.7 b	3.6 a	46 ± 3 b
Aa2E1	Pale green	E1	30	6.1 a	32.8 a	2.7 a	34 ± 6 a

[1]Dissimilar letters indicate values that are significantly different per characteristic per family (α=0.05)
[2]Chlorophyll content in SPAD-units
[3]Plastome types of hybrids were deduced as judged from leaf colour (Table 4.5, Figure 4.1)

Green plants of R1E1 progeny, which contained the plastome of R1 (Table 4.5), had a chlorophyll content of SPAD = 39 ± 5. Virescent tissue of their pseudo-reciprocal hybrids (R2E1), which contained the plastome of E1 (Table 4.5), had less chlorophyll (SPAD = 16 ± 4) (Table 4.6). Green R1E1 progeny were more vigorous than their virescent pseudo-reciprocals that contained the plastome of E1: the latter carried smaller tubers, were smaller and had fewer leaves (Table 4.6). The plastome of E1 thus affected vigour negatively as compared to the plastome of R1.

Leaf tissue of Aa2E1 progeny demonstrated two leaf types: green and pale green.. Pale green plants, which probably contained the plastome of E1 (Table 4.5), contained less chlorophyll (SPAD 34 ± 6) than green plants, which probably contained the plastome of Aa1 ((SPAD 46 ± 3) ; Table 4.6). Green plants carried larger tubers, were taller and had more leaves (Table 4.6). Therefore, the plastome of E1 seems to negatively affect vigour of Aa2E1 progeny as compared to the plastome of Aa2, but the effect was moderate because all plants were vigorous.

In summary, based on the patterns of PGI there are at least three plastomes within the section *Aestivae* (Table 4.7). The incompatibility effects were often quantitative as judged from differences in vigour. The plastome of R1 is compatible with the genomes of Aa1/Aa2, E1 and P, since the respective hybrids were vigorous with unaffected chlorophyll content as compared to their siblings that contained the plastome of the other parent.

Table 4.7. Directions of plastome-genome incompatibility between species of Zantedeschia, *section* Aestivae

Genome[1]	Plastome[2]			
	R	Aa	E	P
RAa	++	±		
RE	++		±	
RP	++			±
AaE		++	+	

[1] Hybrid genomes: R = *Z. rehmannii*, Aa = *Z. albomaculata* subsp. *albomaculata*, E = *Z. elliotiana*, P = *Z. pentlandii*, where RAa = F$_1$ hybrid of *Z. rehmannii* and *Z. albomaculata* subsp. *albomaculata*
[2] '++' = compatible, green leaf tissue; '+'= compatible, pale green leaf tissue; '±' = partially compatible, virescent leaf tissue

The plastome of Aa1 is partially incompatible with the genome of R1, since young seedlings of Aa1 and R1 with the plastome of Aa1 were virescent and less vigorous. The

plastome of E1 was less compatible to the genome of R1 as compared to the plastome of Aa1, reflected by a more severe virescence and poorer growth. The plastomes and genomes of Aa2 and E1 were compatible, since all hybrids were vigorous. Plants with the plastome of Aa2, however, were more vigorous and greener than plants with the plastome of E1.

4.3.3 Plastid inheritance in Aestivae species

The variegation that was demonstrated in some progeny (Table 4.5), originated from biparental inheritance of plastids. To determine modes of plastid inheritance more precisely, hybrids from many crosses were assessed for leaf morphology. Considering the directions of PGI, it is possible to use leaf morphology as an indicator for the plastome type, given a known hybrid background (Table 4.5). The deduced modes of plastid inheritance in these crosses are listed in Table 4.8.

Table 4.8. Percentage of hybrids demonstrating maternal, biparental and paternal plastid inheritance as judged from leaf colour in interspecific hybrids of Z. rehmannii *(R1-R3),* Z. albomaculata *subsp.* albomaculata *(Aa1-Aa4),* Z. elliotiana *(E) and* Z. pentlandii *(P)*

Mother	Father	M[1]	B	P	n
R3	Aa3	83	17		332
Aa3	R3	89	11		37
R3	Aa4	73	27		157
R4	Aa4	58	42		83
Aa4	R4	69	26	6	35
R4	Aa3	77	23		13
R1	Aa1	98		2	41
Aa1	R1	87	11	2	45
R1	A2	95	5		57
Aa2	R2	89	11		57
R1	E1	90	10		58
E1	R1	87	11	2	129
E2	R3	79	21		197
R1	P	89	9	2	66
Aa1	E1	96^2	3^3	1^4	149
E1	Aa2	10^4	90^3		42

[1]M=maternal, B=biparental, P=paternal plastid inheritance
[2]Plants were green and presumably contained the plastome of *Z. albomaculata* subsp. *albomaculata* (Table 4.5)
[3]Plants were variegated green and pale green and presumably contained both plastomes
[4]Plants were pale-green and presumably contained the plastome of *Z. elliotiana* (Table 4.5)

All families showed some degree of biparental plastid inheritance (Table 4.8). Considerable differences existed between families within similar species crossings, however. While R1 × Aa1 had a strong maternal bias (98 %), only 58 % of the offspring of R4 × Aa4 showed maternal plastid inheritance (Table 4.8). A high percentage (90 %) of the offspring of E1 × Aa2 showed biparental plastid inheritance. The pseudo-reciprocal (Aa1 × E1) showed a maternal dominance in plastid inheritance (96 %).

4.4 Discussion

In the present analysis, we have demonstrated that biparental inheritance of plastids and PGI are prevalent among interspecific hybrids of the section *Aestivae*. Biparental inheritance of plastids and PGI were described before in other interspecific hybrids of *Zantedeschia* spp., but this was limited to hybrids of *Z. aethiopica* in the section *Zantedeschia* (Yao and Cohen, 2000) and between cultivars of sections *Aestivae* and *Z. aethiopica* (Yao et al., 1994a). In the present study, we presented more extended data on four *Zantedeschia* species belonging to the section *Aestivae*.

Plastomes and genomes of the different *Aestivae* species showed different levels of compatibility, with different consequences to morphology and vigour (Table 4.6 and Table 4.7). R2E1 progeny that suffered from PGI were initially very pale, greened slowly and had a low vigour, while the plastomes and genomes of Aa1/Aa2 and E1 appeared to be compatible in all directions. Shibuya (1956) also reported low vigour and pale leaves of hybrids of *Z. elliotiana* and *Z. rehmannii*. Reports on unexplained virescence in *Zantedeschia* spp. (New and Paris, 1967) can now be interpreted as PGI (Table 4.9).

Table 4.9. Percentage of hybrids demonstrating maternal, biparental and paternal plastid inheritance in interspecific hybrids of Z. rehmannii *(R),* Z. albomaculata *(A),* Z. elliotiana *(E) as deduced from New and Paris (1967)*

Mother[1]	Father[1,2]											
	R				A				E			
	M	B	P	n	M	B	P	n	M	B	P	n
R					67	31	2	42	100			15
A	83	12	5	42								
E	93	7		15								

[1]Coding as in Table 4.2
[2]M=maternal, B=biparental, P=paternal

Based on the directions of compatibility (Table 4.7) and restriction site polymorphisms (Table 4.4, Figure 4.1), at least three plastomes could be differentiated. Plastomes of E1 and P were similar, but different from the plastomes of R1 and Aa1/Aa2. The decreased vigour of plants with partial incompatible plastome-genome combinations suggests that PGI results in diminished fitness. PGI might therefore promote speciation in the section *Aestivae*, as was demonstrated in *Oenothera* spp. (Hupfer et al., 2000).

Biparental and paternal transmission of plastids occur in many species but the evolutionary significance is uncertain (Birky, 1995; Mogensen, 1996). On the one hand, asexual reproduction and uniparental inheritance inhibit the spread of cytoplasmic parasites. A selfish organelle genome that is inherited biparentally could increase to an equilibrium frequency that significantly reduces the population fitness (Birky, 1995). On the other hand, Muller's ratchet is working in an asexual reproducing population, resulting in an increased amount of mutations on the long term (Muller, 1964). The mutation rate of paternally inherited organelles is higher than of maternally inherited organelles (Whittle and Johnston, 2002), so Muller's ratchet works at a higher pace in plastids that are paternally transmitted. This irreversible building-up of mutations could be slowed down by the presence of biparental inheritance, where selection between plastid types is possible (Birky, 1995).

Many methods have been used to determine plastid inheritance, such as plastid mutations affecting chlorophyll, antibiotic resistances and molecular markers. The method used to identify the plastome type can affect the retrieved plastid inheritance. Medgyesy et al. (1986) used an antibiotic resistance and found no paternal plastids in the offspring of tobacco when seedlings were screened, but could find paternal plastids when callus was screened. It is possible that this difference is caused by an early sorting-out of the paternal plastids during the embryonic stage. If paternal plastids are sorted out in an early stage of development in *Zantedeschia*-seedlings as well, then the actual biparental plastid inheritance ratios are underestimated.

Few genera have been described where biparental inheritance of plastids is as prevalent as in the genus *Zantedeschia* (Table 4.1). Much is known on plastid inheritance in flowering plants, mainly based on *Pelargonium × hortorum* and *Oenothera* spp. (Tilney-Bassett et al., 1992; Stubbe and Steiner, 1999). Most studies have been done on intraspecific crosses (Sager, 1972; Kirk and Tilney-Bassett, 1978; Harris and Ingram, 1991;

Mogensen, 1996), so it is unclear if the modes of plastid inheritance in intraspecific crosses can give insight in plastid inheritance in interspecific hybrids.

Rates of biparental plastid transmission among interspecific hybrids of the section *Aestivae* (Table 4.8) were comparable to rates observed in species crosses of *Rhododendron* and *Oenothera* (Table 4.1). Interestingly, these are genera that exhibit interspecific PGI as well (Chiu and Sears, 1993; Michishita et al., 2002; Ureshino and Miyajima, 2002). An interspecific hybrid might have a higher chance of receiving the compatible plastome type when the plastome is inherited biparentally as compared to uniparentally. In these genera the formation of interspecific hybrids may be promoted by permitting plastids to be inherited biparentally and hereby enabling selection of the most compatible plastome type. Other genera that possibly exhibit PGI and biparental inheritance of plastids are *Geranium* (Dahlgren, 1923; 1925) and *Hypericum* (Noack, 1932).

It appears that the level of PGI between species of the same section is lower than the level of PGI between species of different sections. Plastomes and genomes of species within the section *Aestivae* are incompatible only to some degree, because all plants survived autotrophically. The plastome of *Z. aethiopica* and the hybrid genome of *Z. aethiopica* and *Z. odorata* (section *Zantedeschia*) were also only partially incompatible, because plants survived autotrophically. Intersectional hybrids were incompatible to a high degree, resulting in albino plants that can only survive heterotrophically (Yao et al., 1994a). The level of PGI in interspecific hybrids may therefore be correlated to the relatedness of the parental species in *Zantedeschia* spp.

It is becoming evident that genes have migrated from the plastid to the nucleus during evolution of plants (Martin et al., 2002; Huang et al., 2003). These translocations could result in plastomes containing genes that in other species are restricted to the nucleus. Plastomes and genomes that both do not contain the genes involved, would hence have become incompatible, where the level of incompatibility would depend on the function of the genes involved. The further that species have diverged, the greater the possibility that a translocation has taken place and plastomes and genomes have become incompatible.

In conclusion, all interspecific hybrid families of the genus *Zantedeschia* that were assessed, showed a biparental mode of plastid inheritance with a maternal bias and levels of PGI. Based on the level of PGI, CAPS-markers and directions of compatibility, three

plastome types were differentiated among the section *Aestivae*. Vigour of interspecific hybrids was impaired due to PGI, suggesting that PGI is promoting speciation in the section *Aestivae*. The *Aestivae* species hybridise readily and hybrids are fertile enabling genetic studies. Therefore, we propose the genus *Zantedeschia* and especially the section *Aestivae* as an object for further study of the phenomena of plastome-genome incompatibility and plastid inheritance.

5 Genetic control of resistance to soft rot caused by *Erwinia carotovora* subsp. *carotovora* in *Zantedeschia* spp. (*Araceae*), section *Aestivae*

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Abstract

The pattern of heredity of resistance to *Erwinia carotovora* subsp. *carotovora* in *Zantedeschia* spp. is investigated. Four species with different resistance levels (*Z. albomaculata, Z. elliotiana, Z. pentlandii and Z. rehmannii*) were compared to their reciprocal offspring. The occurrence of plastome-genome incompatibility (PGI) affected plant resistance in all families. Therefore, plants that suffered from PGI were omitted from genetic analyses. Resistance was quantitative and the correlation between resistance levels of parents and offspring (h^2=0.33; r^2=0.66) indicated a genetic basis of resistance. *Z. rehmannii* and *Z. albomaculata* contributed more resistance genes than *Z elliotiana* or *Z. pentlandii*. Transgression among some of the offspring of *Z. rehmannii* and *Z. albomaculata* indicated the presence of complementary resistance genes in these two species and good potential for resistance breeding.

5.1 Introduction

Bacterial soft rot caused by *Erwinia carotovora* subsp. *carotovora* (syn. *Pectobacterium carotovorum* subsp. *carotovorum*) occurs world-wide in many crops. This soil borne, facultative anaerobic pathogen causes maceration and rotting of parenchymatous tissue of all plant organs, eventually resulting in plant death (Pérombelon and Kelman, 1980). The pathogen is difficult to restrain because of many reasons, such as absence of effective bactericides (Blom and Brown, 1999), its genetic variability (Avrova et al., 2002; Gardan et al., 2003), its wide host range, its broad array of virulence factors and because infection can be latent (Pérombelon, 2002). It is an important disease in *Zantedeschia* spp. and the major disease in cultivars of the section *Aestivae* (Kuehny, 2000; Wright and Burge, 2000). Plants turn yellow when disease has initiated, produce a foul smell and can become completely macerated resulting in plant death within a few days (Wright, 1998). Bacteria are spread mechanically during cultivation and symptoms can become visible during all stages of plant growth and development. Conditions are favourable for soft rot when plants are under stress during low soil aeration, high temperature or high relative humidity (Funnell and MacKay, 1999; Wright and Burge, 2000). Cultural measures such as drainage, mulching, soil ventilation and time of tuber lifting can reduce disease development (Funnell, 1993; Wright and Burge, 2000; Wright et al., 2002), but better control of the disease could be achieved by the use of resistant cultivars.

Most commercial cultivars have been developed from interspecific hybrids within the section *Aestivae*, mainly of *Z. albomaculata*, *Z. elliotiana, Z. rehmannii* and *Z. pentlandii* (Funnell, 1993). Cultivars of the section *Aestivae* are susceptible, while wild accessions vary from susceptible to moderately resistant (Snijder et al., 2004a). The level of genetic control of the resistance should be determined in order to value the potential of this resistant germplasm for breeding.

An unusual phenomenon that exists among interspecific hybrids of the section *Aestivae,* is plastome-genome incompatibility (PGI; (Snijder et al., 2004b)). PGI probably results from a disturbed balance between plastids and the nucleus, the severity varies with parental species (Herrmann et al., 2003). Plastids can inherit biparentally among

interspecific hybrids of *Zantedeschia* spp., so if PGI exists between two parents, then PGI will become manifest in hybrids from reciprocal crosses (Snijder et al., 2004b). Plants that show PGI are less vigorous than normal plants (Snijder et al., 2004b), so they may be more susceptible as well.

The aim of the present study was to determine whether resistance is affected by PGI, if resistance is under genetic control and if it is possible to breed for resistance to soft rot. To this end, hybrids were developed of four species of the section *Aestivae* that have different levels of resistance. Resistance levels of offspring that did not suffer from PGI were determined using a non-destructive leaf disk test.

5.2 Materials and methods

5.2.1 Plant material and cultivation

Plants were cultivated as described by Snijder et al. (2004a) with the only modification that tubers of the parents (Table 5.1) were treated with gibberellic acid (100 ppm) before planting, as is common practice in commercial production. Tubers of the reference cultivars (Table 5.1) and of two-year-old seedlings (Table 5.2) were not treated with gibberellic acid before planting, because gibberellic acid can decrease the number of leaves (Brooking and Cohen, 2002), while leaves were needed for the resistance test.

Table 5.1. Accessions of Zantedeschia *spp. of the section* Aestivae *used as parents and two genotypes used as reference in resistance tests.*

PRI-no[1]	Code	Genotype	Use
00060	Aa1	*Z. albomaculata*	Parent
00061	Aa2	*Z. albomaculata*	Parent
00062-1	E1	*Z. elliotiana*	Parent
99017	FG	'Florex Gold'	Susceptible reference
00069-2	P	*Z. pentlandii*	Parent
00063	R1	*Z. rehmannii*	Parent
99022	R3	*Z. rehmannii*	Partial resistant reference

[1]all accessions were clones

5.2.2 Experimental procedure

Hybrids were analysed for resistance to bacterial isolate *E. carotovora* subsp. *carotovora* PD 1784 (obtained from the Dutch Plant Protection Service) in their second growing season using a leaf disk test, with which individual plants can be tested without being destructed (Snijder et al., 2004a). The absence of inbreeding and the effect of age on resistance level was determined by comparing parents (R1 and 'Florex Gold') and their two-year-old selfings..

Table 5.2. Number of hybrids of Zantedeschia rehmannii *(R1, R2),* Z. elliotiana *(E1),* Z. albomaculata *subsp.* albomaculata *(Aa1, Aa2),* Z. pentlandii *(P) and 'Florex Gold'(FG) that were analysed for resistance to soft rot caused by* Erwinia carotovora *subsp.* carotovora *PD 1784.*

Mother	Father					
	Aa1	Aa2	E1	R1	P	FG
Aa1			22	41		
Aa2			1			
E1		36		2		
R1	38	39	13	19	11	
R2			7			
FG						11

The pattern of heredity was determined on interspecific hybrid progenies (Table 5.2) which did not show PGI. Two to four leaves of each seedling were evaluated in one experiment, which was split into eleven plots. Seedlings were randomly distributed over the plots, while leaves of each genotype were observed in separate plots. The observations were done at two or three days after inoculation, depending on the progress of the infection on the reference genotypes 'Florex Gold' and R3, which were represented in every plot with three to five leaves. The resistance levels of the parents were determined in a separate experiment in two replicates each divided over two plots with six leaves per genotype per plot.

5.2.3 Genetic analysis

Genetic analyses were done only with plants that showed a compatible plastome-genome combination. Chimerical plants (with two plastomes) and plants with low vigour because of

PGI (Table 5.4) were omitted from the experiment. Plastome types of the hybrids were determined as judged from CAPS-markers and from the leaf chlorophyll content (Table 5.4). Chlorophyll content was determined using a SPAD-502 instrument (Minolta Camera Ltd.) as by Snijder et al. (2004b). Statistical analyses of resistance tests were done as described by Snijder et al. (2004a) after testing for normality of the logit-transformed observations using a Q-Q plot (Baird, 2002). Results from the different replicates and plots were related to 'Florex Gold', resulting in an index for macerated leaf disk area (M), where 'Florex Gold' was set at 100. All analyses were done using software package Genstat 6, release 6.1 (GenStat, 2002).

5.3 Results

To determine the pattern of heredity of resistance to soft rot in interspecific hybrids of the section *Aestivae*, hybrids were generated of four species (Table 5.2) that have significantly different levels of resistance (Table 5.3). The resistance levels of the interspecific hybrids were compared to their parents to determine patterns of genetic control of resistance to soft rot. R1 and Aa1 were more resistant than Aa2 and E1. P was as susceptible as 'Florex Gold'; more susceptible than any of the other accessions (Table 5.3).

Table 5.3. Index of macerated leaf disk area (M) of Zantedeschia rehmannii *(R1),* Z. elliotiana *(E1),* Z. albomaculata *subsp.* albomaculata *(Aa1, Aa2) and* Z. pentlandii *(P) after inoculation by* Erwinia carotovora *subsp.* carotovora *PD 1784.*

Genotype	Accession	M[1]
Z. albomaculata subsp. *albomaculata*	Aa1	57 a[2]
Z. albomaculata subsp. *albomaculata*	Aa2	86 bc[3]
Z. elliotiana	E1	84 b[2]
Z. rehmannii	R1	44 a[2]
Z. pentlandii	P	100 c[3]

[1] 'Florex Gold' was set at 100, scoring 73%
[2] significant at p=0.95
[3] significant at p=0.90

To determine whether age affects the level of resistance, two-year-old selfings of R1 and 'Florex God' were compared to their parents, which had been clonally propagated for at least four years. Two-year old selfings of R1 (M=68) were more susceptible than R1 (44) itself and two-year-old selfings of 'Florex Gold' (114) were more susceptible than 'Florex Gold' (100). This difference is not likely due to inbreeding, because vigour of these selfings was comparable or higher than vigour of hybrids of the same age (Snijder et al., 2004b). Absence of inbreeding depression was also observed by Shibuya (1956) and Horn (1962), so it appears that young plants are more susceptible than old plants.

5.3.1 Plastome-genome incompatibility affects resistance

Before comparing the hybrids to their parents, the effect of PGI to resistance was determined (Table 5.4). The R1Aa1 progeny that contained the plastome of R1, was more resistant (67) than their siblings with the plastome of Aa1 (80). The R1E1 progeny with the plastome of R1 (76) was more resistant than their half-siblings (R2E1) with the plastome of E1 (91). The Aa2E1 progeny that were green (87), and contained the plastome of Aa2, were less resistant than their pale-green siblings (76), which contained the plastome of E1 (Table 5.4). The plastomes of Aa2 and E1 are both compatible to the hybrid Aa2E1 genome (Snijder et al., 2004b), so PGI cannot be the cause of the difference in resistance for these plastomes. Therefore, other unknown effects of the plastomes of Aa2 or E1 must account for the difference in resistance level. In summary, resistance in R1Aa1 and R1E1/R2E1 progenies has declined if PGI was present. Siblings with different plastomes have different levels of resistance and genetic analyses should be confined to plants that do not suffer from PGI.

Offspring of all combinations except Aa2E1 that did not express PGI, scored more susceptible than their parental means (Figure 5.1). This difference coincides with the age effect that was observed between two-year-old selfings of R1 and 'Florex Gold'. Genetic analysis can still be done, because relative differences subsisted: selfings of R1 were amongst the more resistant as compared to other seedlings (Table 5.5), just as R1 itself is more resistant than the other parents (Table 5.3).

Table 5.4. Effect of plastome-genome combinations in interspecific hybrids of Zantedeschia rehmannii *(R1),* Z. elliotiana *(E1),* Z albomaculata *subsp.* albomaculata *(Aa1, Aa2) and* Z. pentlandii *(P) to leaf colour and chlorophyll content (from Snijder et al,(2004b)) and to index of macerated leaf disk area (M) after inoculation by* Erwinia carotovora *subsp.* carotovora *PD 1784*

Genome	Leaf colour	SPAD ± sd	Inferred Plastome[1]	PGI affects vigour[1]	n	M[2]
R1Aa1	Green	42 ± 6	R1	No	43	67 a
R1Aa1	Virescent	17 ± 6	Aa1	Yes	36	80 b
R1Aa2	Green	38 ± 8	R1	No	39	69 a
R1E1	Green	39 ± 6	R1	No	12	77 a
R2E1	Virescent	16 ± 4	E	Yes	7	91 b
Aa2E1	Green	46 ± 3	Aa2	No?	10	87 b
Aa2E1	Pale green	34 ± 6	E1	No?	27	76 a
R1P	Green	34 ± 5	R1	No	10	78

[1]As described by Snijder et al. (2004b)
[2]'Florex Gold' was set at 100, scoring 88%, letters indicate significant different values at p=0.90 within the family (Student's t-test)

Table 5.5. Index (M) and variance of macerated leaf disk area after inoculation by Erwinia carotovora *subsp.* carotovora *PD 1784 (variance on logit-scale) of interspecific hybrids of* Zantedeschia rehmannii *(R1),* Z. elliotiana *(E1),* Z albomaculata *subsp.* albomaculata *(Aa1, Aa2) and* Z. pentlandii *(P) that did not express plastome-genome incompatibility*

Genome[1]	Plastome[2]	n	M[3]	Variance[4]	Transgression[5]
R1E1	R1	12	77 bc	0.17 a	No
R1P	R1	10	77 bc	0.21 ab	No
R1Aa1	R1	43	67 a	0.42 bc	Yes
R1Aa2	R1	39	69 ab	0.59 c	Yes
Aa1E1	Aa1 or E1	21	76 bc	0.42 bc	?[6]
Aa2E1	Aa2 or E1	37	78 c	0.32 ab	?[6]
R1R1	R1	19	68 a	0.33 b	Not applicable

[1]Two letters indicate the parental genomes: "R1E1" = hybrid genome of R1 and E1
[2]Only plants with a single plastome were included, chimerical plants (with two plastomes) were excluded
[3,4]Different letters indicate significantly different values at p=0.90 [3] by Student's t-test, 'Florex Gold' was set at 100, scoring 88% [4]by F-test
[5]Presence of plants within the family that were more resistant than partial resistant reference R3
[6]*Z. elliotiana* and *Z. albomaculata* subsp. *albomaculata* were not included in disease tests with their offspring.
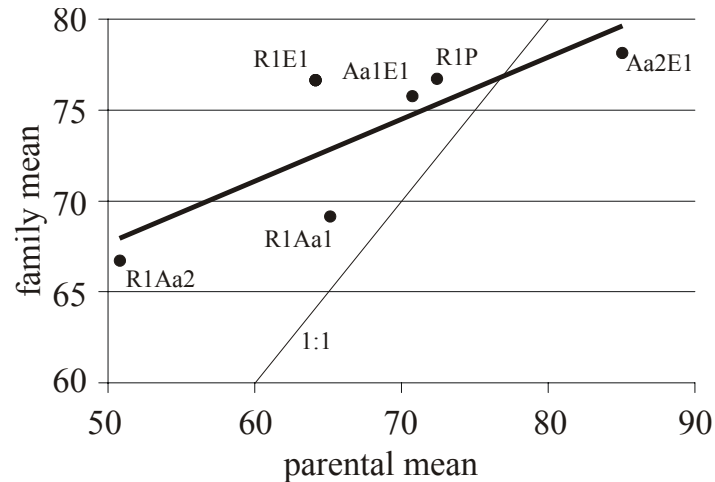
Figure 5.1. Relation between the parental mean and the mean of the corresponding family for level of resistance to Erwinia carotovora *subsp.* carotovora *PD 1784 in interspecific F$_1$ hybrids of four* Zantedeschia *spp. (section* Aestivae*) with a compatible plastome type, as measured by the leaf disk test in index of macerated leaf disk area; a 1:1 line (thin line) is included for reference.*

5.3.2 Positive correlation between parents and offspring

The levels of resistance of parents and offspring were positively correlated ($h^2 = 0.33$, $r^2 = 0.66$, Figure 5.1), so resistance is under genetic control. The R1Aa1-progeny (67) and the R1Aa2-progeny (69) were most resistant, while the R1E1-, Aa2E1-, Aa1E1-, and R1P-progenies were susceptible (76-78) (Table 5.5). This trend suggests that *Z. albomaculata* contributed more resistance genes to its progeny with *Z. rehmannii* than *Z. elliotiana* or *Z. pentlandii* to their progeny with *Z. rehmannii*.

5.3.3 Variation within families

The segregation of resistance within families was examined to explore the pattern of heredity. Resistance was quantitative, because phenotypes within families were distributed continuously (data not shown). Table 5.5 shows that the variances of the level of resistance were not similar among the hybrid families, indicating some differences in transfer of resistance genes by the different parents. The variances varied from 0.59 (R1Aa2) to 0.17

(R1E1). The variance of the R1 selfing was intermediate with 0.33. The variances of the R1E1 progeny (0.17) and the R1P progeny (0.21) were similar, being much smaller than the variances of the other progenies (0.32-0.59). E1 and P are susceptible (Table 5.3), so the smaller variances of their progenies can be related to the absence of resistance genes in E1 and P. Aa2 is also susceptible, but the R1Aa2 progeny had a variance that was comparable to the variance of R1Aa1-hybrids. These putative resistance genes of Aa2 may therefore have had a negative effect.

5.3.4 Transgression

Some seedlings existed that had higher levels of resistance than R3 (Figure 5.2). R3 has a resistance level that is similar to the resistance level of R1 (Snijder et al., 2004a). Therefore, seedlings that have a resistance level that is higher than R3 can be considered more resistant than R1 and hereby more resistant than any of the other parents (Table 5.3). The transgression shown by these seedlings indicates the presence of complementary resistance genes in R1 and Aa1/Aa2. Transgression among Aa1E1 and Aa2E1 could not be determined, because their resistance levels could not be compared to their parents.
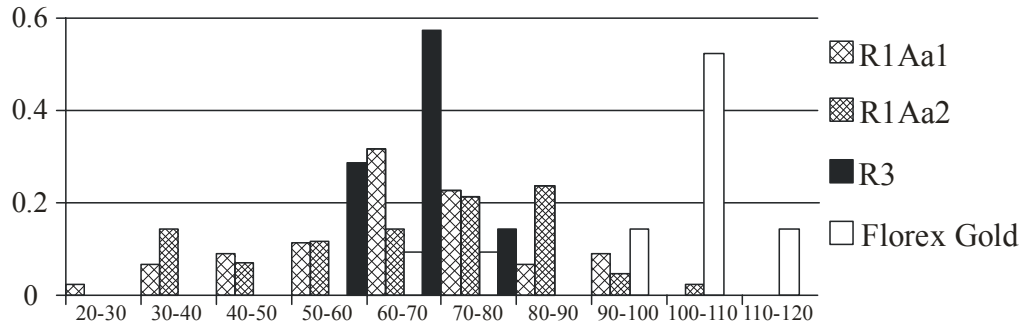

Figure 5.2. Frequency distribution of index of macerated leaf disk area of two interspecific hybrid families of Z. rehmannii *and* Z. albomaculata *subsp.* albomaculata*: R1Aa1 (finely hatched) and R1Aa2 (roughly hatched) containing the plastome of R1, after inoculation by* Erwinia carotovora *subsp.* carotovora *PD 1784; the frequency distributions of 'Florex Gold' (white) and R3 (black) are included as reference.*

5.4 Discussion

Resistance to soft rot in *Zantedeschia* of the section *Aestivae* was quantitative. In other crops, resistance to soft rot caused by *Erwinia carotovora* was also quantitative (Darling et al., 2000; Zimnoch Guzowska et al., 2000; Ren et al., 2001). This quantitative nature might be explained by the wide array of pathogenicity factors of the pathogen (Pérombelon and Salmond, 1995; Toth et al., 2003). The host may need as many genes to resist the pathogen. The quantitative nature can also be caused by the variability of the disease test (Snijder and Van Tuyl, 2002; Snijder et al., 2004a) or by a large sensitivity of resistance genes to changes in the environment.

An estimation of the number of genes could not be made, since Mendelian segregations were not observed; all segregations were continuous. Using a diallel cross analysis, together with backcrossings, Ren et al. (2001) presumed two major resistance loci and a few minor loci in Chinese cabbage. In a QTL analysis, many minor loci were found by Zimnoch Guzowska et al. (2000) in potato.

Occurrence of plastome-genome incompatibility affected the level of resistance of offspring negatively (Table 5.4) and thus veiled breeding value, possibly by negatively affecting plant vigour (Snijder et al., 2004b). Genetic analyses are thus more reliable when they are performed on plants that do not suffer from PGI. The positive relation between levels of resistance of offspring and their parents (Figure 5.1) demonstrates that parental level of resistance is a reliable measure for breeding value.

Aa2E1 progeny that was green (with the plastome of Aa2) was more susceptible than the Aa2E1 progeny that was pale-green (with the plastome of E1). Although Aa2E1-hybrids did not suffer from PGI (Snijder et al., 2004b), the difference in resistance indicates some causality. It is possible that the pale green plants are stressed because of plastome-genome interactions that did not affect vigour significantly, but did affect resistance. If classic stress signals were involved with these interactions, these could have caused induced resistance (Sticher et al., 1997). As a result, the plants could have become more resistant, because of induced resistance by the state of stress. Aa2E1-hybrids that contained the plastome of E1 would then bias the genetic analysis. If these putatively stressed plants are omitted from analysis, the correlation between the parental mean and the family mean is

larger (r^2=0.84), indeed suggesting the presumed bias. These plants were still included in the presented analysis, because we did not demonstrate the presence of induced resistance.

Progenies of E1 and P were susceptible (76-78). Although the relation between the parental mean and the family mean was clear (Figure 5.1), the susceptibility of E1 and P progenies can also be explained by (partial) dominance of susceptibility. If dominance of susceptibility in these species indeed occurred, there should be a shift towards susceptibility in back-crosses with resistant genotypes or a segregation for susceptibility in F_2-plants. This hypothesised dominance remains to be tested.

Some F_1-hybrids of R1 and Aa1/Aa2 were more resistant than the partial resistant control R3 (Figure 5.2) and were thus more resistant than their parents. This transgression suggests that the parents contributed complementary resistance genes. Progeny of these parents showed the largest variance (Table 5.5). Although mechanisms and durability of these putative resistance genes are to be tested further, it can be concluded that there is potential for breeding *Zantedeschia* spp. for resistance to *E. carotovora* subsp. *carotovora*.

6 General Discussion

Soft rot caused by *Erwinia carotovora* subsp. *carotovora* (*Ecc*; syn. *Pectobacterium carotovorum* subsp. *carotovorum*) challenges many crops world-wide. Resistance breeding is one of the possible actions to control the pathogen. The present study was undertaken because introgression of resistance from *Z. aethiopica* into cultivars of the section *Aestivae* appeared to be impossible due to a breeding barrier caused by severe PGI (Yao et al., 1995). Therefore, resistance sources had to be identified within the *Aestivae* species. The possibility to breed *Zantedeschia* spp. of the section *Aestivae* for resistance to soft rot was confirmed. Sources of resistance were identified among the section *Aestivae* using newly developed resistance tests. Resistance appeared to be under genetic control and most likely several genes are involved, resulting in a quantitative pattern of inheritance. The consequences of interspecific plastome-genome incompatibility (PGI) for plant vigour and resistance had to be taken into account to enable genetic analyses.

6.1 Optimisation of the leaf disk test

The leaf disk test (Chapter 2) was chosen to screen the gene pool (see Table 3.6) and to evaluate seedlings for genetic analysis (Chapter 5). The advantage of the leaf disk test is that plants are not destructed and that individual plants can be evaluated, which enables screening of seedlings and rare germplasm. Experience is needed to obtain reproducible results, because visual scoring (Figure 6.1) needs skilled observers.
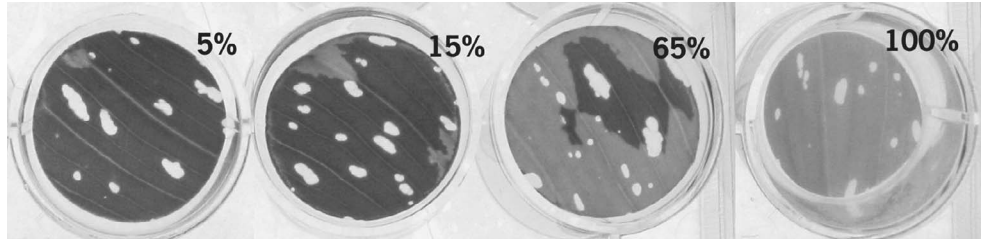

Figure 6.1. Leaf disks test; the dark area is not macerated, the percentage of light area is estimated; the white spots are wild-type maculation (see also Figure 1.1)

Image analysis is therefore preferred, but this was hampered by the low contrast between macerated and unmacerated tissue, as well as by the presence of the (wild type) leaf maculation (Figures 1.1 and 6.1) that interferes with macerated tissue during digital interpretation of the image.

The size of a plot in the leaf disk test is restricted by the number of leaves. The maximum number of disks that was scored per plot in the present study was 864 (144 leaves). A number of independent assays per leaf (disks) is needed to compensate for the variation that exists within a leaf. This number restricts the number of leaves that can be included per plot. A decrease of the number of disks that is sampled per leaf thus enables to increase plot size. We therefore determined how many disks are needed to obtain a precise observation. Hereto, two and four disks were randomly chosen from the dataset that was used to obtain Table 5.5 and the resistance levels were estimated accordingly. The resistance levels obtained from two disks ($r^2=0.88$) and four ($r^2=0.92$) disks were strongly correlated to the levels obtained by observing six disks. It can therefore be concluded that

the number of disks can be reduced to four or even two, thus enabling simultaneous analysis of more leaves or more genotypes.

6.2 Mechanisms of resistance

To our knowledge no study has been done on the nature of resistance to soft rot in *Zantedeschia*. Some indications however are provided by the results presented in Chapters 4 and 5. Hybrids of *Z. elliotiana* (E1) and *Z. albomaculata* (Aa2) with the (compatible) plastome of Aa2 were more vigorous but more susceptible than seedlings with the (also compatible) plastome of E1 (Table 5.4 and Table 4.6). In contrast, hybrids of *Z. rehmannii* and *Z. albomaculata*, *Z. elliotiana*, or *Z. pentlandii* that contained the plastome of *Z. rehmannii* were more vigorous, but less susceptible than their siblings that contained the plastome of the other parent. A similar negative relation between growth rate and resistance was observed between half-sibs of 'Pink Persuasion' and 'Florex Gold'. The half-sib family of 'Florex Gold' carried larger tubers, was taller, had more leaves and the leaves contained more chlorophyll than the half-sib family of 'Pink Persuasion' (Table 6.1). Although the half-sib family of 'Florex Gold' thus appeared more vigorous, it was more susceptible than the half-sib of 'Pink Persuasion'.

Table 6.1. Growth characteristics and level of resistance of two-year-old half-sib families of fathers 'Florex Gold' and 'Pink Persuasion' (mothers were 'Black Magic', 'Sensation', 'Treasure' and 'Florex Gold').

Half-sib family of father	n	M[12]	Tuber Weight[34]	Plant Height[34]	Number of leaves[34]	Chlorophyll Content (SPAD)[34]
'Florex Gold'	89	108b	8.8b	43b	10b	52b
'Pink Persuasion'	60	92a	4.3a	26a	6a	35a

[1]Index of macerated leaf disk area was determined by the leaf disk test, as described in Chapters 2 and 3
[2]Different letters indicate significant different means at p=0.90
[3]Characteristics were determined and analysed as described in Chapter 4
[4]Different letters indicate significant different means at p=0.95

Vigour and susceptibility thus appear to be negatively correlated. Slower growing plants may have a higher dry weight, resulting in sturdy tissue that is more difficult to colonise by

Ecc bacteria, as was observed by Pagel and Heitefuss (1989) in potato and by Hoffland et al. (1996) for *Fusarium* resistance in radish. The less vigorous plants may be in a constant state of stress and therefore have an increased level of induced resistance (Chapter 5). Although this type of plants appears resistant, it is not interesting as source of resistance for breeding, because this resistance is associated with decreased vigour.

Z. aethiopica is more resistant than any genotype of the section *Aestivae* (Chapter 3), but it cannot be deployed as resistance genitor, because of the severe breeding barrier between these taxa (Yao et al., 1994a). The cause of its high level of resistance is unknown. But it is important to get more insight in the mechanisms of resistance to soft rot, in order to study the perspective of breeding for soft rot in the section *Aestivae*. *Z. aethiopica* is a vigorous species that can adapt well to many habitats, so its general vigour may account for its resistance. Singh (1996) described *Z. aethiopica* to contain large air spaces between mesophyll cells of leaves, which could also account for its resistance. Furthermore, *Z. aethiopica* produces substances that are lethal to algae (Kinoshita et al., 1998), so other secondary metabolites may account for its resistance to *Ecc*.

6.3 Limits to resistance breeding

Some genotypes were identified within the section *Aestivae* that have high relative levels of (partial) resistance (Table 3.6b). These include some accessions of *Z. rehmannii* and *Z. albomaculata* and they should be used as sources of resistance in further elevating the level of resistance. The transgression that appeared in some seedlings (Figure 5.2) suggests the presence of complementing resistance genes that could add to a higher level of resistance. A further elevation of the resistance level by pyramiding resistance genes can thus be expected.

The possibility of introgression of resistance depends on PGI, because PGI affects expression of resistance (Chapter 5). The level of PGI between *Z. odorata* and *Z. aethiopica* is correlated to the amount of nuclear genes that are conspecific with the plastome (Yao and Cohen, 2000). If this relation also exists among section *Aestivae*, then the level of resistance of an F_1 is merely indicative for the level of resistance of its backcrossings (BC). If the

recurrent parent in the BC is conspecific with the plastome of the F_1, then the BC will suffer less from PGI and the expression of resistance genes will be higher.

The possibility of introducing resistance genes by genetic modification should not be completely ruled out. Chapter 1 gives a few examples where resistance genes were introduced by genetic modification, but where the resistance level was not raised to a degree that is agronomically relevant. The use of synthetic genes may be promising, because high levels of resistance may be obtained (Osusky et al., 2000). Knowledge that is being developed in human pharmaceutical research to fighting bacterial diseases that also exhibit quorum sensing may be useful in designing an efficient approach. An example is the invention of molecules that competitively inhibit the quorum signal (Reverchon et al., 2002), thus possibly preventing latent bacteria from disease initiation.

6.4 Genetics of plastid inheritance

As plastid inheritance is under genetic control in *Petunia hybrida* (Derepas and Dulieu, 1992), *Pelargonium × hortorum* (Amoatey and Tilney-Bassett, 1994), and *Oenothera* spp. (Chiu and Sears, 1993), it may also be in *Zantedeschia* spp. Some families of *Z. rehmannii* and *Z. albomaculata* originated from different ratios of biparental plastid inheritance (Table 6.2). Both the maternal and the paternal effects were significant, so accessions Aa3 and Aa4 of *Z. albomaculata* and the accessions R3 and R4 of *Z. rehmannii* transmitted a different ratio of plastids to their offspring.

Table 6.2. Percentage of seedlings originating from biparental plastid inheritance of four families of Z. rehmannii *accessions (R3, R4) and* Z. albomaculata *(Aa3, Aa4) and χ^2 values of maternal and paternal effects.*

Mother	Father		Effect	χ^2	P
	Aa3	Aa4	Father	11.07	0.01
R3	17	27	Mother	7.92	0.05
R4	23	42	Mother × Father	6.09	0.11

The mechanisms that govern plastid inheritance may be under genetic control. In a *Petunia hybrida* mutant inbred line, the capacity to transfer plastid DNA via pollen (resulting in 2% biparental inheritance mode) was postulated to be regulated by two genes that are expressed at the male gametophytic level (Derepas and Dulieu, 1992). In *Pelargonium* cultivars, the percentage of paternal plastids that is present in offspring was dependent on the mother and under nuclear control (Kirk and Tilney-Bassett, 1978; Tilney-Bassett, 1994). Two genes were proven to regulate the inheritance of plastids via pollen in *Pelargonium* cultivars, expressed at the female gametophytic level (Amoatey and Tilney-Bassett, 1994). These genes resulted in two phenotypes. A Type I female conferred a segregation pattern in which maternal zygotes are frequent, resulting in M>B>P. A Type II female gives more paternal zygotes, where biparental inheritance is less frequent, resulting in M>B<P. In *Oenothera* spp., the plastid itself has a large effect on its inheritance (Chiu et al., 1989). There were 'stronger' and ' weaker' plastids, where the 'stronger' plastids outgrew or outcompeted the weaker plastids. However, relatedness of plastomes and genomes also affects plastid inheritance in *Oenothera*: the more related the plastome, the higher the preference in its transmission (Chiu and Sears, 1993).

There may be similar differences in 'strength' of plastids in genus *Zantedeschia* as observed in *Oenothera* (Chiu et al., 1989). Since the plastome of *Z. aethiopica* prevailed in interspecific progenies, it appeared to be 'stronger' than the plastome of *Z. odorata*. This difference in 'strength' could account for the reciprocal difference in the mode of plastid inheritance in F_1-hybrids of *Z. aethiopica* and *Z. odorata*. The plastome of *Z. aethiopica* also dominated in progenies of the section *Aestivae*-cultivars and *Z. aethiopica* (Yao et al., 1994a; 1995). The plastome of *Z. aethiopica* thus appeared to be 'stronger' than the plastomes of *Z. odorata* and the section *Aestivae*-cultivars. In hybrids of *Z. albomaculata* and *Z. elliotiana*, there presumably was a dominance of *Z. albomaculata* plastids (Table 4.8), implying a preference for the *Z. albomaculata* plastome in these hybrids.

6.5 Relation between the mode of plastid inheritance of intraspecific and interspecific crosses

Zantedeschia spp. are unusual in that plastids inherit biparentally in interspecific crosses and PGI occurs (Yao et al., 1994a; 1995) and Chapter 4). These phenomena not only hamper breeding and genetic analyses in *Zantedeschia* (Chapter 4 and Chapter 5), but also indicate that the evolution of *Zantedeschia* is unusual in that plastids do not inherit uniparentally as in most other plant genera (Birky, 2001).

The mode of plastid inheritance in intraspecific crosses of *Zantedeschia* spp. had not been studied before the present study started and was therefore unknown. The modes of plastid inheritance in interspecific and intraspecific crosses might be similar, but an unnatural genomic background of interspecific hybrids may affect the ability to distinguish parental plastids and hence may affect the mode of plastid inheritance. To verify the similarity between intraspecific and interspecific modes of plastid inheritance, a comparison was made between the modes of plastid inheritance among interspecific and intraspecific hybrids among flowering plants (Table 4.1 and Table 6.3).

Only results of studies that could proof plastid inheritance with plastid markers such as plastid mutants or other genetic markers (mainly RFLP markers) were included. The reliability of the determination of plastid inheritance mode was added, to avoid misinterpretation of unreliable data.

Many adaptations exist among flowering plants to inherit plastids uniparentally (Birky, 2001). In any case, parental plastids must be differentiated to ensure uniparental inheritance. It is unknown how this differentiation takes place in flowering plants. In *Chlamydomonas reinhardtii*, methylation of the inherited plastid DNA plays a central role in the preferential digestion of the not-inherited plastid (Umen and Goodenough, 2001). In mammals, where mitochondria are mainly inherited maternally, there is strong evidence that the protein ubiquitin is bound on sperm mitochondria and are thus differentiated from maternal mitochondria. Ubiquitination was not observed in interspecific crosses of cow, in contrast to intraspecific crosses (Birky, 2001).

Table 6.3. Mode of plastid inheritance in intraspecific hybrids of flowering plant species determined with genetic evidence, also see Table 4.1.

Mother species	M[1]	B[1]	P[1]	n[2]	ß[3]	Family (-ceae	Reference[4]
Actinidia deliciosa			100	143	0.51	*Actinidia*	Chat et al., 1999
Brassica napus	100			>20	>0.09	*Brassica*	Kao et al., 1991
Brassica rapa (syn. *B. campestris*	100			8224	~1	*Brassica*	Souza Machado et al., 1978; Souza Machado and Bandeen, 1982
Chlorophytum elatum	96	3	1	151		*Lilia*	Sager, 1972; K
Coffea canephora	100			?		*Rubia*	Lashermes et al., 1996
Daucus carota subsp. *sativus*	100			330	0.81	*Apia*	Vivek et al., 1999
Epilobium hirsutum	99.93		0.07	1451		*Onagra*	Schmitz and Kowallik, 1986
Epilobium hirsutum	99.77	0.23		1774		*Onagra*	Michaelis, 1954
Epilobium hirsutum	100			301	0.78	*Onagra*	Michaelis, 1958
Epilobium parviflorum	100			295	0.77	*Onagra*	Michaelis, 1954
Eucalyptus globulus	100			194	0.62	*Myrta*	McKinnon et al., 2001
Eucalyptus nitens	100			41	0.19	*Myrta*	Song et al., 1993
Gossypium hirsutum	100			1895	~1	*Malva*	K
Helianthus annuus	100			3209	~1	*Astera*	Triboush et al., 1999; Rieseberg et al., 1994
Hordeum vulgare	100			195	0.62	*Poa*	Sager, 1972; Ahokas, 1976
Nicotiana plumbaginifolia	100			6800	~1	*Solana*	Medgyesy et al., 1986
Nicotiana plumbaginifolia	97.5	2.5 B+P		1500		*Solana*	Medgyesy et al., 1986
Nicotiana tabacum	100			257900	~1	*Solana*	Huang et al., 2003
Nicotiana tabacum	100			500	0.92	*Solana*	Sager, 1972, K
Nicotiana tabacum	100			4005	~1	*Solana*	Sager, 1972, K
Nicotiana tabacum	98.5	1.5		7460		*Solana*	Avni and Edelman, 1991
Oenothera spp.	97-14	3-86		5827		*Onagra*	Chiu and Sears, 1993
Oryza rufipogon	100			?		*Poa*	Harris and Ingram, 1991
Oryza sativa	100			?		*Poa*	Réboud and Zeyl, 1994
Oryza sativa	+		R	?		*Poa*	Dally and Second, 1990
Secale cereale	73	27		193		*Poa*	Fröst et al., 1970
Stellaria longifolia	86	9	5	22		*Caryophylla*	Chong et al., 1994
Triticum aestivum	100			49	0.22	*Poa*	Briggle, 1966
Zea mays	100			16675	~1	*Poa*	Sager, 1972, K
Zea mays	100			1219	0.998	*Poa*	K

[1]M=maternal, B = biparental, P = paternal, R = rarely
[2]number of plants analysed; "?" indicates that the number of plants was not mentioned in the reference.
[3] Power of the test that the frequency of uniparental plastid inheritance is > 99.5%, assuming a binomial distribution of the mode of inheritance among the offspring, so ß = 1- $(0.995)^n$.
[4]K=Kirk and Tilney-Bassett, 1978

In flowering plants, little evidence exists upon a difference between intraspecific and interspecific plastid inheritance. Therefore, Table 6.4 was constructed from Table 6.3 and Table 4.1 by showing data from species of which plastid inheritance in both crossing

types could be retrieved from literature. Only uniparental inheritance was reported in intraspecific and interspecific crosses of *Actinidia deliciosa, Brassica napus, B. rapa, Coffea canephora, Daucus carota, Eucalyptus globulus*, *E. nitens, Gossypium hirsutum*, *Helianthus annuus, Hordeum vulgare Oryza rufipogon*, *Oryza sativa* (Table 6.4); in *Oryza sativa* paternal inheritance of plastids was very rare in a male sterile form (Dally and Second, 1990)), *Triticum aestivum* and *Zea mays*.

Table 6.4. Relation between intraspecific and interspecific plastid inheritance in flowering plant species as deduced from Table 6.3 and Table 4.1.

Species	Intra[12]	ß[4]	Inter (mother)[1]	ß[4]	Inter (father)[12]	ß[4]
Actinidia deliciosa	P	0.51			P	0.23[5a]
Brassica napus	M	0.09			M	0.10
Brassica rapa	M	~1	M>B		M	0.18[5b]
Chlorophytum elatum	M>B>P		M>B>P		M>B>P	
Coffea canephora	M+		M+		M+	
Daucus carota	M	0.81	M+		M+	
Epilobium hirsutum	M>>B		M>>B			
Epilobium parviflorum	M	0.77			M>>B	
Eucalyptus globulus	M	0.62	M	0.69		
Eucalyptus nitens	M	0.19			M	0.69
Gossypium hirsutum	M	~1			M+	
Helianthus annuus	M	~1	M	0.42		
Hordeum vulgare	M	0.62	M+B+P+			
Nicotiana tabacum	M>>B				M>>B	
N. plumbaginifolia	M>>B		M>>B			
Oenothera spp.	M>B to M<B		M>>B to M<B[3]		M>>B to M<B[3]	
Oryza rufipogon	M+				M+	
Oryza sativa	M+P+				M+	
Secale cereale	M>B				M+B+P+ (mother is *H. vulgare*) M+ (mother is *Triticum* spp.)	
Stellaria longifolia	M>B>P		M	0.11	M>B=P	
Triticum aestivum	M	0.22	M+			
Zea mays	M	~1	M+		M+	

[1] M=maternal, B=biparental, P=paternal;
[2] ">>" = much more frequent (>90% difference); ">" = more frequent (up to 90% difference); "+"= only qualitative data known
[3] ratio depends on phylogenic distance between parental species (Chiu and Sears, 1993)
[4] Power of the test that the frequency of uniparental plastid inheritance is > 99.5%, assuming a binomial distribution of the mode of inheritance among the offspring, so ß = 1- (0.995).
[5a] Cipriani et al. (1995) [5b] Song et al. (1993), Oszminkowski and Jourdan (1994)

The power of the test that plastids inherit uniparentally in interspecific hybrids is never higher than 0.7, though. Other species transmitted plastids in intraspecific and interspecific crosses biparentally to a wide range of degrees: *Chlorophytum elatum* (3-5%), *Epilobium hirsutum* (<1%)*, Nicotiana tabacum* (0-2%)*, N. plumbaginifolium* (0-2.5%), *Oenothera* spp.(0-86%), *Secale cereale* (29%) and *Stellaria longifolia* (13-33%). In *Brassica rapa, Epilobium parviflorum, Stellaria longifolia, Hordeum vulgare* (Table 6.4), the plastid inheritance in intraspecific and interspecific crosses was reported different.

The mode of plastid inheritance in intraspecific and interspecific crosses was similar in most of the reported cases (21 out of 25). Four out of 25 cases exhibited a plastid inheritance in interspecific crosses that was different from intraspecific crosses. Some of these cases can be explained by biparental inheritance that is present in the other parent. In *Epilobium hirsutum × E. parviflorum*, there may have been influence of *E. hirsutum*, which rarely inherits plastid biparentally (Table 6.3). Similarly, in *Hordeum spp. × Secale* spp. there may have been influence of *Secale cereale* which can show male plastid inheritance as well (Table 6.3). In *Stellaria longifolia × S. porsildii*, the maternal inheritance pattern may be explained by *S. porsildii*, of which the plastid inheritance pattern is not known. The difference in biparental inheritance in interspecific and intraspecific hybrids for *Brassica rapa* cannot be explained, however. In this last case, the unusual genomic background of interspecific hybrids may have influenced plastid inheritance.

In conclusion, the plastids inherited similarly in intraspecific and interspecific hybrids in 21 out of 25 cases. From the remaining four cases where the difference in intra- and interspecific was different, three could be explained by the plastid inheritance of the other parent. Only one case (*Brassica rapa × B. oleracea*; Table 4.1), could not be explained and may be caused by the hybrid nuclear background. It is not known how plastids inherit intraspecifically in *Zantedeschia*. There appears to be a strong correlation between the mode of plastid inheritance in intraspecific and interspecific hybrids, so I expect that plastids inherit biparentally in intraspecific crosses of *Zantedeschia* spp.

6.6 Overcoming intersectional PGI

A large potential for improving resistance and to improve the crop *Zantedeschia* in general would be to introgress characteristics of *Z. aethiopica* into genotypes of the section *Aestivae*. It has not been possible to achieve this goal, because of severe PGI, resulting in hybrid albinism (Yao et al., 1994a; Yao and Cohen, 2000). More knowledge on PGI and communication between the plastid and the nucleus is needed to overcome this breeding barrier.

Recent studies in other genera have clarified on the mechanism of PGI in albino hybrids. Studies on PGI between *Atropa belladonna* and *Nicotiana tabacum* showed that plants suffering from PGI could not process plastid RNA correctly and suffered from impaired transcription (Herrmann et al., 2003). The impaired transcription was due to interspecific sequence variation of the plastid encoded (PEP) RNA polymerases. Plastid RNA could not be correctly processed due to the inability of the *Atropa* genome to process four of five tobacco specific RNA editing sites, resulting in defective polypeptides (Herrmann et al., 2003). Similar mechanisms may exist in other genera. The rRNA genes of only one parent in the progeny were expressed in interspecific hybrids of *Brassica* and were associated with specific nuclear genomes (Redinbaugh et al., 2000). Similarly, rRNA genes of wheat were dominant over rye rRNA genes in triticale (Neves et al. (1997) in Redinbaugh et al. (2000)) and the rDNA complement of *F. pratensis* was lost completely in a vital intergeneric hybrid of (*Festuca pratensis* × *Lolium perenne*) × *L. perenne* (Kiang et al., 1994). Similar RNA transcription and editing problems may cause the albinism in hybrids of *Z. aethiopica* and *Aestivae* cultivars.

Detailed sequence data of all *Zantedeschia* plastomes is needed to give insight on the mechanisms and evolution of PGI. An alternative to sequencing the plastomes would be to amplify the entire plastome by PCR as described by Yamagishi (2002).

6.7 Taxonomical status of *Z. elliotiana*

The status of *Z. elliotiana* is unclear, because it has not been observed in the wild (Traub, 1948; Letty, 1973; Singh et al., 1996). No restriction site polymorphism was observed between the plastomes of *Z. elliotiana* and *Z. pentlandii* (Table 4.4). This made it impossible to differentiate these plastomes, but it indicated a close genetic relationship between the plastomes, because the plastome of *Z. rehmannii* differed by at least three polymorphisms from *Z. elliotiana* and *Z. pentlandii*. Another indication of the status of *Z. elliotiana* is given by the compatibility of its plastome to other species' genomes, because the level of PGI may be related to genetic distance (Chapter 4). While the plastome of *Z. elliotiana* and the genome of *Z. rehmannii* were partially incompatible, the plastomes and genomes of *Z. albomaculata* and *Z. elliotiana* appeared to be compatible reciprocally (Table 4.7). Therefore, *Z. elliotiana* probably is more related to *Z. pentlandii* and *Z. albomaculata* than to *Z. rehmannii*.

6.8 Effects of PGI to cultivation of *Zantedeschia*

Peculiar phenomena that are associated with *Zantedeschia* hybrids may be explained by PGI. The phenomenon of spontaneous variegation that sometimes arises after propagation by tissue culture of some cultivars, may be explained by PGI. This spontaneous variegation resembles the hybrid variegation in hybrids of *Z. rehmannii* and *Z. elliotiana* that is caused by PGI and biparental plastid inheritance (Table 4.5). Chimerism or even heteroplasmy could thus be the cause of the variegation, making purification of the clone the solution to overcoming the variegation. Markers for the different plastomes have been developed (Table 4.4), so identification of the chimerical or heteroplasmic tissue should be possible.

Another phenomenon is spontaneous variation within clones. Some cultivars that are vegetatively propagated and should therefore be genetically homogenous, show unexpected variation. Various characters such as flower colour, leaf colour, number of leaves (bushiness) and leaf size can be affected. The bushiness could not be explained by hormonal after-effect from tissue culture (D'Arth et al., 2002). On the one hand, unknown

viruses or virus-like bodies may cause some of these variations. A virus that only occurs in *Zantedeschia* spp. has only previously been described (Kwon et al., 2002), so other unknown pathogens may be present as well. On the other hand, the spontaneous variation may have a genetic background, as it shows similarity with high mutation rates that were described in *Oenothera* spp. The plastomes of *Oenothera* spp., which also inherit biparentally and cause PGI, showed an unusual high mutation rate (Sears et al., 1996).

6.9 Closure

The breeding barrier that is caused by PGI plays a central role in breeding of *Zantedeschia* spp. Genetic analyses for resistance could be done only with knowledge of the consequences of PGI and biparental plastid inheritance. Selection of the most resistant offspring is possible using the leaf disk test (Chapter 2), but the introgression of resistance genes into commercial cultivars depends on breeding barriers, mainly PGI.

The region of origin, Southern Africa, is relatively under-represented in botanical research and could therefore harbour much unknown genetic variation. The genetic variation for resistance to soft rot that was determined in the section *Aestivae* (Table 3.6b) shows that there is much variation in wild germplasm that has not been used in breeding. The discovery of a pink-flowered variation among the pure-white flowering *Z. aethiopica* (Singh, 1996) and the recent discovery of *Z. odorata* (Perry, 1989) prove furthermore that there is still much unknown variation in the wild. In conclusion, *Zantedeschia* spp. is a rich source for biological science and there is a large potential for breeding.

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Samenvatting

Zachtrot veroorzaakt door *Erwinia carotovora* subsp. *carotovora* (syn. *Pectobacterium carotovorum* subsp. *carotovorum*) is de belangrijkste ziekte van *Zantedeschia* spp. Cultuurmaatregelen kunnen het gewas met mate beschermen, maar een combinatie met resistente cultivars zou een betere controle kunnen bewerkstelligen. Resistente cultivars zijn echter niet beschikbaar. In dit proefschrift is bepaald dat het mogelijk is om te veredelen voor resistentie tegen *Erwinia*. Hiertoe zijn ziektetoetsen ontwikkeld, evenals een collectie van *Zantedeschia* spp. om de genetische variatie voor resistentie tegen *E. carotovora* subsp. *carotovora* te bepalen. De resultaten van vier verschillende ziektetoetsen die gebruik maken van gehele knollen, knolschijven, bladstelen en bladponsen kwamen over het algemeen met elkaar overeen en met ervaringen uit de praktijk. Hoewel de twee knoltoetsen het meest reproduceerbaar waren, is de bladponstoets gebruikt voor verder onderzoek. De bladponstoets is namelijk niet destructief, zodat zaailingen en zeldzaam plantmateriaal geëvalueerd konden worden. Binnen de sectie *Zantedeschia* bleken genotypen van *Z. aethiopica* resistent tot matig resistent, terwijl *Z. odorata* vatbaar bleek. Binnen de sectie *Aestivae* waren slechts enkele cultivars significant minder vatbaar ('Neroli', 'Coral Sunset' en 'Hazel Marie') dan de zeer vatbare controle 'Florex Gold'. Partiële resistentie werd gevonden in *Z. albomaculata* subsp. *macrocarpa* en in *Z. rehmannii*. Om de genetische basis van de partiële resistentie te achterhalen, zijn hybriden ontwikkeld van soorten die in resistentienivo verschillen: *Z. albomaculata* (vatbaar tot partieel resistent), *Z. rehmannii* (partieel resistent), *Z. elliottiana* (vatbaar) en *Z. pentlandii* (zeer vatbaar). De families van deze interspecifieke kruisingen (F_1-hybriden) splitsten niet-mendeliaans uit voor bladkleur (chlorofylgehalte) en groeikracht, afhankelijk van de ouders en de kruisingsrichting. Zo waren nakomelingen van *Z. rehmannii* als moeder voornamelijk groen (met een chlorofylgehalte gelijk aan selfings van de ouders) en groeikrachtig. Daarentegen waren nakomelingen van *Z. elliotiana* als moeder voornamelijk bleek en deze bleven al dan niet sterk achter in groei, afhankelijk van de vader. De oorzaak van deze reciproke verschillen is bepaald om te verkennen of ze het nivo van resistentie konden beïnvloeden. Aan de hand van plastoom-specifieke CAPS-merkers (Cleaved Amplified Polymorphic Sequence) is bepaald dat er minstens drie plastomen bestaan met verschillende compatibiliteit met de

genomen van de verschillende soorten van de sectie *Aestivae*. De uitsplitsingen van bladkleur (chlorofylgehalte) bleken voort te komen uit plastiden die via pollen waren overgedragen. Deze paternale invloed in plastidenvererving kwam voor in 24 soorthybride families van *Z. rehmannii*, *Z. albomaculata*, *Z. elliotiana* en *Z. pentlandii*. Aldus werd geconcludeerd dat biparentale vererving van plastiden een algemeen optredend verschijnsel is in interspecifieke kruisingen binnen sectie *Aestivae*. Hierbij bleek het plastoom van *Z. rehmannii* het meest compatibel met de andere genomen. Planten met een plastoom-genoom combinatie die niet compatibel was, waren minder groeikrachtig en minder resistent. Daarom is een genetische analyse voor resistentie alleen gedaan op de nakomelingen die niet te lijden hadden van verlaagde groeikracht die veroorzaakt was door plastoom-genoom incompatibiliteit. Het resistentienivo van deze F_1-hybriden was gecorreleerd aan het resistentienivo van hun ouders en er trad transgressie op in zaailingen van *Z. rehmannii* en *Z. albomaculata*. Deze positieve relatie en de transgressie geven aan dat er een genetische basis bestaat voor resistentie, met complementerende of additieve genen en een groot potentieel voor resistentieveredeling.

Summary

Soft rot caused by *Erwinia carotovora* subsp. *carotovora* (*Pectobacterium carotovorum* subsp. *carotovorum*) is the most important disease of *Zantedeschia* spp. Cultivation measures can protect the crop partially, but a combination with resistant cultivars could result in better control. Resistant cultivars are not available, however. It is determined in this thesis that it is possible to breed for resistance caused by *Erwinia*. Resistance tests as well as a collection of plant material were developed to determine the genetic variation for resistance to *Erwinia carotovora* subsp. *carotovora*. The results of four resistance tests were in general concordance with experiences from practice. Although the tuber tests were most reproducible, the leaf disk test was used further research. The leaf disk test was not destructive, so seedlings and rare germplasm could be evaluated. Among the section *Zantedeschia, Z.. aethiopica* appeared resistant to moderately resistant, while *Z. odorata* appeared susceptible. Only some cultivars of the section *Aestivae* ('Neroli', 'Coral Sunset' and 'Hazel Marie') appeared significantly less susceptible than the very susceptible reference 'Florex Gold'. Partial resistance was observed in *Z. albomaculata* subsp. *macrocarpa* and in *Z. rehmannii*. To determine the genetic basis of the partial resistance, hybrids were developed of species that have different resistance levels: *Z. albomaculata* (susceptible to partially resistant), *Z. rehmannii* (partially resistant), *Z. elliotiana* (susceptible) and *Z. pentlandii* (very susceptible). The families of these interspecific crosses (F$_1$-hybrids) segregated for leaf colour (chlorophyll content) and vigour, dependent on the parents and the crossing direction. This segregation was not Mendelian, but depended on the crossing direction. Descendents of *Z. rehmannii* as mother were green (with a chlorophyll content that was similar as selfings of the parents) and vigorous. Descendents of *Z. elliotiana* as mother, however, were mainly pale green and these were less vigorous in some cases, depending on the father. The cause of these reciprocal differences was determined to evaluate the effect to the level of resistance. Plastome-specific CAPS-markers (Cleaved Amplified Polymorphic Sequence) were used to determine that there existed at leat three plastomes that differed in the level of compatibility to the genomes of the different species of section *Aestivae*. The segregation in leaf colour (chlorophyll content) within families of the same crossing direction originated from plastids that had

inherited via pollen. This paternal influence in plastid inheritance was observed in 24 intespecific families of *Z. rehmannii*, *Z. albomaculata*, *Z. elliotiana* and *Z. pentlandii.* Thus was concluded that biparental ininheritance of plastids is common common within interspecific crossings among section *Aestivae*. The plastome of *Z. rehmannii* was the most compatible to the other genomes. Plants with not fully compatible plastome-genome combinations had decreased vigour and level of resistance. Genetic analyses were therefore performed only on plants that did not suffer from plastome-genome incompatibility. The level of resistance of F_1-hybrids was correlated to the level of resistance of the parents en transgression was observed in seedlings of *Z. rehmannii* and *Z. albomaculata*. This relation indicates a genetic basis for resistance with complementary or additive genes with great potential for resistance breeding.

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Ronald
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Related publications

Snijder, R. C., J. M. van Tuyl and M. van Paassen. 2000. Veredeling brengt inzicht in Erwinia-resistentie. Bloembollencultuur (5): 29.

Snijder, R. C. and J. M. van Tuyl. 2002. Evaluation of tests to determine resistance in *Zantedeschia* spp. (*Araceae*) to soft rot caused by *Erwinia carotovora* subsp. *carotovora*. European Journal of Plant Pathology 108: 565-571.

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Ronald Christiaan Snijder was born in Harderwijk, The Netherlands on 24 June 1976. After obtaining the Atheneum (with Latin) diploma in June 1994 at the Minkema Scholengemeenschap in Woerden, he obtained a Master's degree on Plant Breeding and Crop Protection at Wageningen Agricultural University (now Wageningen University) in September 1999. During this study, he specialised in classical plant breeding and genetic resources by obtaining experience with classical breeding of *Tulipa*, interspecific hybridisation of *Lilium,* genetic variation and molecular techniques with *Alstroemeria* and *Hordeum,* and cultivar identification of *Syringa*. From November 1999 to September 2003, Ronald had a position as Junior Researcher at CPRO (now Plant Research International) on the project '*Erwinia* resistance in *Zantedeschia*'. The present thesis is a direct result of the research that was conducted in this position.

The author and his subjects in their habitat.