Unraveling the genetics of *Botrytis cinerea* resistance in *Gerbera hybrida*

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This research was conducted under the auspices of the graduate school Experimental Plant Sciences.

Unraveling the genetics of *Botrytis cinerea* resistance in *Gerbera hybrida*

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Thesis

Submitted in fulfilment of the requirements for the doctor degree
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr A.P.J. Mol
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 19 June 2017
at 11 a.m. in the Aula

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Unraveling the genetics of *Botrytis cinerea* resistance in *Gerbera hybrida*
160 pages

PhD thesis, Wageningen University, Wageningen, NL (2017)
With references, with summary in English
ISBN: 978-94-6343-181-1
DOI: 10.18174/413048

Content

This thesis is dedicated

to my dearest grandmothers, Yanzhen Kang and Sumei Zhu, who grew the *Magnolia*, *Rosa*, *Rhododendron*, *Camellia*, *Osmanthus* in my heart.

Chapter 1 General introduction

The genus *Gerbera* that currently includes about 42 accepted species (Flann 2009) belongs to the *Compositae* (*Asteraceae*) family in the tribe of Mutisieae. Most species in this genus are distributed in Africa and Asia (Hansen 1985; Tseng 1996). The generic name *Gerbera* was established in 1737 after the German medical doctor Traugott Gerber (Hansen 1985), and is well-known to people since the commercial gerbera hybrid has been consistently ranked among the top 5 of most used cut flowers worldwide (Hansen 1999; Tourjee *et al.* 1994). In 2014, a total number of 1,890 million gerbera stems were sold at the Dutch Flower Auctions (FloraHolland 2015) and the annual quantity of gerbera sold in the USA domestically in 2015 was about 106 million stems based on the statistics of 15 states (USDA 2016).

Origin of *Gerbera hybrida*

The present-day commercial gerbera cultivars, which are designated as *Gerbera hybrida*, are believed to have been derived from the artificially hybridized progenies of the two species with African origin, *G. jamesonii* and *G. viridifolia* (Fig. 1-1) by Richard Irwin Lynch, curator of the Cambridge Botanic Garden during 1879 to 1919 (Hansen 1985, 1999; Johnson 2010; Tourjee *et al.* 1994). *G. jamesonii* is a perennial herbaceous plant with large stemless runcinated-pinnatifid leaves growing in a rosette from the crown. The large composite flower head (up to 680 mm in diameter) which is borne on a long single peduncle/stem also from the rosette, with noticeable ray florets varying in color (Hansen 1985; Hansen 1999). The species is named after Robert Jameson who brought plants from Barberton region in South Africa to Durban Botanical Garden from where they were sent to England (Hansen 1985; Hansen 1999). *G. viridifolia* is relatively small in comparison to *G. jamesonii* and has light-purple flower heads of about 13–37 mm (Hansen 1999; Johnson 2010). The specific epithet name *viridifolia* means 'green leaves' characterizing the lack of white-felting on the back of leaf (Johnson 2010). The distribution of *G. jamesonii* is restricted to a relatively small area in southern Africa, while the *G. viridifolia* is widespread in eastern Africa and part of southern Africa (Hansen 1985). It seems there is a geographic overlap of the two species, yet natural hybrids have not been reported (Hansen 1985).

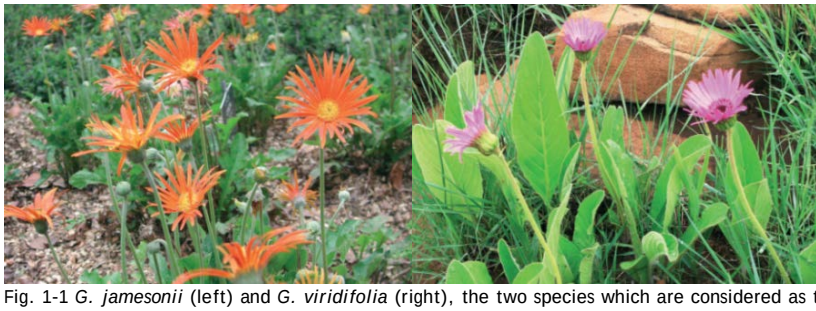

Fig. 1-1 *G. jamesonii* (left) and *G. viridifolia* (right), the two species which are considered as the origins of commercial cultivars. Pictures kindly provided by I. Johnson (2010).

Breeding of commercial gerbera

The breeding of gerbera can be traced back from a wild scarlet-colored *G. jamesonii* that *Lynch* received and cultivated at the end of the 19th century in England (Hansen 1999). A new yellow gerbera from his first recorded breeding program was produced and named *G. jamesonii* 'Sir Michael' which was identified as the first gerbera cultivar (Hansen 1999; Upson 2006). Lynch's crossing works were done afterward

between these *G. jamesonii* individuals and also with *G. viridifolia*, which was introduced to England earlier. Interspecific hybridization of the two species could generate completely fertile offspring with new color combinations and hybrids were found more easily to grow (Hansen 1999).

However, the specimen of *G. viridifolia* used for the first hybridization experiments perished and the hybridizations later on were mainly performed by backcrossing with *G. jamesonii* or crossings among the hybrid offspring (Hansen 1999; Tourjee *et al.* 1994; Upson 2006). Although *G. viridifolia* was involved in the commercial gerbera more or less, *G. jamesonii* is therefore considered as the primary contributor (Hansen 1999). It might be the reason *G. hybrida* was often referred to as *G. jamesonii*, even in scientific publications nowadays (Baas *et al.* 1995; Bhatia *et al.* 2011; Caballero *et al.* 2009; Gantait *et al.* 2011; Minerdi *et al.* 2008; Nhut *et al.* 2007). Seeds from the pioneering work of Lynch were distributed to different places and breeders (Hansen 1999; Tourjee *et al.* 1994). After getting the hybrid plants and seeds from Lynch's stock, a gardener named Adnet in France made 2,700 crosses and created a fourth-generation population with about 25,000 seedlings, which varied in color (Tourjee *et al.* 1994). Breeding was subsequently done in Italy, Belgium, and the Netherlands by crossing the wild gerbera directly from South Africa with the processed materials probably also obtained from Lynch directly or indirectly (Hansen 1999; Tourjee *et al.* 1994). Meanwhile, growers in Europe and US also made efforts for the gerbera hybrids to become adapted to and survive in new climates, for instance by acquiring cold hardiness, by using horticultural techniques (Tourjee *et al.* 1994).

Gerbera was identified as a commercial crop in the US around 1930 and there were already commercial gerbera growers in the Netherlands in 1951 (Tourjee *et al.* 1994). Breeding gerbera as a commercial crop faced new challenges. A breeding project was started in 1963 at IVT (former Institute for Horticultural Plant Breeding, Wageningen), aiming to introduce resistance against o the soil-borne fungus *Phytophthora cryptogea* into the commercial gerbera when plant production switched to glasshouses (Sparnaaij and Lamers 1971; Sparnaaij *et al.* 1975). Gerbera cut flowers with high quality, like longer vase life, were also preferred by customers. Gerbera vase life was counted from the first one or two circles of disc florets showing pollen to when stem folded or petals wilted (de Jong and Garretsen 1985). Van Meeteren (1978a, b, 1979a, b, 1980) researched quite intensively the relations between water content and gerbera flower quality after cutting. Besides the physiological and pathogenic reasons, the shortened vase life could be explained by genetic variance as well and selecting parents with strong stem could reduce the problem (de Jong 1978; de Jong 1986; de Jong and Garretsen 1985). A gerbera *Davis Population* was produced from random mating in the 1970s and was initially created to increase the yield (Drennan *et al.* 1980; Harding *et al.* 1981b). Plants in each generation with the highest yield were chosen as the parents of the next generation. Harding *et al.* (1981b) found that the averaged broad-sense heritability of gerbera yield was 42% and concluded that most genetic variance was additive and selectable. Except the yield, vase life and consumer preference were also used as selection indices for parents for each generation (Drennan *et al.* 1980; Harding *et al.* 1981a; Harding *et al.* 1981b; Harding *et al.* 1985; Harding *et al.* 1987).

Using classical breeding methods, breeding for improvement of some specific traits in gerbera, like disease resistance, is not easy. Gerbera breeders are confronted with several restrictions of these artificial hybrids: it is an outcrossing crop with high degree of genetic variability and thus highly heterozygote for most

characteristics for which there is an interest; high self-incompatibility with noticeable inbreeding depression *etc*. (Barigozzi and Quagliotti 1979). To overcome some of these barriers, Jenkin carried out a recurrent selection program for gerbera in 1940 to eliminate lethal and sub-lethal genes to prevent inbreeding depression and reduced survival and fertility in offspring; and by doing this he found that the fertility (seed production) of certain genotypes increased after only one generation (Schiva 1979). Since inbreeding depression makes F1 hybrid development difficult, Hondelman suggested making a sib crossing to render the crop more homogenous (Harding *et al*. 1981b).

With the early success of tissue culture in gerbera by using rhizome (Leffring 1971), midrib (Pierik and Segers 1973), capitulum (Pierik *et al*. 1975; Pierik *et al*. 1973) and shoot tip (Murashige *et al*. 1974) as explants, several alternative breeding methods combined with this *in vitro* technique have been applied to improve gerbera. Jain *et al*. (1998) reviewed several technologies, which could be used to produce genetic variability in breeding options for gerbera improvement:

- mutagenesis to induce mutations on flower morphology or color by X-ray or gamma ray irradiation and chemical (EMS) mutagens (Laneri *et al*. 1989; Walther and Sauer 1986);
- production of haploids by *in vitro* culture of unfertilized ovules or anthers (Ahmim and Vieth 1986; Cappadocia *et al*. 1988; Cappadocia and Vieth 1990; Gidrol *et al*. 1984; Preil W *et al*. 1977; Sitbon 1981);
- production of doubled haploid homozygous lines or tetraploids from chromosome doubling using colchicine; (Miyoshi and Asakura 1996; Tosca *et al*. 1995; Gantait *et al*. 2011; Honkanen *et al*. 1990);
- somaclonal variation developed from *in vitro* propagated material (Buiatti and Gimelli 1993; Vitti 1996);
- genetic transformation to modify certain traits, like flower color and flower pattern (Elomaa *et al*. 1996; Elomaa *et al*. 1993; Helariutta *et al*. 1993; Yu *et al*. 1999).

Till now, gerbera breeders are active in this floricultural crop to achieve improved characteristics of interests to satisfy customers, like new flower colors, variation in morphology and size, vase life extension and improved resistance to diseases (Barigozzi and Quagliotti 1979; Jain *et al*. 1998). Those methods mentioned above could still provide valuable contributions for gerbera development (Jain *et al*. 1998). Breeding companies in general invest on average about 15% of their turnover in R&D per year (Plantum 2014) and also want their varieties to get protection. More than 4800 gerbera varieties have been applied for Plant Breeder's Right according to the plant variety database of the International Union for the Protection of New Varieties of Plant (UPOV) with applications from amongst The Netherlands, Germany, Denmark, Brazil, France, Israel, Italy, Japan and United States (UPOV 2016).

Propagation of commercial gerbera

Most commercial used cut flower gerberas and some garden and pot flower gerbera are primarily vegetatively propagated from tissue culture (Hamrick 2005). Gerbera can be easily multiplied by a division of rhizomes with buds and attached roots, yet less than 10-12 progeny plants might be produced in this way for each mature plant per year and cut pieces are easily infected (Rogers and Tjia 1990). Therefore, for

large-scale commercial gerbera production, tissue culture is widely used to result in uniform, vigorous and pathogen-free plantlets (Kanwar and Kumar 2008; Rogers and Tjia 1990).

Gerbera cultivars can also be produced by sexual propagation (Kanwar and Kumar 2008). Several ornamental (gerbera specific) breeding companies developed female and male parental inbred lines to produce F1 hybrid seeds for potted and garden used gerbera because of economic and phytopathogenic reasons (Hamrick 2005; Reimann-Philipp 1983). More importantly, the F1 hybrid seeds possess so-called 'hybrid vigor', uniformity and high yield, and it could be used against illegal reproduction since the hybrids segregate and produce variable offspring (Horn 2002; Reimann-Philipp 1983). Sakata, PanAmerican Seeds and Florist have released their own series/lines (http://www.durora.com/; http://www.panamseed.com/revolution.aspx ; http://www.floristholland.nl/en/products/flori_line_pot_plants).

Ornamental diversity of gerbera

Owing to the continuous activities of commercial and hobbyists gerbera breeders, there are thousands of gerbera varieties with a wide range of variation. The flower of gerbera in fact is a composite of small florets forming an inflorescence. The most attractive part of the inflorescences is the surrounding 'petals' of a gerbera flower that are actually individual strap-shaped florets and called 'ray florets'. The central part, the 'heart', consists of so called 'disc florets'. Between these two types of florets, some gerbera inflorescences also possess 'trans florets' (Fig. 1-2).

Fig. 1-2 The three different types of florets in the gerbera inflorescence (shown is the cultivar named Soap® from Schreurs B.V., http://www.schreurs.nl).

According to the inflorescence structure, commercial gerbera can be simply classified by presence or absence of trans florets, size and cycle of trans florets, as single, semi-double and double types. Further classification is by ray floret shape such as spider type (Fig. 1-3). The color of corolla (ray florets and trans florets) in a single flower can be quite diverse and vary from simplex white, cream, yellow, orange, salmon, pink, lilac, purple or red, with contrasting color combinations of ray and trans florets or with bicolor in ray florets only. The disc florets can be either black or green, with slightly different shades.

Fig. 1-3 From left to right, there are single flower type gerbera cultivar (Harumi® from Schreurs B.V.), semi-double flower type (Café® from Schreurs B.V.), double type (Tattoo® from Florist B.V., http://www.floristholland.nl) and spider type (Stryker® from Florist B.V.).

Based on the flower/capitulum size, commercial gerbera used for cut flowers can mainly be divided into two types: standard gerbera with flower size around 10-14 cm (Fig. 1-4) and mini gerbera (germini) with a size around 6-9 cm (Fig. 1-5). The stem length for both two types of gerbera can reach to 60-70 cm with a vase life of around 2-3 weeks. Pot gerbera (Fig. 1-6) for indoor or outdoor use, usually have the same flower pattern of cut flower gerbera however with relatively shorter stem and smaller flower size.

Fig. 1-4 Different standard gerbera cultivars from Florist B.V. and Schreurs B.V.. The commercial names of cultivars are White Jewel, Alliance, Alma, Volia, Spotlight, Hollywood, La Vida; Edelweiss, Barista, Damask, Benidorm, Palestra, Oilila, Kaiser. Bar, 1 cm.

Fig. 1-5 Different mini gerbera (Germini) cultivars from Florist B.V. and Schreurs B.V.. Cultivars are Noxx, Jimmies, Honky tonk, Bolero BonBon, Shayna, Babydoll, yell, Verdana; Frozen, Babyface, Bride, Sylvie, Mamamia, Garfield, Nacho, Purple Wonder, Smoothie, Cassis. Bar, 1 cm.

Fig. 1-6. Different pot gerbera cultivars Sweet Memories®, Sophie®, Sunny®, Eyecatcher Purple BC, Dark Fireball BC, Orange from Florist B.V..

Model for *Compositae* family with regards to molecular studies

Gerbera is attractive not only to consumers for their diverse colors but also for biochemical, developmental and genetic research (Teeri *et al.* 2006). Gerbera is a member of the *Compositae* that exclusively form capitula and show a large variation in flower shapes. Studies of floral development and secondary metabolites synthesis in gerbera as a model plant could be representative of a large number of plants species since the *Compositae* are the largest family of the flowering plants with a number of economically important crops, like sunflower, lettuce, chrysanthemum and gerbera itself (Teeri *et al.* 2006).

The diversity of inflorescence patterns and colors in gerbera are evidence for the beauty of gerbera. The inflorescence of gerbera is composed of hundreds of small flowers that are differentiated into ray and disc florets (sometimes also trans florets, see the previous section). These small flowers are morphologically different (Fig. 1-7). The stamens of gerbera flowers are aborted in ray florets and trans florets, yet the stamens and pistils in disc florets are completely developed (Teeri *et al.* 2006). A series of flower traits were investigated and analyzed in gerbera morphologically (Drennan *et al.* 1986; Harding *et al.* 1990; Huang and Harding 1998; Huang *et al.* 1990). By looking for the homologous genes from the ABC(E) model found in Arabidopsis or snapdragon, several homologous genes were identified in gerbera and genetic transformation with these genes resulted in a phenotypic change in flower type (Broholm *et al.* 2010; Ruokolainen *et al.* 2010, 2011; Teeri *et al.* 2006). However, floral development in gerberas is much more complicated because of multiple homologous genes with subfunctionalization (Teeri *et al.* 2006).

Fig. 1-7 Three different types of florets in gerbera inflorescence. The pigmented ligulate ray and trans florets (left and middle) are female. The disc florets (right) are completely developed with both stamens and carpels. Stamens (arrow) fuse post genitally and cover the carpels in disc flowers. Bar, 1 cm. Reprinted from Teeri *et al*. (2006), with the permission from the John Wiley & Sons.

Moreover, wide ranges of gerbera flower colors already exist in wild gerbera species and varied colors can also be present on a single capitulum or even on single ray florets. The color of the capitulum of gerbera can also be divided into three distinct parts according to morphology based on the color of disc florets, ray florets and/or trans florets. Disc color, which comes from pappus bristles, could be simplified distinguished as black or green (Barigozzi and Quagliotti 1979), although varying colors as black-purple, brown-black, or green-yellow, light-yellow also exist. Kloos *et al.* (2005) indicated the segregation of disc color is according to Mendel's law and showed that it is determined by a dominant gene named *Dc*.

However, the color of ray florets (including trans florets) seems a much more complex trait. Ray floret color can be simply grouped into several categories, like *white*, *cream*, *yellow, orange*, *pink*, *lilac*, *purple*, *red* or *bi-color* (indicates the presence of two colors in a single ray floret or two different colors of ray and

trans florets) for marketing purposes. In reality, there is no clear boundary for the shades of the color, and it is observed like a quantitative trait with continuous variables (Tourjee *et al*. 1995a, b). Flavonoids and carotenoids together determine the color of gerbera (Tyrach and Horn 1997). Pigmented flavonoids include anthocyanins, flavones and flavonols (Tyrach and Horn 1997). A series of genes/enzymes in the secondary metabolite synthesis, such as the general phenylpropanoids, flavonoids, anthocyanins, carotenoid biosynthesis pathway and transcription factors, like BHLH- and MYB-type TF, were confirmed contributing to the understanding of pigmentation in the gerbera flower (Ainasoja 2008; Bashandy *et al*. 2015; Deng *et al*. 2014; Elomaa *et al*. 1996; Elomaa *et al*. 1993; Helariutta *et al*. 1993; Teeri *et al*. 2006).

Molecular genetic resources for gerbera development

At the NCBI database, about 60,000 nucleotide sequences related to gerbera have been published in the recent 20 years. The increasing amounts of sequencing data that have been made available in gerbera provide genetic resources for research in gerbera as a model plant or for improvement of itself as a floriculture crop. Quite a lot of researches on flower development and flower color synthesis mentioned before were based on the EST data submitted by the Gerbera Laboratory at University of Helsinki (http://blogs.helsinki.fi/gerberalab/). In the last 25 years, each year around 40 new gerbera cultivars from the Netherlands alone and around 120 from other countries were put forward for Plant Breeder's Rights application (UPOV 2016). Many new cultivars are with little morphological differences and that makes DUS testing (examining new cultivars for compliance with the Distinctness, Uniformity and Stability requirements) difficult. Some researches were done by using molecular markers which were developed from the NCBI database to study genetic diversity/variation and evaluate the genetic fidelity of certain gerbera cultivars (Da Mata *et al*. 2009; Minerva *et al*. 2012; Rezende *et al*. 2009; Bhatia *et al*. 2011; Bhatia *et al*. 2009; Gong and Deng 2012). These results could be applied in cultivar identification, potential collection undoubling and detection of possible infringements.

Genetic resources in gerbera could also help gerbera improvement in breeding by enabling indirect selection for traits. In field and vegetable crops, marker assisted selection/breeding is not a new story, while the use of molecular markers in ornamental crops is still lagging far behind (Arens *et al*. 2012; Smulders *et al*. 2012). Only a small amount of SSR and RGA (resistance gene analog) markers have been developed in gerbera (Gong and Deng 2010 2012; Song *et al*. 2012). The lag-behind situation in ornamentals is partly because some ornamental traits, like flower color, flower pattern, flower size or flower scent, themselves are perfect morphological markers and indeed widely used in breeding for a long time and selection programs. However, nowadays, new cultivars, in which disease resistance and novel ornamental traits are integrated, are demanded by growers and consumers. Disease resistance is often controlled by multiple genes and/or is strongly influenced by the environment. Selection on phenotype instead of genotype is therefore difficult. Thus, developing genetic tools will assist the breeding of gerbera for these traits.

Gerbera gray mold

Gerbera is mainly used for cut flower production with plants that are grown commercially in greenhouses for year round production. However, during cultivation in the greenhouse, especially in winter, the

heating easily results in high humidity and in condensation on plant surfaces, which form ideal conditions for *Botrytis cinerea* conidial germination causing gerbera gray mold (Salinas *et al*. 1989). After cutting, gerbera is generally transported either in closed cardboard boxes or in plastic containers filled with water. In both transport systems, high relative humidity and condensation are unavoidable. Therefore, during every stage of gerbera flower production and transport botrytis infection can occur and form a considerable threat to the commercial gerbera flower production chain.

Problem of gerbera gray mold

Botrytis cinerea is an airborne plant pathogen, and spores could be detected everywhere in gerbera growing greenhouses using a selective medium (Kerssies 1990, 1993a). Botrytis spores can be spread by air currents, water droplets and/or irrigation systems (Cole *et al*. 1996). They can remain dormant on the plant surfaces for a long time. *B. cinerea* lesion numbers on gerbera flowers counted under the microscope after cutting were observed with a seasonal pattern: few lesions in spring and early summer, many lesions at other times of the year (Kerssies 1993b). This was mainly related to the effects of relative humidity (Kerssies and Frinking 1996). Once the humidity reaches to 90% or higher, Botrytis spores will germinate and invasion will start at any temperature either in pre-harvest or post-harvest stage (Eden *et al*. 1996).

To control gray mold, keeping good greenhouse sanitation, removing faded or blighted flowers, and using air circulation to reduce high humidity levels are required (Dreistadt 2001; Elad *et al*. 2016b). Chemical control, by applying fungicides, is also a common method to control Botrytis, although it increases the risk of fungicide resistance in Botrytis, of damaging flowers and of chemical residues on the flowers (Dean *et al*. 2012; Kerssies 1993b; Salinas and Verhoeff 1995; van Kan *et al*. 1997). Particularly in the post-harvest stage, it is difficult to avoid Botrytis infections (Kerssies 1993b), when gerbera stems are cut from the plant and experience senescence.

The Dutch Flower Auction Association (VBN) practice a zero tolerance on gerbera affected by Botrytis, and all gerbera flowers with detectable Botrytis infections will be rejected and destroyed (VBN 2009). In 2002, a total number of 759 million gerbera stems were sold at the Dutch Flower Auction. However, 0.26 % of the stems were found to be infected by Botrytis causing at least 200,000 euro economic losses. It has to be stated here that this is just the visible Botrytis infection found during quality inspection at the Auction (Vrind 2005). Based on the vase life test of *FloraHolland* during the period 2000-2003, vase life of Botrytis-infected gerbera was dropped from 10.2 to 6.8 days. Quality losses due to gerbera gray mold might affect the seller (a breeder's reputation), the grower (reduction in profits) (Bastiaan-Net *et al*. 2007) and the consumer (dissatisfied with short vase life, flower reputation) (Dean *et al*. 2012). Breeding for a Botrytis resistance gerbera will benefit breeding companies, growers and consumers.

Wide host range of Botrytis cinerea and disease cycle

Botrytis-induced infections in gerbera manifest itself by brown stripes and spots on ray florets (Fig. 1-8 Left), by rotting inside of disc florets (Fig. 1-8 Right) and/or also by overgrowing gray fungus hyphae on anthers and unripe disc florets. *B. cinerea*, the cause of gerbera gray mold, is a typical necrotrophic fungal pathogen. This notorious fungus causes devastating diseases and considerable quality/quantity losses in a

wide range of plant species. Widely cited literature from Jarvis (1977) estimated that *B. cinerea* could infect over 200 plant species. However, up to now, the species *B. cinerea* is reported to attack at least 586 genera including far more than thousand plant species (Elad *et al.* 2016a). From the host list of *B. cinerea*, commonly used ornamental plants, such as, *Alstoemeria*, *Anemone*, *Antirrhinum*, *Aquilegia*, *Aster*, *Begonia*, *Bellis*, *Calendula*, *Callistephus*, *Camellia*, *Canna*, *Chrysanthemum*, *Cymbidium*, *Dahlia*, *Delphinium*, *Dendrobium*, *Dianthus*, *Echinacea*, *Eschscholzia*, *Freesia*, *Gladiolus*, *Helianthus*, *Hemerocallis*, *Hibiscus*, *Hosta*, *Hyacinthus*, *Hydrangea*, *Hymenocallis*, *Impatiens*, *Iris*, *Jasminum*, *Lavandula*, *Malus*, *Narcissus*, *Paeonia*, *Papaver*, *Phalaenopsis*, *Phlox*, *Primula*, *Ranunculus*, *Rhododendron*, *Rosa*, *Syringa*, *Tagetes*, *Viola*, *Zinnia* and also *Gerbera* are all covered. Like for *Gerbera*, the symptoms of disease caused by *B. cinerea* on different ornamental plants are visible as necrotic spots on leaves or petals, as gray mold rot on leaves or bulbs, blossom blight, stem canker and damping off (Elad *et al.* 2016a and Fig. 1-9.).

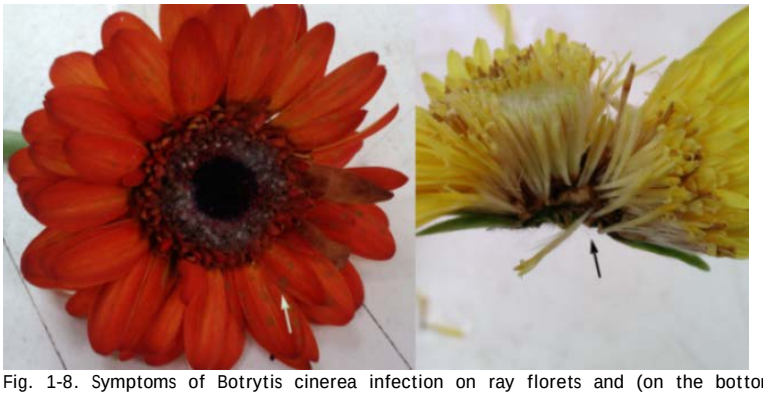

Fig. 1-8. Symptoms of Botrytis cinerea infection on ray florets and (on the bottom of) disc florets of gerbera inflorescence. The brown (necrotic) spots could be seen on ray florets (left, the arrow shown). Inside rot of disc florets could be observed after cutting (right, the arrow shown).

B. cinerea overwinters either as sclerotia or mycelia in/on plant debris and in soil and the growth of the pathogen can be activated by favorable weather conditions. Germinated infectious spores mainly target senescent or damaged tissue (Fig. 1-9). When Botrytis lands on the surface of host plants, it secretes enzymes to degrade host plant cell walls and induce cell death. Alternatively, spores might also remain quiescent for a while waiting for suitable conditions for development (van Kan 2006; Williamson *et al.* 2007). Botrytis is considered an opportunistic invader (Corbaz 1978; Prins *et al.* 2000), and decomposing plant biomass for its own use (van Kan 2006). With lesion expansion, the surface of infected plants forms visible gray molds of conidiophores and conidia which are the infection source for the next round of disease (Agrios 2005).

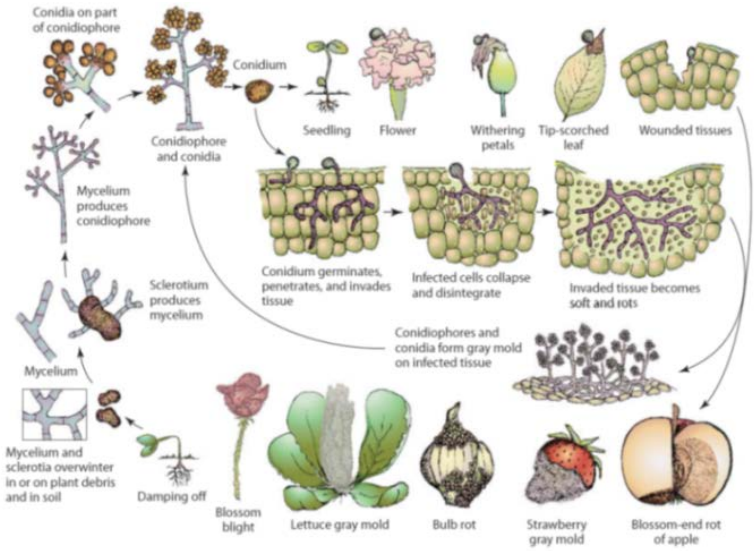

Fig. 1-9. Disease cycle of gray mold disease. Reprinted from Plant Pathology, Agrios (2005), with the permission from the Elsevier.

Genetic basis of resistance to Botrytis cinerea

Botrytis cinerea, and some other necrotrophic pathogens, with a broad range of host plants are considered to possess versatile tools to acquire a successful infection. *B. cinerea* takes advantage of these tools to invade host plants, and then to degrade plant cells as the carbon source for their own growth (van Kan 2006). Although *B. cinerea* is considered to carry such subtle mechanisms, host plants also developed corresponding strategies to cope with the invasion from this necrotrophic pathogen. A total number of 621 Arabidopsis genes were induced upon *B. cinerea* inoculation and the expression of these genes increased at least twofold (AbuQamar *et al.* 2006). A series of plant hormones, ethylene, salicylic acid, jasmonic acid and abscisic acid, are found to influence tomato resistance to *B. cinerea* (Diaz *et al.* 2002) and partial resistance against *B. cinerea* in wild tomato stem and leaf was described (ten Have *et al.* 2007).

The genetic basis for resistance to *B. cinerea* in Arabidopsis and tomato is regarded as polygenic, which needing the involvement of multiple loci to confer a reduction in disease instead of complete resistance (St.Clair 2010). Furthermore, the developmental process and outbreak of *B. cinerea* infection is greatly affected by environment. Compared with the effect of environment, the effect of the allelic difference from any individual gene on phenotypic variation is small. The genetic basis of Arabidopsis, tomato and other crops as well as the response of gerbera to *B. cinerea* all indicate the genetic basis of gerbera resistance to *B. cinerea* would also be quantitative.

Breeding for resistance to Botrytis cinerea

Sax (1923) first presented in common bean the genetic linkage between the polymorphism of seed color pattern, which can be used as a visible marker, with a quantitative trait (seed weight). It provided a possible solution for identifying individual polygenes of quantitative traits and treating these complex traits like Mendelian traits. The location in the chromosomal region of the causal gene(s) is defined as

quantitative trait loci (QTLs) (Collard *et al*. 2005; Mackay *et al*. 2009; St.Clair 2010). Allelic differences in genes underlying the QTL affect the quantitative trait variation. Although the idea of QTL mapping was known since the 1920s, limited morphological and isozyme markers were available which restricted the application of QTL mapping (Tanksley 1993). With the introduction of abundant DNA markers, the pioneering work from Lander and Botstein (1989) and Paterson *et al*. (1988) inspired researchers to use this tool to study the molecular quantitative genetics and resolve complex traits into single genetic components (Paterson 1997).

Mapping quantitative resistance loci (QRLs) is an effective tool to identify the genes that account for QDR (Quantitative Disease Resistance; St.Clair 2010). DNA markers tightly linked to QTLs/QRLs can predict a direct linkage of phenotype and genotype. The process of using DNA markers for tracking and selection of traits of interesting in breeding is called marker-assisted selection (MAS). DNA markers with a clear Mendelian segregation can be applied as the visible indicators in breeding programs for quantitative traits, such as improved crop disease resistance. MAS for single or multiple QRLs have been deployed for QDR in some important crops for disease reduction after years of efforts. A major QRL (*Fhb1)* for fusarium head blight resistance in wheat was identified and using flanking markers, near-isogenic lines (NILs) for validation were developed and the lines with the *Fhb1* allele were found to have a significant reduction of FHB disease (Pumphrey *et al*. 2007). In common bean, recombinant inbred lines (RILs) selected with two major-QTLs resistance alleles for white mold were less diseased than those with susceptible alleles (Ender *et al*. 2008).

In crops with well-annotated genomes, candidate genes underlying QTL regions would be easily located and the quantitative trait nucleotides, the causal molecular variants in or outside of the coding region of the causal gene, could also be identified by targeted-sequencing. Using the specific polymorphisms in the causal gene for MAS is more precise than using the flanking random DNA markers at some distance because of the complete linkage between target gene allele with the marker (Andersen and Lubberstedt 2003). For crops without genome information, candidate genes could also be predicted by prior knowledge from model species. Identification of the causal gene(s) for MAS breeding will produce selection gain and minimize linkage drag (St.Clair 2010).

Outline of this thesis

In this chapter (*Chapter 1*), I introduced the origin of commercial gerbera hybrids; the breeding and propagation methods used in gerbera and reviewed some researches of inflorescence development and floral color development performed in gerbera. As an important floricultural crop, gerbera is easy infected by gray mold during transportation, which causes significant losses. The aim of this research was to unravel the genetics of Botrytis resistance in Gerbera and develop genetic tools for gerbera breeding against this serious disease.

In *Chapter 2*, the leaf and flower bud transcriptomes of the four parents of two populations segregating for botrytis resistance were sequenced using Illumina paired-end sequencing and consensus contig sequences were generated. Reads from the four parents were mapped to the consensus contig sequences to identify genotype and population specific SNPs. After annotation, gerbera transcripts associated with possible

processes of disease resistance, Botrytis resistance in particular, were *in silico* demonstrated. In *Chapter 3*, genotype and population specific SNP markers were selected for KASP genotyping in the two segregating populations. Four parental linkage maps were constructed using the SNP markers and the maps were integrated per population as well as over both populations to derive at a consensus map. Botrytis disease severity was evaluated by *whole inflorescence*, *bottom* (of disc florets) and *ray floret* tests on the parents and progenies of the two populations. QTLs were identified on the parental maps of the two populations using these three different tests. *Chapter 4* describes a candidate gene approach to narrow down QTL regions. By screening recently published literature, several candidate genes related to Botrytis resistance were identified and used to find corresponding homologs in gerbera. These candidate gene homologs were mapped on the previously constructed gerbera linkage maps to check for co-localization with the found QTLs. The allelic diversity and gene expression of promising candidate genes were analyzed. In *Chapter 5*, two candidate genes which were mapped in a QTL region for *ray floret* were characterized using virus induced gene silencing (VIGS) and inoculated with Botrytis spores to evaluate the function of candidate genes. The General discussion in *Chapter 6*, summarizes the results from the above experimental chapters and discusses them in view of literature. Some propositions to the use of molecular markers and genetic tools to increase the resistance of gerbera to botrytis are made.

Acknowledgments

I also would like to express my appreciation to Hans V. Hansen for the original paper of *A story of the cultivated Gerbera* and Isabel Johnson for providing the pictures of *Gerbera jamesonii* and *G. viridifolia*.

Statement

Wageningen UR does not promote the sales or use of the gerbera cultivars which were described in this chapter.

Chapter 2 Transcriptome analysis of *Gerbera hybrida* including *in silico* confirmation of defense genes found

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This chapter has been published in Frontier in Plant Science (2016) 7: 247.

Abstract

For the ornamental crop *Gerbera hybrida*, breeding at the moment is done using conventional methods. As this has drawbacks in breeding speed and efficiency, especially for complex traits like disease resistance, we set out to develop genomic resources. The leaf and flower bud transcriptomes of four parents, used to generate two gerbera populations, were sequenced using Illumina paired-end sequencing. In total, 36,770 contigs with an average length of 1397 bp were generated and these have been the starting point for SNP identification and annotation. The consensus contig sequences were used to map reads of individual parents, to identify genotype specific SNPs, and to assess the presence of common SNPs between genotypes.

Comparison with the non-redundant protein database (nr) showed that 29,146 contigs gave BLAST hits. Of sequences with blast results, 73.3% obtained a clear gene ontology (GO) annotation. EST contigs coding for enzymes were found in Kyoto Encyclopedia of Genes and Genomes maps (KEGG). Through these annotated data and KEGG molecular interaction network, transcripts associated with the phenylpropanoid metabolism, other secondary metabolite biosynthesis pathways, phytohormone biosynthesis and signal transduction were analyzed in more detail. Identifying genes involved in these processes could provide genetic and genomic resources for studying the mechanism of disease resistance in gerbera.

Keywords

EST, candidate gene, annotation, disease pathway, gerbera gray mold

Introduction

Gerbera hybrida (2n = 2x = 50) is one of the most important ornamental plants and belongs to the Compositae family. Cultivated gerbera, which probably originates from a crossing of two wild species from Africa (*G. jamesonii* and *G. viridifolia*; Hansen 1999), is highly heterozygous. Commercial gerbera cultivars are mainly produced in greenhouses for year round cut flower production (Moyer and Peres 2008; Simpson 2009) and ranked fifth in the cut flower sale at the Dutch flower auctions 2014 (https://www.floraholland.com/media/3949227/Kengetallen-2014-Engels.pdf). Gerbera became a model plant to study flower development in composed (*Compositae*) flowers (Teeri *et al.* 2006). Furthermore, the high variation in flower color and patterning of ray and disc florets as well as the high levels of secondary metabolites derived from connected pathways make it a putative model crop for biosynthetic research (Teeri *et al.* 2006). Given the importance of gerbera in floriculture and breeding as well as its potential for fundamental research on flower developmental and regulation of secondary metabolites, there is a demand for genomic resources.

In general, the use of molecular markers in breeding for ornamental crops has been lagging behind other agricultural and horticultural crops (Arens *et al.* 2012; Smulders *et al.* 2012). This is partly due to some breeding traits for ornamentals like flower color that are themselves easily visible markers. Also, it is more complicated to develop molecular markers for ornamental crops since they are highly heterozygous with a complex genetic background (Debener 2009). In gerbera, there are only a small amount of SSR and RGA (resistance gene analog) markers (Gong and Deng 2010, 2012; Seo *et al.* 2012) available for genetic studies in this species.

With the rapid progress in high-throughput next-generation sequencing (NGS) technologies, new possibilities for creating genomic resources and identifying (SNP) markers have become feasible. Transcriptome RNA sequencing (RNA-seq) provides significant advantages for ornamental crops where genomic resources are still scarce and high levels of heterozygosity are expected. Because gerbera has a relatively large genome size, sequencing transcripts as a genome complexity reduction not only reduces cost and time significantly, but also contributes to establishment of resources by the focus on genes. Furthermore, in species with a very high diversity, many SNPs may not be useable markers because of flanking SNPs. Targeting genic regions which have a lower expected SNP diversity may reduce this and result in more widely applicable markers. At present only two studies have contributed to genomic resources building in gerbera. Using Sanger sequencing, an ESTs database with nearly 17,000 cDNA sequences was already constructed for mining genes involved in gerbera floral development (Laitinen *et al.* 2005). A transcriptome of the gerbera ray floret sequenced by NGS sequencing was constructed to predict genes involved in gibberellin metabolism and signal transduction (Kuang *et al.* 2013). Although these transcriptome analyzes have been reported in gerbera, these studies were not focussed on finding SNP markers and focussed strictly on flowers.

SNPs that can be discovered from expressed sequence tags (ESTs) NGS-sequencing are valuable resources for genetic research and accepted as markers for MAS in ornamentals (Koning-Boucoiran *et al.* 2015; Shahin *et al.* 2012). RNA-Seq can generate numerous transcripts with sufficient read-depth to guarantee

high quality SNP identification (Kim *et al.* 2014). Development of SNP markers for the highly heterozygous ornamentals is very feasible and 200 - 1000 SNP markers will be sufficient to construct a genetic map for QTL mapping (Smulders *et al.* 2012).

In this study, we aim for the identification of SNP markers from the transcriptomes of four gerbera genotypes based on leaf and flower tissues using NGS sequencing. Through alignment of reads from four genotypes with consensus contigs constructed by *de novo* assembly, we expect to identify SNPs within and between cultivars and detect reliable SNPs markers that can be used for mapping and other genetic studies. Transcriptomes are analyzed by gene annotation and predicted candidate genes that relate to disease resistance pathways, and to gerbera gray mold in particular, will be shown as examples. Gerbera gray mold is a main problem in gerbera production in greenhouses which is caused by *Botrytis cinerea*. As a necrotrophic pathogen, a series of plant secondary metabolites from the phenylpropanoid and flavonoid biosynthesis pathway are considered to be involved in plant defense responses (Dixon 2001; Dixon *et al.* 2002). Phytohormone jasmonate (JA) and ethylene (ET) also play a role in plant defense against *B. cinerea* (Thomma *et al.* 2001). Identifying the gene sequences involved in these pathways will help us to study their gene function in gerbera upon Botrytis infestation. SNPs found will provide genetic tools for gerbera breeding that may help in efficient gerbera improvement.

Material and Methods

Plant materials, RNA isolation and cDNA library construction

Three Mini Gerbera breeding lines ('SP1', 'SP2', 'FP1') and a garden gerbera breeding line ('FP2') that are also the parents of two gerbera populations were used for cDNA sequencing. The selected 4 parental genotypes show different symptoms on Botrytis susceptibility and the two populations of these parents showed the largest variation on Botrytis susceptibility among 20 populations tested. Young leaves and floral buds of the four parents were collected and stored at -80°C upon RNA isolation.

Total RNA of the leaves and floral buds for the four parents was isolated according to the standard TRIZol reagent protocol (Life Technologies, USA) followed by purification using the RNeasy isolation Kit (Qiagen, Germany). Total RNA of leaves and floral buds was mixed in equal amounts and sent to GATC Biotech (Germany) for sequence library preparation.

Sequencing, assembly and SNP detection

The cDNA libraries of all four genotypes were sequenced using 2 × 100 bp paired-end sequencing on an Illumina HiSeq platform (Illumina, USA). Reads were pre-processed using ConDeTri (Content Dependent Read Trimmer) (Smeds and Künstner 2011) with default settings to trim adapter sequences from the 3' and 5' ends from reads and to filter reads with low quality. To improve the quality of assemblies, FLASH (Fast Length Adjustment of Short reads) (Magoč and Salzberg 2011) was used with default settings to merge overlapping read pairs. For *de novo* assembly, transcripts of four parents were constructed separately by Trinity (Grabherr *et al.* 2011) from the merged, single-end and paired-end reads.

Construction of a reference transcriptome was performed in a stepwise procedure. In short, transcriptome of SP1 was assembled *de novo* and redundancy was removed by reassembling the transcriptome using CAP3 (Huang and Madan 1999) with default setting and an identity (-p) of 95%. Next, the transcripts of SP2 were added to the CAP3 contigs and singlets of SP1 and assembled again with the same settings. In a similar way the transcripts of FP1 and FP2 were added and contigs were reassembled. The final consensus contig sequences were used as a reference transcriptome for SNP detection.

For SNP detection the raw reads were pre-processed using Prinseq-lite (vs. 0.20.3) which included the trimming of nucleotides having a phred score lower than 25, the trimming of poly A/T tails, the removal of duplicate reads, of low complexity reads (DUST approach), of reads shorter than 50 bp and of reads with more than one ambiguous nucleotide. The remaining reads of each genotype where aligned to the reference transcriptome using Bowtie2 (--very-sensitive setting) and filtered for mapping quality (>2) using SAMtools (Li *et al.* 2009). The resulting .sam files were merged and used for SNP calling using QualitySNPng (Nijveen *et al.* 2013) with default settings. Retrieved SNP regions were blasted (BLASTn, e-value: 1E-30) to the contigs derived from the EST sequences as a control for possible paralog presence.

GO annotation, enzyme code annotation and KEGG annotation

To predict function, assembled unigene contigs were annotated. Gene ontology (GO) annotation in Blast2GO (Conesa *et al.* 2005) consisted of three steps: blasting, mapping and annotation. The assembled contigs were compared by BLASTX against the NCBI non-redundant protein database (nr) using Blast2GO V.3.0. The expectation value (E-value) threshold was set at 1E-3 for reporting matches and the number of retrieved hits at 20 (default value). After blasting, Gene Ontology (GO) terms associated to the hits were mapped. When a BLAST result is successfully mapped to one or several GO terms, GO annotations were assigned.

Enzyme code (EC) annotation was available only for contig sequences with GO annotations with EC numbers. Additionally, the KEGG mapping was done to display enzymatic functions in the context of the metabolic pathways in which they participate.

Results

Sequencing and SNP detection

The transcriptome reads of four genotypes were obtained using Illumina 2 × 100 bp paired-end sequencing. For genotype 'SP1', sequencing the cDNA library resulted in a total of 114,519,206 raw paired end reads. After trimming and removing reads with low quality 80,182,250 (70%) paired end reads remained. Merging connected paired-end reads using FLASH software resulted in 46,043,245 single reads and 5,931,379 paired end reads for *de novo* assembly. The *de novo* assembly for 'SP1' resulted into 113,970 transcripts longer than 200 bp. Results of sequencing assembly data for all four genotypes are shown in Table 2-1. All raw data has been donated to the SRA (Short Read Archive) and can be found under accession numbers PRJEB12127.

Table 2-1 Summary of the sequence assembly data

Parents	# bases	# paired end reads	Pre-processing			*De novo* assembly	CAP3 Assembly	
			# paired end reads after trimming	# paired end reads after merging	# single end reads after merging	# transcripts (>200 bp)	# contigs	# singlets
SP1	11,566,439,806	114,519,206	80,182,250	5,931,379	46,043,245	113,970	36,770	144,356
SP2	11,885,597,584	117,679,184	82,276,886	6,964,989	46,331,282	119,675		
FP1	14,435,581,146	142,926,546	99,901,704	9,400,798	55,225,869	130,234		
FP2	9,416,840,444	93,236,044	73,156,762	6,104,039	38,065,122	73,634		

Transcripts of parent 'SP1' were first used to construct a reference transcriptome after which transcripts of the other genotypes were one by one added to reach an overall consensus assembly (Fig. 2-1). The final consensus transcriptome yielded 36,770 consensus contigs and 144,356 singletons. The average length of consensus contigs was 1397 bp, and the N50 was equal to 1889 bp (The minimum length of 201 bp, median length of 1130 bp and maximum length of 15746 bp). This consensus transcriptome (named 'Cap3Contigs_All') was the starting point for SNP identification and annotation. All paired-end and single-end reads of the four genotypes were mapped to the 36,770 'Cap3Contigs_All' consensus contigs for SNP detection. In total 398,917 SNPs polymorphic within or between the four genotypes were detected (Table 2-2). Genetic diversity of the consensus sequences on average is 7.8 SNPs per kb. The average SNP density within the four genotypes varied from 3.7 - 4.8 SNPs per kb of sequence. They all harbour quite a lot parent specific SNPs and population specific SNPs polymorphic in only that specific parent or population (Table 2-2).

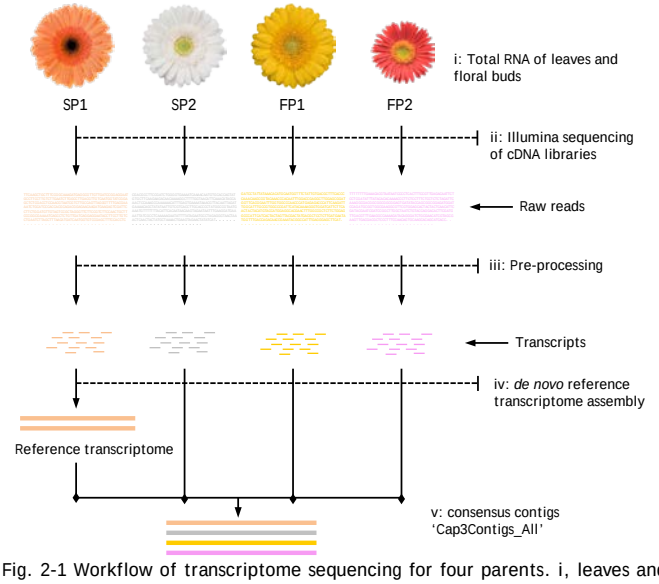

Fig. 2-1 Workflow of transcriptome sequencing for four parents. i, leaves and floral buds of the four gerbera genotypes used to isolate RNA; ii, mixed cDNA libraries of four genotypes sequenced on an Illumina platform; iii, raw reads used for pro-processing to trim adapter sequences and to filter reads with low quality; iv, transcripts of SP1 assembled as first step towards reference transcriptome construction; v, transcripts of other genotypes mapped to reference to yield consensus contigs named 'Cap3Contigs_All' for SNP detection and annotation.

Table 2-2 SNPs on parents and consensus sequences

Population	Parents	#SNPs present in parent[1]	SNPs per kb[2]	#SNP genotype specific[3]	#SNP population specific[4]	Total polymorphic SNPs[5]	Average polymorphic position per kb[6]
S	SP1	218,049	4.2	16,490	19,762	398,917	7.8
	SP2	220,590	4.3	20,852			
F	FP1	245,063	4.8	27,780	17,189		
	FP2	190,047	3.7	23,741			

[1] Total number of SNPs present in each parental genotype; [2] SNPs per kb of sequenced data in four parental genotype; [3] Number of unique SNPs in each parental genotype; [4] Number of unique SNPs in each population; [5] Total number of SNPs within and between the four parental genotypes in the 36,770 consensus contigs; [6] Average polymorphic site per kb of the four parental genotypes combined.

GO, EC, KEGG annotation

The 36,770 consensus contigs 'Cap3Contigs_All' were used for blasting against the NCBI non-redundant protein database (nr) to look for the most similar proteins for each contig (Table 2-3). A total number of 29,198 contigs gave BLAST hits (79.4%). Out of the best-hit for every contig with BLAST result (See Suppl. Table S2-1), the E-value of 9398 contigs (~32%) is below an value of $1e^{-180}$, and 2481 contigs (8.5%) with the E-value greater than $1e^{-20}$, others are in between. Sequences similarity distribution chart (see Suppl. Fig. S2-1A) shows that most (91.2%) of the BLAST hits have sequence similarity values higher than 60% and half of them (50.4%) higher than 80% with our gerbera consensus contigs. Most blast hits were found from grape (*Vitis vinifera*), soybean (*Glycine max*), poplar (*Populus trichocarpa*), potato (*Solanum tuberosum*), tomato (*S. lycopersicum*) and cacao tree (*Theobroma cacao*) (Suppl. Fig. S2-1B). Most of these species also feature in the top hits distribution with grape as main contributor (Suppl. Fig. S2-1C).

Table 2-3 Transcriptomes annotation process results

Annotation step	contig no.
contigs blasted without blast hit	7,572
contigs with Blast hit, without GO mapping	3,025
contigs with GO Mapping, without GO annotation	4,787
contigs B2G Annotated	21,386
total contigs	36,770

The GO terms were obtained during the mapping step. Out of sequences with blast results, 73.3% (21,357 contigs) could be GO annotated. The sequences with GO annotation were described in terms of biological processes, cellular components and molecular functions. Top 20 GO terms of the three separate aspects were listed in Suppl. Table S2-2.

Enzyme annotations were also done in contigs with GO annotations. A total of 8,761 contigs eventually showed EC numbers and the enzyme code distribution is shown in Table 2-4. KEGG mapping displayed enzymatic functions in the context of the metabolic pathways in which they participate. The EC annotated contigs are involved in a total of 144 different metabolic pathways, including all kinds of carbohydrate metabolic pathways, amino acid metabolic pathways, nitrogen metabolic pathways, as well as a series of

secondary metabolic biosynthesis pathways. The top 30 pathways in overall sequence coverage and the details of all 144 pathways with contig identity and enzyme code can be found in Suppl. Table S2-3 and Suppl. Table S2-4, respectively.

Table 2-4 Gerbera transcripts enzyme code distribution

EC Classes	#Contigs
1.- Oxidoreductases	1,769
2.- Transferases	3,404
3.- Hydrolases	2,403
4.- Lyases	421
5.- Isomerases	281
6.- Ligases	483

Transcripts related to phenylpropanoid biosynthesis and flavonoid biosynthesis pathway

Based on the EC annotated sequences, enzymes involved in phenylpropanoid and flavonoid biosynthesis pathway that are considered to be involved in flower color and disease resistance were retrieved and highlighted in different colors in the pathway-maps from KEGG (see Suppl. Fig. S2-2 and Suppl. Fig. S2-3). There are 137 contigs that translate to 14 enzymes in the phenylpropanoid biosynthesis pathway. Eleven enzymes represented by 71 contigs were found for the flavonoid biosynthesis pathway. These two pathway-maps loaded from KEGG (see Suppl. Fig. S2-2 and Suppl. Fig. S2-3) included all possible enzymes and metabolites in a broad perspective, but we can see clearly from the simplified phenylpropanoid and flavonoid biosynthetic pathway (Fig. 2-2) that the key enzymes in the pathways are well represented. The three key regulatory enzymes in the phenylpropanoid biosynthesis are phenylalanine ammonia lyase (PAL, EC:4.3.1.24), cinnamate-4-hydroxylase (C4H, EC:1.14.13.11), 4-coumarate-CoA ligase (4CL, EC:6.2.1.12), which were represented by 11, 2 and 12 homologous contigs respectively. The sequence similarities of the best-hit for these 25 contigs are above 90% and 17 of the best-hits come from other species within the Compositae like, *Helianthus tuberosus*, *Lactuca sativa*, *Artemisia sieberi*, *Cynara cardunculus*, *etc* indication that these pathways are well conserved within the family. After the formation of p-coumaroyl-CoA, the next step is into the central flavonoid pathway. Chalcone synthase (CHS, EC:2.3.1.74) and chalcone isomerase (chalcone-flavanone isomerase, CHI, EC:5.5.1.6.) are the first two enzymes in the flavonoid pathway leading to subsequent metabolite synthesis. Eight out of the 10 contigs which are predicted as *CHS* gene are highly identical to the gerbera *CHS* genes in public databases (Z38096.1, Z38097.2, Z38098.1, AM906210.1, AM906211.1, X91339.1) with a very low (or zero) E-value and similarity close to 100%. No contig were found specific for stilbene synthase (STS, EC:2.3.1.95) which is the key enzyme for stilbene synthesis. Enzymes in this pathway and the number of contigs homologous to these enzymes are show in Fig. 2-2.

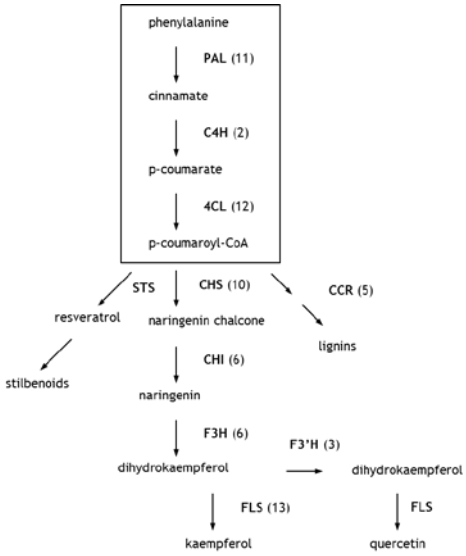

Fig. 2-2 Distribution of Gerbera transcripts in the simplified phenylpropanoid and flavonoid biosynthesis pathway (Ainasoja 2008; Ali *et al*. 2011; Boubakri *et al*. 2013; Dixon *et al*. 2002; Ferreyra *et al*. 2012). The processes in the box indicate the general phenylpropanoid pathway and the rest is flavonoid pathway. Each enzyme name is followed with the number of contigs homologous to the gene family encoding this enzyme between brackets. PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; STS, stilbenes synthase; CCR, cinnamoyl-CoA reductase; CHI, chalcone isomerase/chalcone-flavanone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase.

Transcripts related to phytohormone biosynthesis and signalling

The initial precursor for ethylene synthesis is the amino acid methionine. The three key regulatory enzymes in the pathway are S-adenosyl-l-methionine synthase (SAMS, EC:2.5.1.6), ACC synthase (ACS, EC:4.4.1.14) and ACC oxidase (AOC, EC:1.14.17.4). Our gerbera EST database contains multiple contigs encoding these three enzymes (see Fig. 2-3). Jasmonate biosynthesis start from linolenic acid. Lipoxygenase (LOX, EC:1.13.11.12), allene oxide synthase (AOS, EC:4.2.1.92), allene oxide cyclase (AOC, EC:5.3.99.6) and 12-oxo-phytodienoic acid reductase (OPDR, EC:1.3.1.42) participant in the synthesis. Numbers of contigs encoding these enzymes are show in Fig. 2-4. We also found the multiple contigs connected with these two plant hormone signalling pathway which were shown in Fig. 2-5, although some of them still remained without coverage.

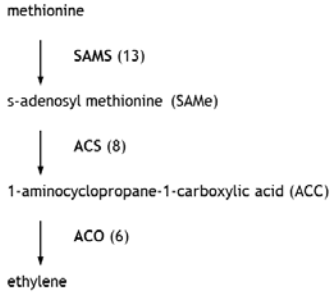

Fig. 2-3 Distribution of Gerbera transcripts in the ethylene biosynthetic pathway (Wang *et al*. 2002). Each enzyme name is followed with the number of contigs homologous to the gene family encoding this enzyme between brackets. SAMS, S-adenosyl methionine synthase; ACS, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase; ACO, ACC oxidase.

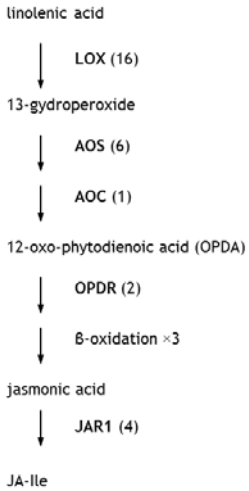

Fig. 2-4 Distribution of Gerbera transcripts in the Jasmonic acid biosynthetic pathway (Howe 2001; Wasternack 2007). Each enzyme name is followed with the number of contigs homologous to the gene family encoding this enzyme between brackets. LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPDR, 12-oxo-phytodienoic acid reductase; JAR1, JA amino acid conjugate synthase; JA-Ile, jasmonate-isoleucine.

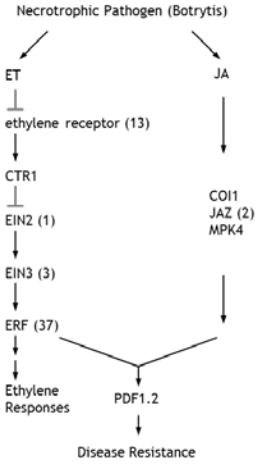

Fig. 2-5 Simplified signaling ethylene and jasmonic acid signal transduction during Botrytis infection (Wang *et al.* 2002; Katsir *et al.* 2008a). Each enzyme name is followed with the number of contigs homologous to the gene family encoding this enzyme between brackets. ET, ethylene; CTR1, constitutive triple response 1; EIN2, ethylene insensitive 2; EIN3, Ethylene insensitive 3; ERF; ethylene response factor; JA, jasmonate; JA-Ile, jasmonate-isoleucine; SCF, Skp/Cullin/F-box; COI1, coronatine-insensitive 1; JAZ, jasmonate ZIM domain; bHLH TFs, basic-helix-loop-helix transcription factors; PDF1.2, plant defensin 1.2.

Discussion

In this study, we developed transcriptomes (based on leaf and flower tissues) of gerbera using RNA sequencing in four gerbera genotypes. After *de novo* assembly, we generated 36,770 consensus contigs with an average length of 1397 bp and a N50 length of 1889 bp. Average length and N50 length are markedly larger than a transcriptome of flower development (845 bp and 1321 bp respectively; Kuang *et al.* 2013). This is likely because four gerbera genotypes were used instead of one which increases chances for higher read coverage and wider sequence overlap.

SNPs present within genotypes and between genotypes were detected from the alignment of reads of all parents with the consensus contigs. In all those consensus contigs with a total length of 51,360,054 bp, 398,917 polymorphic loci (SNPs present either in parent or between parents) were identified with an average SNP density of 7.8 SNPs per 1kb whereas within genotype SNP density ranged from 3.7 - 4.8 SNPs per 1 kb. These numbers are comparable to for instance rose 4 - 6 SNP/kb (Koning-Boucoiran *et al.* 2015)

and sunflower 6.1 SNP/kb (Bachlava *et al.* 2012). In eleven safflower (*Carthamus tinctorius*) individuals exons and introns sequences of 7 genes showed a SNP density of 10.5 SNP/kb (Chapman and Burke 2007) whereas SNP densities varied from 2.6 to 26.9 SNP/kb in ten intron regions from eight safflower accessions (Chapman *et al.* 2007). The frequency of polymorphisms in safflower seems higher than that in overall consensus gene sequences of gerbera but this could be biased because of the small set of genes studied in safflower. Furthermore, polymorphism rates in intron are higher than in exons since introns are under less strict selection pressure (Tamura *et al.* 2013).

The highest numbers of homologs were found with *Vitis vinifera* (grapevine). Interestingly, grapevine is a crop that also is known for its high number of secondary metabolites and its interaction with *B. cinerea*. Many studies have been performed on this crop-pathogen from multiple aspects (Bézier *et al.* 2002; Coutos-Thevenot *et al.* 2001; Deytieux-Belleau *et al.* 2009; Goetz *et al.* 1999; Hain *et al.* 1993; Jeandet *et al.* 1991; Poinssot *et al.* 2003; Timperio *et al.* 2012; Trotel-Aziz *et al.* 2006) that could be instructive for the interaction of the pathogen with gerbera as well.

There is general recognition that various natural secondary metabolites play an important role in plant defense (Dixon 2001; Dixon *et al.* 2002; Howlett 2006; van Baarlen *et al.* 2007). Plants combating the necrotrophic pathogen *Botrytis* especially utilize this tool (Glazebrook 2005; Oliver and Ipcho 2004). The precursors for these compounds, which are involved in physical and chemical barriers, such as lignin and phytoalexins, are derived from the phenylpropanoid pathway. Enzymes involved in the biosynthesis of the general phenylpropanoid pathway have been well studied (see Dixon *et al.* 2002). For instance, enzyme activities increased lignification in wheat upon *B. cinerea* infection (Maule and Ride 1976, 1983). In our study, we identified homologs for the three key genes (PAL, C4H and 4CL) in the core of the phenylpropanoid pathway in gerbera, and found multiple transcripts encoding the three enzymes. For 17 out of the 25 best-hits homologs come from *Compositae* species.

The flavonoid pathway is closely linked with the phenylpropanoid pathway and the precursor is a phenylpropanoid-derived compound. Flavonoid biosynthesis will yield in different flower color pigments (Winkel-Shirley 2001) but may also produce a range of plant defense compounds (Treutter 2005). For instance, chalcone synthase (CHS) belongs to the type III polyketide synthase (PKS) superfamily (Abe and Morita 2010; Austin and Noel 2003) and is the key enzyme towards the flavonoid biosynthesis. Members in the type III PKS superfamily, including stilbene synthases (STS) and 2-pyrone synthase (2-PS) in gerbera, share high amino acid similarities and are highly correlated with Botrytis resistance. Grapevine synthesizes stilbenes upon Botrytis infection (Goetz *et al.* 1999; Jeandet *et al.* 1991). Tobacco transformed with a stilbene synthase gene from grapevine showed increased resistance to *B. cinerea* (Hain *et al.* 1993). The 2-pyrone synthase (2-PS) codes for polyketide synthase which synthesizes a putative precursor for two phytoalexins in gerbera. Knocking out this gene resulted in increased susceptibility to *B. cinerea* (Koskela *et al.* 2011). Deng *et al.* (2014) confirmed that CHS enzymes in gerbera are encoded by a family of three genes. We found at least 10 transcripts annotated to the chalcone and stilbene synthase family protein. Eight of them showed high similarity at nucleotide level with gerbera *CHS* or *CHS*-like genes in public databases, whereas the other two showed only low amino acid similarity (40%) to known gerbera sequences. The latter two give the best hits to chalcone and stilbene synthases from *T. cacao*

(XP_007041944.1, 90%) and a putative chalcone synthase from *Artemisia annua* (ACY74337.1, 93%). Based on the phylogenetic tree of the amino acid sequences of chalcone- and stilbene-like synthases (Suppl. Fig. S2-4 and Suppl. Table S2-5), these two transcripts (GhCHS1 and GhCHS2 in Suppl. Fig. S2-4) belong to a clearly separate group of putative chalcone and stilbene synthases.

In this study, we also exampled the possibilities of the presented transcriptome on plant hormone ethylene and jasmonate biosynthesis and signaling networks that are considered to play an important role in plant resistance in general and Botrytis in specific. For instance, ethylene production plays an important role in plant resistance against *B. cinerea* (Broekaert *et al.* 2006). The rate-limiting step of ethylene synthesis is the conversion of *S*AMe to ACC by ACC synthase (ACS; Kende 1993). A multigene family codes for ACC synthase in plants. Nine contigs were found in our data related with ACS genes. Similarly, the Arabidopsis and tomato genomes contain nine ACS genes (Harpaz-Saad *et al.* 2012). Two knockout mutants of type I ACS isoforms in Arabidopsis, *acs2* and *acs6*, reduced *B. cinerea*-induced ethylene biosynthesis (Han *et al.* 2010). ACS-silenced apple fruit was more susceptible to *B. cinerea* than untransformed apple (Akagi *et al.* 2011). The activity of lipoxygenase (LOX), a key JA biosynthetic enzyme, is also highly related to Botrytis resistance (Azami-Sardooei *et al.* 2010).

Our gerbera EST database contained multiple transcripts encoding key enzymes in ethylene and jasmonate synthesis pathways. An efficient defense response to Botrytis also need genes in signaling transduction pathways, such as EIN2 in ethylene signaling (Thomma *et al.* 1999), COI1 and JAZ in jasmonate signaling (Cerrudo *et al.* 2012). COI1 protein was shown to mediate JAZ degradation to release its bound downstream TFs (e.g. MYC2) for defense gene expression (Katsir *et al.* 2008b, Kazan and Manners 2013). Jasmonoyl–isoleucine (JA-Ile), which is the only bioactive jasmonates derivative by a JA conjugate synthase (JAR1) confirmed so far, directly promotes their interaction (Katsir *et al.* 2008a; Wasternack 2007). In Arabidopsis, the *coi1* and other mutations that block functional JA signaling, showed increased susceptibility to Botrytis and decreased induction of the plant antimicrobial metabolite camalexin after infection (Rowe *et al.* 2010). Some of these components in the signal transduction pathway still remained without coverage which may be related to the RNA-seq source that is from unchallenged material as the main focus was building generic genomic resources and SNP detection.

Through analysis of the large gerbera EST database that was generated from next-generation sequencing, we identified a series of SNP markers for further linkage mapping and also identified transcripts that might be involved in interesting pathways for both fundamental as well as applied studies as was exampled for Botrytis resistance. We expect these genes can provide genetic resources for studying the mechanism of disease resistance and developing markers for gerbera breeding in the future.

Acknowledgments

We are thankful for the support from the Foundation Technological Top Institute Green Genetics (3CFL030RP) and from gerbera breeding companies Florist Holland BV and Schreurs Holland BV.

Supplementary Tables and Figures

Table S2-1 The best-hit of contigs with BLAST result (part)

Sequence name	Sequence description	Sequence length	Hit description	Hit ACC	E-Value	Similarity	Bit-Score	Alignment length	Positives
Contig1	polyubiquitin	1138	gi|2760347|gb|AAB95251.1|ubiquitin [Arabidopsis thaliana]	AAB95251	0	99	745.732	379	378
Contig2	beta subunit isoform 1	2256	gi|297743300|emb|CBI36167.3|unnamed protein product [Vitis vinifera]	CBI36167	0	91	976.467	582	530
Contig3	beta-galactosidase 3-like	1358	gi|567865941|ref|XP_006425593.1|hypothetical protein CICLE_v10024881mg [Citrus clem	XP_006425	0	88	768.074	452	402
Contig4	nuclear transport factor 2-like	825	gi|225425388|ref|XP_002276841.1|PREDICTED: nuclear transport factor 2 isoform 1 [Vitis	XP_002276	9.15E-78	96	243.047	123	119
Contig5	leucine aminopeptidase chloroplastic-like	2378	gi|225445867|ref|XP_002276114.1|PREDICTED: leucine aminopeptidase 2, chloroplastic isc	XP_002276	0	88	889.797	559	492
Contig6	werner syndrome-like exonuclease-like	652	gi|255573515|ref|XP_002527682.1|3-5 exonuclease, putative [Ricinus communis]>gi|223	XP_002527	1.46E-71	76	228.024	182	139
Contig7	dna mismatch repair protein	2791	gi|565361589|ref|XP_006347538.1|PREDICTED: uncharacterized protein LOC102596319 [	XP_006347	0	77	704.901	559	433
Contig8	diaminopimelate chloroplastic-like	2426	gi|225439868|ref|XP_002278566.1|PREDICTED: diaminopimelate epimerase, chloroplastic [	XP_002278	4.75E-176	90	523.087	318	289
Contig9	acid phosphatase 1-like	1064	gi|418730703|gb|AFX66999.1|putative acid phosphatase [Solanum tuberosum]	AFX66999	4.95E-106	71	324.324	267	191
Contig10	betaine aldehyde dehydrogenase	2517	gi|256260278|gb|ACU65243.1|betaine aldehyde dehydrogenase [Helianthus annuus]	ACU65243	0	96	899.812	501	484
Contig11	40s ribosomal protein s4-like	1076	gi|568870787|ref|XP_006488578.1|PREDICTED: 40S ribosomal protein S4-like [Citrus sinen	XP_006488	1.78E-164	98	472.626	256	253
Contig12	omega-6 fatty acid desaturase	1505	gi|339779634|gb|AEK06374.1|microsomal oleate desaturase FAD2-5 [Carthamus tinctorius	AEK06374	0	89	660.218	376	338
Contig13	oligopeptidase a	2807	gi|460372323|ref|XP_004231980.1|PREDICTED: oligopeptidase A-like [Solanum lycopersicu	XP_004231	0	88	1222.61	782	689
Contig15	dihydrodipicolinate reductase chloroplast	1920	gi|225434245|ref|XP_002280402.1|PREDICTED: dihydrodipicolinate reductase 1, chloropla	XP_002280	6.74E-165	82	488.034	350	287
Contig16	enoyl-acp reductase 1 family protein	1690	gi|317737789|gb|ADV16377.1|enoyl-ACP reductase 2 [Helianthus annuus]	ADV16377	0	93	669.078	392	365
Contig17	epoxide hydrolase	1483	gi|568214599|ref|NP_001274907.1|epoxide hydrolase [Solanum tuberosum] >gi|407942|gb	NP_001274	3.72E-161	83	471.855	322	268
Contig18	coatomer subunit gamma-like	3171	gi|449465393|ref|XP_004150412.1|PREDICTED: coatomer subunit gamma-like [Cucumis sa	XP_004150	0	96	1640.94	886	855
Contig19	murus 4 family protein	1919	gi|255578061|ref|XP_002529901.1|UDP-glucose 4-epimerase, putative [Ricinus communis]	XP_002529	0	93	756.903	411	385
Contig22	udp-glycosyltransferase 76f1-like	931	gi|225449296|ref|XP_002281324.1|PREDICTED: UDP-glycosyltransferase 76C4 [Vitis vinife	XP_002281	3.25E-102	70	318.931	312	220
Contig23	udp-glycosyltransferase superfamily	1500	gi|224112637|ref|XP_002316247.1|UDP-glucuronosyl/UDP-glucosyltransferase family prot	XP_002316	0	75	548.895	447	336
Contig24	integral membrane family protein	788	gi|462397312|gb|EMJ03111.1|hypothetical protein PRUPE_ppa013304mg [Prunus persica]	EMJ03111	1.13E-62	83	204.527	130	109
Contig25	early nodulin-like protein 2-like	807	gi|502082781|ref|XP_004487271.1|PREDICTED: early nodulin-like protein 2-like [Cicer arie	XP_004487	8.65E-41	79	149.828	105	83
Contig26	cryptochrome 1	3500	gi|537845445|gb|AGU91427.1|cryptochrome 1.2 [Chrysanthemum boreale]	AGU91427	0	94	788.489	466	439
Contig27	glutamate-1-semialdehyde- -aminomutase	1983	gi|449438260|ref|XP_004136907.1|PREDICTED: glutamate-1-semialdehyde 2,1-aminomutas	XP_004136	0	93	764.992	440	413
Contig28	serine arginine-rich splicing factor rs22a	1514	gi|596014212|ref|XP_007218729.1|hypothetical protein PRUPE_ppa009074mg [Prunus per	XP_007218	8.19E-51	85	186.422	119	102
Contig29	global transcription factor group isoform	3756	gi|568215711|ref|NP_001275266.1|bromodomain-containing RNA-binding protein 1 [Solanu	NP_001275	2.73E-150	67	478.019	513	345
Contig30	myosin-1-like isoform x2	755	gi|567894110|ref|XP_006439543.1|hypothetical protein CICLE_v10022634mg [Citrus clem	XP_006439	2.04E-42	89	152.91	110	98
Contig31	phd finger family protein	1222	gi|566214377|ref|XP_006371893.1|hypothetical protein POPTR_0018s05470g [Populus trit	XP_006371	4.96E-120	93	360.918	254	238
Contig32	lon protease homolog peroxisomal-like	1000	gi|587940117|gb|EXC26738.1|Lon protease-2-like protein [Morus notabilis]	EXC26738	1.23E-162	93	488.034	284	266
Contig33	leucine-rich repeat protein	1148	gi|308943845|gb|ADO51751.1|leucine rich repeat protein [Camellia sinensis]	ADO51751	1.77E-119	90	358.607	210	189
Contig34	abc transporter g family member 9-like	1113	gi|508720959|gb|EOY12856.1|White-brown-complex ABC transporter family [Theobroma c	EOY12856	2.17E-64	80	226.868	192	154
Contig35	abc transporter g family member 9-like	760	gi|147802534|emb|CAN62144.1|hypothetical protein VITISV_007841 [Vitis vinifera]	CAN62144	1.41E-133	86	401.749	251	217
Contig36	ferredoxin-dependent glutamate synthas	5451	gi|225441236|ref|XP_002267056.1|PREDICTED: ferredoxin-dependent glutamate synthase	XP_002267	0	92	2921.72	1608	1490
Contig37	cyclin-dependent protein	1405	gi|470143150|ref|XP_004307245.1|PREDICTED: cyclin-U4-1-like [Fragaria vesca subsp. ver	XP_004307	1.21E-93	91	293.893	163	149
Contig38	60s ribosomal protein l14-2-like	1529	gi|460393540|ref|XP_004242367.1|PREDICTED: 60S ribosomal protein L14-1-like isoform 1	XP_004242	1.79E-75	96	245.358	133	129
Contig39	beta-glucosidase 18-like	1959	gi|567919862|ref|XP_006451937.1|hypothetical protein CICLE_v10007996mg [Citrus clem	XP_006451	0	84	718.768	479	405
Contig40	calcium-dependent protein kinase 13-like	2284	gi|565404157|ref|XP_006367515.1|PREDICTED: calcium-dependent protein kinase 13-like [	XP_006367	0	95	989.949	531	508
Contig41	ascorbate peroxidase	1301	gi|9065881.4|gb|ABD97259.1|ascorbate peroxidase [Camellia sinensis]	ABD97259	9.91E-130	91	386.726	250	228
Contig42	heterogeneous nuclear ribonucleoprotein	1728	gi|359480808|ref|XP_002282572.2|PREDICTED: uncharacterized RNA-binding protein C66(	XP_002282	0	89	549.28	348	311
Contig43	copper ion binding isoform 1	1456	gi|460368229|ref|XP_004229970.1|PREDICTED: uncharacterized protein LOC101248615 [	XP_004229	7.90E-100	79	311.612	252	200
Contig44	abc transporter g family member 1-like	2708	gi|449464204|ref|XP_004149819.1|PREDICTED: ABC transporter G family member 16-like [	XP_004149	0	89	1038.48	722	646
Contig45	topless-related protein 3	3964	gi|565355675|ref|XP_006344708.1|PREDICTED: topless-related protein 3-like isoform X2 [	XP_006344	0	89	1824.68	1108	995
Contig46	udp-galactose transporter 2-like	1869	gi|502118909|ref|XP_004496438.1|PREDICTED: UDP-galactose transporter 2-like [Cicer ar	XP_004496	2.02E-164	91	484.952	310	283
Contig47	uncharacterized membrane protein at1g1	1996	gi|460381890|ref|XP_004236674.1|PREDICTED: uncharacterized membrane protein At1g1	XP_004236	0	83	568.155	437	363
Contig48	chaperonin cpn60- mitochondrial-like	2072	gi|568878328|ref|XP_006492150.1|PREDICTED: chaperonin CPN60-2, mitochondrial-like [C	XP_006492	0	97	1028.85	564	549
Contig49	kinase family protein	2228	gi|359484594|ref|XP_002281124.2|PREDICTED: uncharacterized protein LOC100254414 [	XP_002281	0	83	949.118	623	523
Contig50	transport inhibitor response 1	2843	gi|595800765|ref|XP_007201723.1|hypothetical protein PRUPE_ppa003344mg [Prunus per	XP_007201	0	89	555.058	327	294

Table S2-1 (continued)

Sequence name	Sequence description	Sequence length	Hit description	Hit ACC	E-Value	Similarity	Bit-Score	Alignment length	Positives
Contig51	udp-d-glucose dehydrogenase	1861	gi|460375158|ref|XP_004233374.1|PREDICTED: UDP-glucose 6-dehydrogenase-like isoform	XP_0042333	0	90	795.423	482	434
Contig52	probable glutathione peroxidase 8-like	961	gi|3913794|sp|O23970.1|GPX1_HELANRecName: Full=Glutathione peroxidase 1 >gi|232645 O23970	7.60E-97	95	295.049	165	157
Contig53	gdp-mannose-dependent alpha-mannosyltr	2067	gi|356496170|ref|XP_003516943.1|PREDICTED: uncharacterized protein LOC100780899 | XP_0035169	0	83	754.977	508	422
Contig54	probable trr receptor-like serine threonin	2642	gi|566180602|ref|XP_006380687.1|hypothetical protein POPTR_0007s10500g [Populus trix XP_0063806	0	73	835.099	748	547
Contig55	serine threonine protein phosphatase 2a	1926	gi|462411273|gb|EMJ16322.1|hypothetical protein PRUPE_ppa004338mg [Prunus persica] EMJ16322	0	94	721.465	455	429
Contig56	serine threonine protein phosphatase 2a	1420	gi|571514969|ref|XP_006597185.1|PREDICTED: serine/threonine protein phosphatase 2A XP_0065971	0	93	717.613	424	396
Contig57	probable phosphatidylinositol 4-kinase tyr	1187	gi|460386663|ref|XP_004239016.1|PREDICTED: probable phosphatidylinositol 4-kinase typ XP_0042390	3.78E-52	89	194.512	121	108
Contig58	serine threonine-protein kinase afc2-like	2579	gi|255540473|ref|XP_002511301.1|afc, putative [Ricinus communis] >gi|223550416|gb|EI XP_0025113	0	96	629.402	320	310
Contig59	system protein c	537	gi|565385211|ref|XP_006358507.1|PREDICTED: uncharacterized protein LOC102600962| XP_0063585	2.57E-19	74	88.5817	87	65
Contig60	60s ribosomal protein l27-like	818	gi|225462170|ref|XP_002267478.1|PREDICTED: 60S ribosomal protein L27 [Vitis vinifera] XP_0022674	2.81E-73	96	231.876	135	130
Contig61	wd-40 repeat-containing protein msi4-like	1792	gi|357449789|ref|XP_003595171.1|Nucleosome/chromatin assembly factor group [Medica XP_0035951	0	87	707.212	441	388
Contig62	myb family transcription factor family pro	3107	gi|462416773|gb|EMJ21510.1|hypothetical protein PRUPE_ppa000126mg [Prunus persica] EMJ21510	0	67	609.757	704	477
Contig63	probable trr receptor-like serine threonin	974	gi|567886376|ref|XP_006435710.1|hypothetical protein CICLE_v10033293mg [Citrus clem XP_0064357	6.36E-68	70	239.195	234	164
Contig64	cyclobartenol synthase	2657	gi|484847896|gb|AGK62447.1|cycloartenol synthase [Panax quinquefolius] AGK62447	0	92	1375.53	757	700
Contig65	proliferation-associated protein 2g4-like	2483	gi|462400945|gb|EMJ06502.1|hypothetical protein PRUPE_ppa006778mg [Prunus persica] EMJ06502	0	95	656.366	390	371
Contig66	uncharacterized loc101202927	4034	gi|225450585|ref|XP_002282109.1|PREDICTED: uncharacterized protein LOC100264846| XP_0022821	0	75	951.044	846	636
Contig67	glutathione s-transferase t1-like	1146	gi|255537437|ref|XP_002509785.1|glutathione-s-transferase theta, gst, putative [Ricinus XP_0025097	1.39E-110	76	335.88	250	192
Contig68	serine threonine-protein kinase at5g0102	2196	gi|565393317|ref|XP_006362327.1|PREDICTED: serine/threonine-protein kinase At5g0102 XP_0063623	0	93	696.041	379	356
Contig69	aconitate hydratase mitochondrial-like	1036	gi|565402239|ref|XP_006366590.1|PREDICTED: aconitate hydratase 2, mitochondrial-like XP_0063665	3.70E-133	94	415.616	218	206
Contig70	duf246 domain-containing protein at1g04	2937	gi|587900812|gb|EXB89105.1|hypothetical protein L484_016665 [Morus notabilis] EXB89105	0	93	869.381	486	454
Contig71	epoxide hydrolase	1290	gi|568214641|ref|NP_001275417.1|epoxide hydrolase [Solanum tuberosum] >gi|407944|gb NP_0012754	1.46E-76	86	252.677	161	140
Contig72	PREDICTED: uncharacterized protein LOC	1897	gi|359480951|ref|XP_002264842.2|PREDICTED: uncharacterized protein LOC100243295|x XP_0022648	0	87	702.975	436	380
Contig73	sumo-activating enzyme subunit 1a-like	1391	gi|225456343|ref|XP_002283880.1|PREDICTED: SUMO-activating enzyme subunit 1B [Vitis XP_0022838	0	87	532.332	323	284
Contig74	cinnamoyl- reductase 1-like	1246	gi|225434488|ref|XP_002275195.1|PREDICTED: dihydroflavonol-4-reductase [Vitis vinifer XP_0022751	0	91	526.168	327	298
Contig75	calcium-dependent phosphotriesterase su	1191	gi|568364957|ref|XP_006349184.1|PREDICTED: uncharacterized protein LOC102598953| XP_0063491	3.66E-123	77	371.318	319	246
Contig76	uncharacterized partial	2937	gi|449474556|ref|XP_004154213.1|PREDICTED: uncharacterized protein LOC101207800_| XP_0041542	2.00E-79	94	270.781	140	132
Contig77	kinase family protein	3618	gi|255568924|ref|XP_002525432.1|casein kinase, putative [Ricinus communis] >gi|22535\$ XP_0025254	0	93	1257.28	708	660
Contig78	pleiotropic drug resistance protein 1-like	3662	gi|75326590|sp|Q76CU2.1|PDR1_TOBACRecName: Full=Pleiotropic drug resistance protein Q76CU2	0	86	1819.67	1182	1024
Contig79	pleiotropic drug resistance protein 1-like	2381	gi|460388418|ref|XP_004239864.1|PREDICTED: pleiotropic drug resistance protein 1-like XP_0042398	0	88	927.932	595	528
Contig80	splicing factor 1-like protein	2956	gi|225461650|ref|XP_002283115.1|PREDICTED: uncharacterized protein LOC100267539| XP_0022831	0	73	845.884	779	573
Contig81	clathrin interactor epsin 2	2094	gi|255583860|ref|XP_002532681.1|Epsin-2, putative [Ricinus communis] >gi|223527594|g XP_0025326	5.52E-154	63	481.1	593	378
Contig82	protein executer chloroplastic-like	5192	gi|225464089|ref|XP_002271011.1|PREDICTED: protein EXECUTER 1, chloroplastic [Vitis v XP_0022710	0	72	721.079	697	506
Contig83	uncharacterized membrane protein ymr11	1022	gi|565348785|ref|XP_006341383.1|PREDICTED: uncharacterized protein LOC102606368| XP_0063413	6.99E-140	85	420.239	261	224
Contig84	protein berm6-like	1364	gi|590575522|ref|XP_007012710.1|Alpha/beta-Hydrolases superfamily protein isoform 2| XP_0070127	0	91	550.436	314	286
Contig86	-fructan: -fructan 1-fructosyltransferase	2560	gi|378407622|gb|AFB83199.1|fructan-fructan 1-fructosyltransferase [Cichorium intybus] AFB83199	0	85	969.148	612	522
Contig87	glutamate binding protein	1174	gi|470108658|ref|XP_004290629.1|PREDICTED: Bl1-like protein-like [Fragaria vesca subsp XP_0042906	7.31E-102	87	313.538	240	211
Contig88	gata transcription factor 24-like	1586	gi|255572874|ref|XP_002527369.1|GATA transcription factor, putative [Ricinus communis XP_0025273	6.59E-105	81	329.331	218	177
Contig89	transcription elongation factor spt6-like	4413	gi|297741709|emb|CBI32841.3|unnamed protein product [Vitis vinifera] CBI32841	0	83	1895.94	1366	1134
Contig90	high mobility group protein	813	gi|297849910|ref|XP_002892836.1|hypothetical protein ARALYDRAFT_888880 [Arabidopsis XP_0028928	1.49E-50	67	176.022	184	124
Contig91	methyl- -binding domain-containing protei	3053	gi|571498685|ref|XP_006594288.1|PREDICTED: methyl-CpG-binding domain-containing prc XP_0065942	2.47E-132	63	452.595	544	344
Contig92	anaphase-promoting complex subunit 1-lik	7841	gi|508725846|gb|EOY17743.1|E3 ubiquitin ligase isoform 1 [Theobroma cacao] EOY17743	0	83	2254.17	1605	1347
Contig93	probable inactive leucine-rich repeat rec	2990	gi|225456272|ref|XP_002279697.1|PREDICTED: probable inactive leucine-rich repeat rece XP_0022796	0	71	728.398	762	543
Contig94	protein nsp-interacting kinase 1-like	3304	gi|566183369|ref|XP_002311408.2|hypothetical protein POPTR_0008s10970g [Populus trix XP_0023114	0	90	759.599	430	391
Contig95	serine threonine-protein kinase edr1-like	3166	gi|502082392|ref|XP_004487152.1|PREDICTED: serine/threonine-protein kinase EDR1-like XP_0044871	0	71	890.567	816	581
Contig96	flavonoid 3-monooxygenase-like	1965	gi|565378114|ref|XP_006355507.1|PREDICTED: cytochrome P450 93A1-like [Solanum tuber XP_0063555	0	83	716.457	499	418

The best-hit of all contigs with BLAST result can be found in the link:
http://journal.frontiersin.org/file/downloadfile/167778_supplementary-materials_tables_1_xlsx/octet-stream/Table%201.XLSX/1/167778

Table S2-2 Transcriptomes annotation process results

GO-id	GO-term	Score	
Biological Process			
GO:0006950	response to stress	3186	9.10%
GO:0006464	cellular protein modification process	2824	8.07%
GO:0005975	carbohydrate metabolic process	2442	6.98%
GO:0006520	cellular amino acid metabolic process	2185	6.24%
GO:0006629	lipid metabolic process	1849	5.28%
GO:0007165	signal transduction	1757	5.02%
GO:0006259	DNA metabolic process	1114	3.18%
GO:0055085	transmembrane transport	1075	3.07%
GO:0006091	generation of precursor metabolites and energy	933	2.67%
GO:0051186	cofactor metabolic process	874	2.50%
GO:0040007	growth	867	2.48%
GO:0030154	cell differentiation	842	2.41%
GO:0071554	cell wall organization or biogenesis	836	2.39%
GO:0006412	translation	794	2.27%
GO:0006461	protein complex assembly	777	2.22%
GO:0034655	nucleobase-containing compound catabolic process	763	2.18%
GO:0016192	vesicle-mediated transport	753	2.15%
GO:0051276	chromosome organization	746	2.13%
GO:0006605	protein targeting	733	2.09%
GO:0006790	sulfur compound metabolic process	726	2.07%
Molecular Function			
GO:0043167	ion binding	6190	28.54%
GO:0016301	kinase activity	2121	9.78%
GO:0016491	oxidoreductase activity	2114	9.75%
GO:0003677	DNA binding	1267	5.84%
GO:0022857	transmembrane transporter activity	1192	5.50%
GO:0016887	ATPase activity	758	3.49%
GO:0016757	transferase activity, transferring glycosyl groups	729	3.36%
GO:0008233	peptidase activity	608	2.80%
GO:0001071	nucleic acid binding transcription factor activity	487	2.25%
GO:0016829	lyase activity	467	2.15%
GO:0016791	phosphatase activity	459	2.12%
GO:0016798	hydrolase activity, acting on glycosyl bonds	456	2.10%
GO:0003735	structural constituent of ribosome	417	1.92%
GO:0016746	transferase activity, transferring acyl groups	387	1.78%
GO:0016874	ligase activity	377	1.74%
GO:0004871	signal transducer activity	338	1.56%

Table S2-2 (continued)

GO-id	GO-term	Score	
GO:0008168	methyltransferase activity	321	1.48%
GO:0016853	isomerase activity	315	1.45%
GO:0016779	nucleotidyltransferase activity	270	1.24%
GO:0008092	cytoskeletal protein binding	266	1.23%
GO:0004386	helicase activity	258	1.19%
Cellular component			
GO:0009536	plastid	3314	17.29%
GO:0043234	protein complex	2986	15.58%
GO:0005886	plasma membrane	2769	14.45%
GO:0005739	mitochondrion	1839	9.59%
GO:0005829	cytosol	1831	9.55%
GO:0005794	Golgi apparatus	1064	5.55%
GO:0005773	vacuole	950	4.96%
GO:0005840	ribosome	740	3.86%
GO:0005783	endoplasmic reticulum	679	3.54%
GO:0005618	cell wall	630	3.29%
GO:0009579	thylakoid	565	2.95%
GO:0005730	nucleolus	517	2.70%
GO:0005768	endosome	342	1.78%
GO:0016023	cytoplasmic membrane-bounded vesicle	303	1.58%
GO:0005777	peroxisome	230	1.20%
GO:0005654	nucleoplasm	212	1.11%
GO:0005635	nuclear envelope	85	0.44%
GO:0000228	nuclear chromosome	64	0.33%
GO:0000229	cytoplasmic chromosome	22	0.11%
GO:0005815	microtubule organizing center	18	0.09%
GO:0044421	extracellular region part	7.8	0.04%

Table S2-3 Annotated contigs involved in top 30 metabolic pathway

Pathway	#Seqs in Pathway	#Enzyme
Biosynthesis of antibiotics	668	175
Starch and sucrose metabolism	478	40
Purine metabolism	435	52
T cell receptor signaling pathway	198	2
Glycolysis / Gluconeogenesis	196	25
Amino sugar and nucleotide sugar metabolism	195	42
Phenylalanine metabolism	182	22
Pyrimidine metabolism	171	28
Glycerophospholipid metabolism	168	29
Glycerolipid metabolism	151	21
Galactose metabolism	146	19
Pyruvate metabolism	146	26
Drug metabolism - other enzymes	137	14
Phenylpropanoid biosynthesis	137	14
Oxidative phosphorylation	135	17
Phosphatidylinositol signaling system	135	7
Pentose and glucuronate interconversions	133	16
Carbon fixation in photosynthetic organisms	126	21
Arginine and proline metabolism	121	34
Aminobenzoate degradation	119	34
Cysteine and methionine metabolism	119	11
Glycine, serine and threonine metabolism	117	32
Pentose phosphate pathway	113	18
Methane metabolism	106	19
Carbon fixation pathways in prokaryotes	104	18
Glyoxylate and dicarboxylate metabolism	104	27
Citrate cycle (TCA cycle)	101	16
Inositol phosphate metabolism	95	22
Thiamine metabolism	94	8
Ascorbate and aldarate metabolism	93	14

Table S2-4 Details of contigs involved in secondary metabolic biosynthesis pathways (part)

Pathway	#Seqs in Pathway	Enzyme	Ezyme ID	#Seqs of Enzyme	Seqs involved	Pathway ID
Steroid hormone biosynthesis	43	O-methyltransferase	ec:2.1.1.6	2	Contig27880, Contig27881	map00140
Steroid hormone biosynthesis	43	5beta-reductase	ec:1.3.1.3	2	Contig23923, Contig25515	map00140
Steroid hormone biosynthesis	43	dehydrogenase	ec:1.1.1.149	1	Contig15861	map00140
Steroid hormone biosynthesis	43	dehydrogenase	ec:1.1.1.145	9	Contig4658, Contig6532, Contig6865, Contig11024, Contig20432, Contig23923, Contig25515, Contig3402	map00140
Steroid hormone biosynthesis	43	17-dehydrogenase	ec:1.1.1.62	5	Contig4866, Contig5424, Contig13099, Contig23008, Contig24044	map00140
Steroid hormone biosynthesis	43	20beta)-hydroxysteroid dehydr	ec:1.1.1.53	1	Contig19083	map00140
Steroid hormone biosynthesis	43	1-naphthol glucuronyltransferas	ec:2.4.1.17	4	Contig2607, Contig12837, Contig12925, Contig15080	map00140
Steroid hormone biosynthesis	43	monooxygenase	ec:1.14.14.1	19	Contig388, Contig2578, Contig10619, Contig12431, Contig12622, Contig12776, Contig13139, Contig1347	map00140
Steroid hormone biosynthesis	43	sulfotransferase	ec:2.8.2.4	1	Contig6053	map00140
Steroid hormone biosynthesis	43	4-dehydrogenase (NADP+)	ec:1.3.1.22	1	Contig11232	map00140
Caprolactam degradation	26	hydratase	ec:4.2.1.17	23	Contig440, Contig2746, Contig2936, Contig2953, Contig5767, Contig12793, Contig12794, Contig12879, '	map00930
Caprolactam degradation	26	dehydrogenase (NADP+)	ec:1.1.1.2	2	Contig1555, Contig8457	map00930
Caprolactam degradation	26	dehydrogenase	ec:1.1.1.35	11	Contig2746, Contig2936, Contig12793, Contig12794, Contig16800, Contig18154, Contig20252, Contig272	map00930
Thiamine metabolism	94	diphosphokinase	ec:2.7.6.2	2	Contig4851, Contig4852	map00730
Thiamine metabolism	94	kinase	ec:2.7.4.7	2	Contig5840, Contig21356	map00730
Thiamine metabolism	94	synthase	ec:2.2.1.7	6	Contig5602, Contig5670, Contig12428, Contig13581, Contig19682, Contig21452	map00730
Thiamine metabolism	94	diphosphorylase	ec:2.5.1.3	4	Contig5771, Contig5840, Contig21356, Contig26429	map00730
Thiamine metabolism	94	desulfurase	ec:2.8.1.7	4	Contig1754, Contig5236, Contig5237, Contig7344	map00730
Thiamine metabolism	94	phosphatase	ec:3.6.1.15	77	Contig561, Contig753, Contig1286, Contig2794, Contig3813, Contig4215, Contig5008, Contig5173, Cont	map00730
Thiamine metabolism	94	kinase	ec:2.7.1.50	1	Contig28590	map00730
Thiamine metabolism	94	kinase	ec:2.7.1.49	2	Contig5840, Contig21356	map00730
Glycosaminoglycan biosynthesis -	45	xylosyltransferase	ec:2.4.2.26	10	Contig2083, Contig4968, Contig6078, Contig6880, Contig13708, Contig18268, Contig30171, Contig3073	map00534
Glycosaminoglycan biosynthesis -	45	3-beta-glucuronosyltransferase	ec:2.4.1.135	4	Contig1534, Contig3130, Contig5531, Contig6654	map00534
Glycosaminoglycan biosynthesis -	45	3-beta-galactosyltransferase	ec:2.4.1.134	14	Contig2517, Contig10476, Contig11031, Contig11159, Contig11168, Contig19551, Contig21765, Contig21	map00534
Glycosaminoglycan biosynthesis -	45	4-alpha-N-acetylglucosaminyltra	ec:2.4.1.224	10	Contig2588, Contig5850, Contig9395, Contig11371, Contig18981, Contig20055, Contig23411, Contig247	map00534
Glycosaminoglycan biosynthesis -	45	4-alpha-N-acetylglucosaminyltra	ec:2.4.1.223	7	Contig3817, Contig5869, Contig15150, Contig21360, Contig27136, Contig27137, Contig31401	map00534
Glycosaminoglycan biosynthesis -	28	xylosyltransferase	ec:2.4.2.26	10	Contig2083, Contig4968, Contig6078, Contig6880, Contig13708, Contig18268, Contig30171, Contig3073	map00532
Glycosaminoglycan biosynthesis -	28	3-beta-glucuronosyltransferase	ec:2.4.1.135	4	Contig1534, Contig3130, Contig5531, Contig6654	map00532
Glycosaminoglycan biosynthesis -	28	3-beta-galactosyltransferase	ec:2.4.1.134	14	Contig2517, Contig10476, Contig11031, Contig11159, Contig11168, Contig19551, Contig21765, Contig21	map00532
Glycosaminoglycan degradation	32	hexosaminidase	ec:3.2.1.52	3	Contig20287, Contig30110, Contig34002	map00531
Glycosaminoglycan degradation	32	alpha-acetylglucosaminidase	ec:3.2.1.50	3	Contig15636, Contig24556, Contig24557	map00531
Glycosaminoglycan degradation	32	beta-glucuronide glucuronohyd	ec:3.2.1.31	1	Contig29348	map00531
Glycosaminoglycan degradation	32	lactase (ambiguous)	ec:3.2.1.23	25	Contig3, Contig1178, Contig1432, Contig2272, Contig2450, Contig6386, Contig6617, Contig7180, Conti	map00531

Details of all contigs involved in secondary metabolic biosynthesis pathways can be found in the link: http://journal.frontiersin.org/file/downloadfile/167778_supplementary-materials_tables_4_xlsx/octet-stream/Table%204.XLSX/1/167778

Table S2-5 Chalcone and stilbene synthase protein sequences obtained from NCBI for phylogenetic analyzes

Species	accession no.	abbreviation	note
Antirrhinum majus	BAE80511.1	AmCHS	
Arabidopsis halleri subsp. *gemmifera*	AAZ23684.1	AhCHS	
A. thaliana	NP_196897.1	AtCHS	
A. thaliana	AAZ23705.1	AtPKSB	
Arachis hypogaea	P20178.1	AhSS	
Artemisia annua	ACY74337.1	AaCHS3	
Betula pendula	CAA71904.1	BpCHS	
Bromheadia finlaysoniana	AAB62876.1	BfCHS	
Callistephus chinensis	CAA91930.1	CcCHS	
Chrysanthemum x *morifolium*	ABF69124.1	CmCHS	
Chrysosplenium americanum	AAB54075.1	CaCHS	
Dahlia pinnata	BAJ14768.1	DpCHS1	
Gerbera hybrida	CAA86219.2	Gh2PS	
G. hybrida	CAA86218.1	GhCHS1	
G. hybrida	CAA86220.1	GhCHS3	
G. hybrida	CAP20328.1	GhCHS4	
Glycine max	P24826.1	GmCHS1	
G. soja	KHN32314.1	GsCHS	
Helianthus annuus	Ha_DY911847	Ha_DY911847	1
H. annuus	Ha_DY921724	Ha_DY921724	1
H. annuus	Ha_DY922225	Ha_DY922225	1
H. annuus	Ha_GE514800	Ha_GE514800	1
H. annuus	Ha_TC39714	Ha_TC39714	1
H. annuus	Ha_TC40680	Ha_TC40680	1
H. annuus	ALL34489.1	HaCHS2	1
Ipomoea perperea	AAB02620.1	IpCHSA	
I. perperea	AAC49030.1	IpCHSB	
I. perperea	AAC49031.1	IpCHSC	
I. perperea	ABW69675.1	IpCHSD	
I. perperea	BAA87337.1	IpCHSE	
I. perperea	AAB41103.1	IpCHSF-1	
Juglans nigra x Juglans regia	CAA64452.1	JCHS	
Lactuca sativa	Ls_DY962354	Ls_DY962354	1
L. sativa	Ls_DY970700	Ls_DY970700	1
L. sativa	Ls_TC16147	Ls_TC16147	1
L. sativa	Ls_TC27150	Ls_TC27150	1
L. sativa	AAP03003.1	LsCHS	1

Table S2-5 (continued)

Species	accession no.	abbreviation	note
Medicago sativa	AAA02823.1	MsCHS1	
M. sativa	AAA02824.1	MsCHS2	
M. sativa	AAA02825.1	MsCHS4	
M. sativa	AAA02826.1	MsCHS8	
M. sativa	AAA02827.1	MsCHS9	
M. sativa	CAA48226.1	MsCHSI	
M. truncatula	XP_013456566.1	MtCHS	
Morus notabilis	XP_010088676.1	MnCHS9	
Oryza sativa	BAA19186.2	OsCHS	
Perilla frutescens	BAA19656.1	PfCHS	
Petunia hybrida	CAA32731.1	PhCHSA	
P. hybrida	CAA32732.1	PhCHSB	
P. hybrida	CAA32733	PhCHSD	
P. hybrida	CAA32734	PhCHSF	
P. hybrida	CAA32735	PhCHSG	
P. hybrida	CAA32737	PhCHSJ	
Pinus strobus	O65872.1	PsCHS	
P. strobus	CAA06077.1	PsSS	
P. sylvestris	P30079.1	PsCHS2	
P. sylvestris	CAA43166.1	PsSS2	
Populus trichocarpa	XP_006379273.1	PtCHS	
Raphanus sativus	AAB87072.1	RsCHS	
Scutellaria baicalensis	BAA23373.1	SbCHS	
Solanum tuberosum	AAB67734.1	StCHS1a	
Theobroma cacao	XM_007041882.1	TcCHS	
Vitis riparia	AAF00586.1	VrSS	
V. vinifera	BAA31259.1	VvCHS	
V. vinifera	AAB72091.1	VvCHS2	
V. vinifera	CAA54221.1	VvSS	
V. vinifera	NP_001267939.1	VvSS1	

1 sequences of sunflower (Helianthus annuus) and lettice (Lactuca sativa) are retrieved from the TGI databases (ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/) using the key word 'chalcone synthase'.

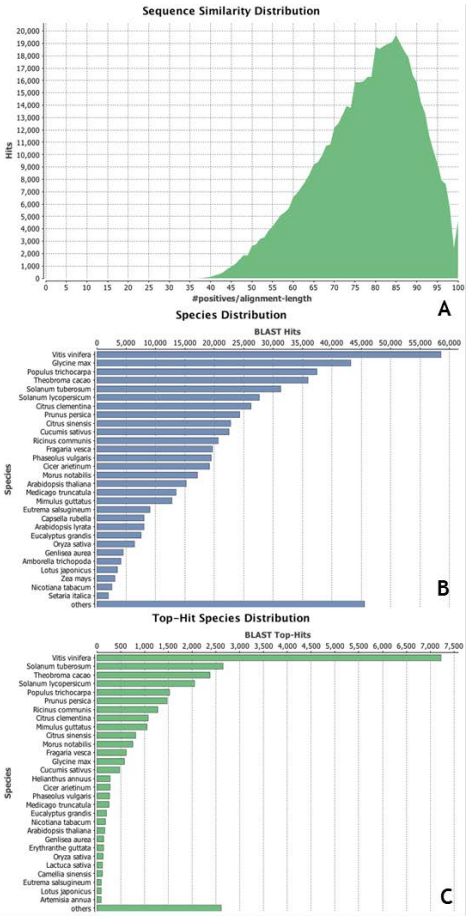

Fig. S2-1 Charts of Sequences Similarity Distribution (A), (Blast hit) Species Distribution (B) and Top-hits Species Distribution (C).

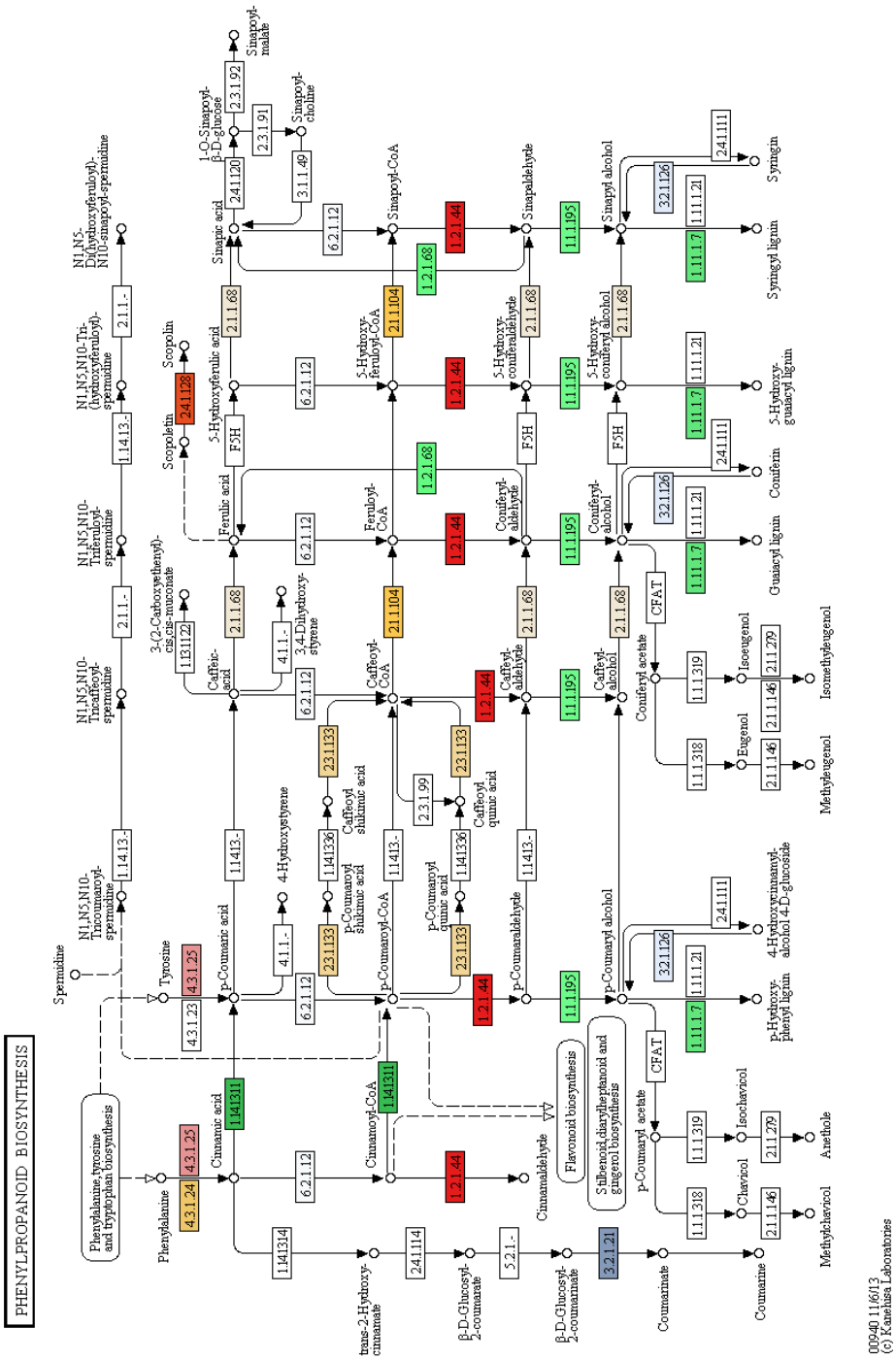

Fig. S2-2 Details of enzymes involved in phenylpropanoid biosynthesis pathway (from KEGG, Kyoto Encyclopedia of Genes and Genomes). Annotated enzymes are highlighted in different colors.

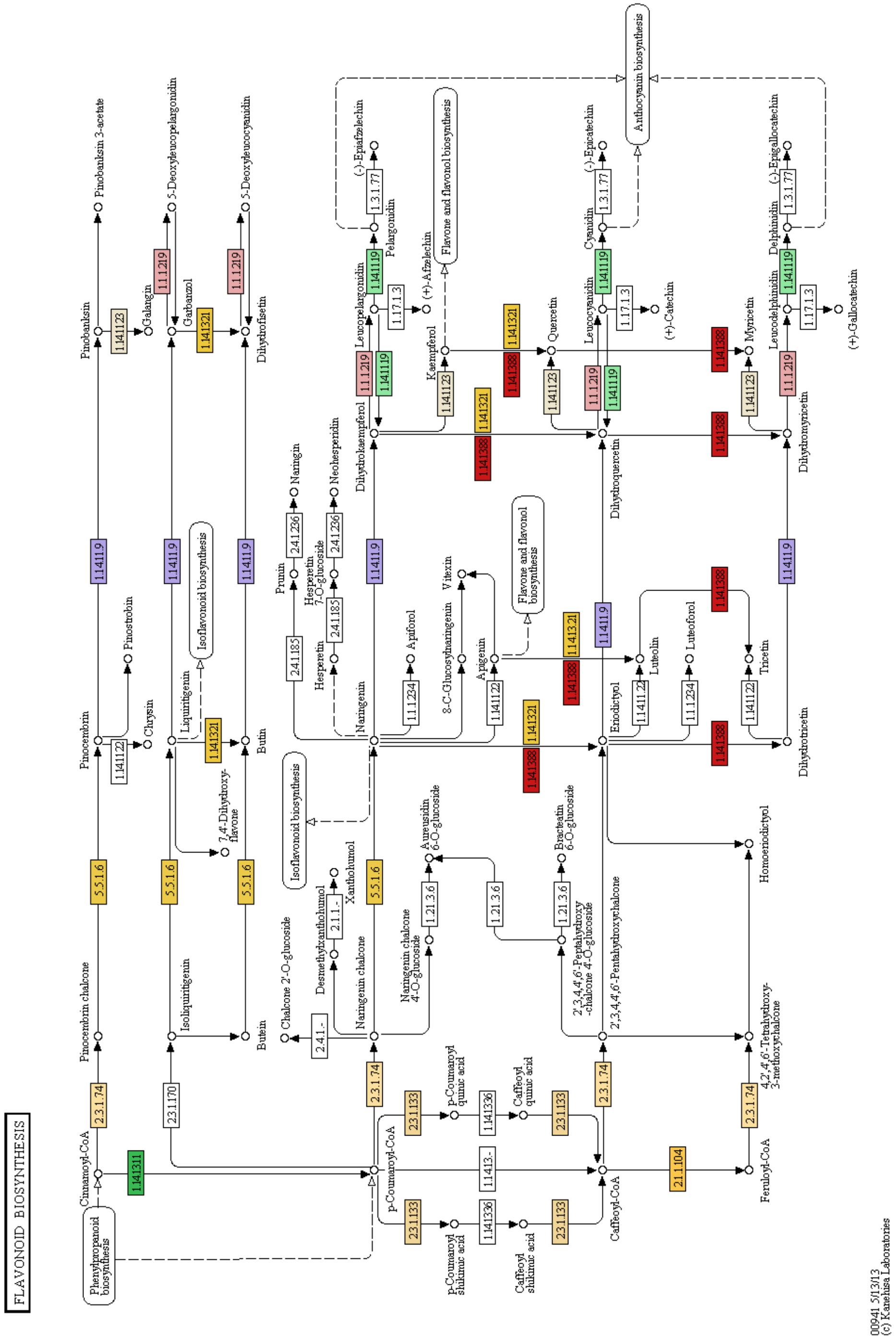

Fig. S2-3 Details of enzymes involved in flavonoid biosynthesis pathway (from KEGG). Annotated enzymes are highlighted in different colors.

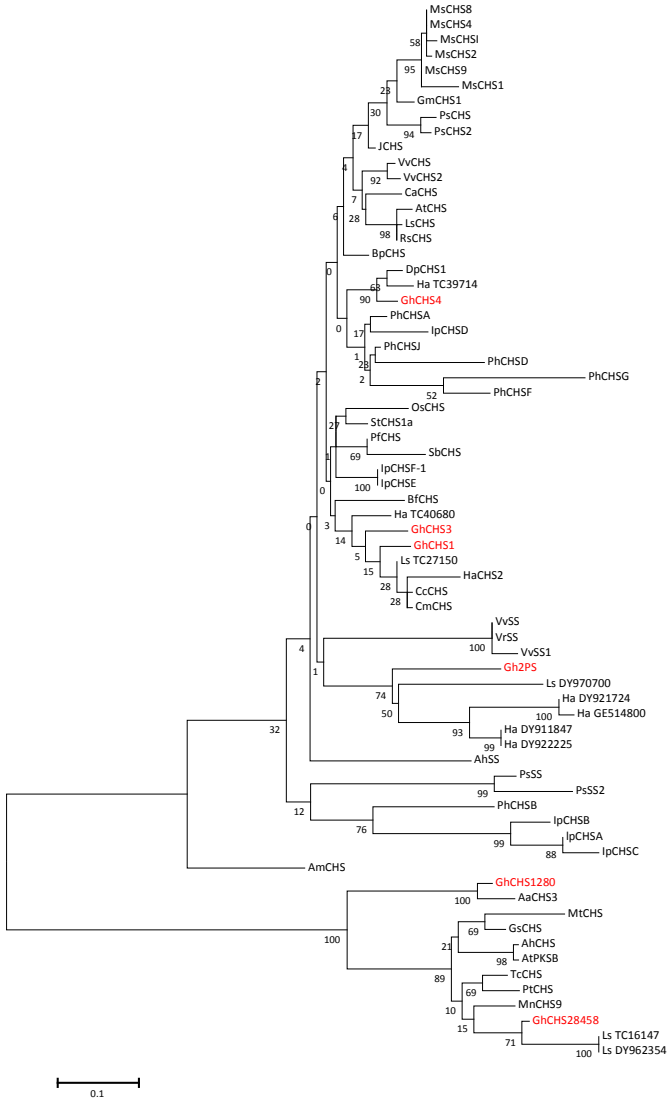

Fig. S2-4 Phylogenetic tree of chalcone and stilbene synthase family protein from different species. The tree generated with MEGA 6.0 using the Maximum-likelihood after sequence alignment. The numbers indicate bootstrap probabilities. NCBI accession numbers of the chalcone and stilbene synthase family protein sequences used in the tree are given in suppl.Table S2-5.

Chapter 3 Genetic mapping and QTL analysis of Botrytis resistance in *Gerbera hybrida*

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This chapter has been published in Molecular Breeding (2017) 37: 13.

Abstract

Gerbera hybrida is an economically important cut flower. In the production and transportation of gerbera with unavoidable periods of high relative humidity, gray mold occurs and results in losses in quality and quantity of flowers. Considering the limitations of chemical use in greenhouses and the impossibility to use these chemicals in auction or after sale, breeding for resistant gerbera cultivars is considered as the best practical approach. In this study, we developed two segregating F1 populations (called S and F). Four parental linkage maps were constructed using common and parental specific SNP markers developed from EST sequencing. Parental genetic maps, contained 30, 29, 27 and 28 linkage groups and a consensus map covering 24 of the 25 expected chromosomes could be constructed. After evaluation of Botrytis disease severity using three different tests, *whole inflorescence*, *bottom* (of disc florets) and *ray floret*. QTL mapping was performed using the four individual parental maps. A total of 20 QTLs (including one identical QTL locus for *whole inflorescence* and *bottom* test) were identified in the parental maps two populations. The number of QTLs found and the explained variance of most QTLs detected reflects the complex mechanism of Botrytis disease response.

Keywords

gerbera gray mold, SNP, linkage group, QTL mapping

Introduction

Gerbera hybrida belongs to the *Compositae* family and is known for its abundant flower colors, capitula sizes and shapes. As one of the most economical important ornamental plants, gerbera is mainly used as cut flower, and ranked fifth in cut flower sales on Dutch flower auctions in 2014 (https://www.floraholland.com/media/3949227/Kengetallen-2014-Engels.pdf). The cultivated commercial gerberas are highly heterozygous almost complete obligatory outcrossing diploid (2n = 50) plants and probably originated from crossings between two wild species, *G. jamesonii* and *G. viridifolia*, from Africa (Hansen 1999).

Gerbera as a cut flower is mainly grown in greenhouses for year-round production. However, during gerbera cultivation especially in winter and during the process of post-harvest transportation, the high relative humidity is ideal for gray mold infestation. Gerbera gray mold is caused by the necrotrophic fungus *Botrytis cinerea*, a notorious fungal pathogen with a wide range of plant host species (Elad *et al.* 2016a). *B. cinerea* infection leads to direct damage on gerbera. Necrotic lesions (spotting) in early infection occurs on flower buds and ray florets, and these symptoms are strengthened when flowers are packed in boxes, in which a high relative humidity develops during cold storage and transport (Bastiaan-Net *et al.* 2007; Kerssies 1993a; Kerssies 1993b; Salinas and Verhoeff 1995). Control of gerbera gray mold in greenhouses frequently relies on spraying chemicals (Prins *et al.* 2000), but using chemicals may cause environmental issues and increase resistance to fungicides (Leroux 2007) whereas the use of some compounds has been restricted and banned in a number of countries. Moreover, quality loss due to gerbera gray mold occurring in post-harvest (in auction or after sale) transport is hard to avoid by chemical treatments, affecting both the buyer (reduction in profits) and the seller (a breeder's reputation) (Bastiaan-Net *et al.* 2007). Thus, breeding for Botrytis resistant varieties is needed to reduce current and future problems due to this devastating pathogen in gerbera.

A number of studies on Arabidopsis indicated that the positive responses regulated by JA/ET (jasmonic acid/ethylene) signalling (Glazebrook 2005; Thomma *et al.* 1998; Thomma *et al.* 1999) and production of camalexin (Kliebenstein *et al.* 2005; van Baarlen *et al.* 2007; Williamson *et al.* 2007) enhance a plant's resistance to *B. cinerea*. Similarly, in *Brassica rapa*, glucosinolate defensive metabolite accumulation coincided with *B. cinerea* QTLs (Zhang *et al.* 2016a). Catecheol from onion scales inhibited *B. cinerea* growth *in vitro* (Clark and Lorbeer 1975). However, none of these metabolites in different species can confer full resistance. Plant resistance to Botrytis is considered conditioned by multiple genes with partial effects and likely requires the contribution of multiple loci to reduce disease severity (Mengiste *et al.* 2003) and to obtain acceptable levels of resistance under standard conditions. This kind of complex resistance is polygenic and can be referred to as quantitative disease resistance (St.Clair 2010). DNA markers tightly linked to quantitative resistance loci can be used for marker-assisted selection (MAS) and desirable QTLs can be then subsequently introgressed into commercial cultivars.

Up to now, QTL analysis for Botrytis resistance has been primarily assayed in Arabidopsis and tomato. Denby *et al.* (2004) identified 12 small- to medium-effect QTL governing Botrytis susceptibility as to lesion size in Arabidopsis using 104 individuals from a Ler × Col-0 recombinant inbred population and

several interesting candidate genes were found co-located in the QTL regions of the genome. Rowe and Kliebenstein (2008) found that several separate QTLs influenced lesion size and camalexin accumulation on Arabidopsis leaves using a larger RILs population with 411 individuals. They suggested that the plant defense against *B. cinerea* is mainly quantitative and genetically complex. Finkers *et al*. (2007b) calculated disease incidence and lesion growth rate in tomato populations, and detected three QTLs that explained 12%, 15% and 7% of the total phenotypic variation. They also analyzed two QTLs in BC2S1 progeny and found additive effects for progeny with homozygous resistance QTL alleles present.

No gerbera genetic maps are published to date. In this study, we developed two F1 populations segregating for Botrytis resistance in order to obtain the first genetic maps for this highly heterozygous ornamental crop. Through next generation sequencing of the transcriptomes of the parental genotypes (*Chapter 2*; Fu *et al*. 2016), SNP markers have been developed. These SNP markers have been used for linkage map construction and QTL mapping of Botrytis resistance in gerbera.

Materials and methods

Mapping populations

Two gerbera segregating F1 populations from heterozygous parents were used in this study. The two mapping populations were derived from four parental genotypes with different resistance levels against *B. cinerea* infection and the selected (unrelated) two populations showed the largest variation among 20 F1 populations (4 half sibs of crosses with a line with known Botrytis infection problems) which were tested for Botrytis susceptibility on 50 individuals. Population Schreurs (hereafter referred to as population S), containing 276 offspring, was obtained from a cross between the gerbera genotypes, SP1 and SP2. Population Florist (here after referred to as population F) was produced by a cross between FP1 and FP2. Population F consisted of 270 progeny. All individuals from both populations were used for linkage mapping, disease tests and QTL analysis.

Phenotypic measurements

The head-like inflorescence of gerbera is composed of different flower types, the marginal ray florets, the central disc florets and the intermediate trans florets. Botrytis infected lesion symptoms vary in these gerbera florets: spotting on ray florets and rotting on disc florets. To assess Botrytis resistance levels on different gerbera inflorescences of all F1 progeny and four parents in the two populations, phenotypic data was collected using three tests based on visual inspection of Botrytis infestation: on whole inflorescence (further referred to as *whole inflorescence* or *WI* test), on the bottom of disc florets in the capitulum (further referred to as *bottom* test) and on ray florets (further referred to as *ray floret* or *RF* test), respectively.

B. cinerea (strain B05.10 obtained from Dr. J. van Kan, Laboratory of Phytopathology, Wageningen University) was grown for 1 week on potato dextrose agar (PDA) medium after which conidia were transferred onto fresh PDA medium and grown until sporulation (about one week). A spore suspension of 1×10^7 conidial spores per ml in sterile distilled water was prepared as stock suspension. For the Botrytis disease test on *whole inflorescence* and *bottom*, the spore suspension was diluted to a concentration of

1×10^5/ml with water and sprayed on the inflorescence with a fine plant sprayer. After inoculation, inflorescences were incubated for five days in a climate cell at 20°C and a R.H. of 90%. Because ripe flowers (anthesis of the first whorl of disk floret) are not available from single plants in abundance testing was done over a period of 10 consecutive weeks (8-10 inflorescences tested on average). Inflorescence testing was done simultaneously for *whole inflorescence* and *bottom* test on the same inflorescence. First, *whole inflorescence*s were visually evaluated to score, after which, the bottom of the capitulum was cut (horizontal cross section) to check (score) fungal growth inside the capitulum for the *bottom* test. The response to Botrytis infection on *whole inflorescence* and *bottom* were scored ranging from 0 (no symptom) to 5 (completely rotten).

For the *ray floret* test, inoculation was performed by pipetting 2 µl of spore suspension that was diluted to a concentration of 3×10^5/ml in potato dextrose (to guarantee 100% spore germination), on the upper surface of single marginal ray floret. Twenty ray florets were incubated for 48 hours at nearly 100% relative humidity. After 48h, the disease score was assessed as follow: 0 = no visible symptoms, 1 = infection limited in inoculation droplet size, 2 = lesion extended twice to forth times the droplet size, 3 = large lesion area but still smaller than ½ of the ray floret, 4= lesion area larger than ½ of the ray floret, and 5 = complete necrosis.

SNP selection and genotyping

EST database establishment and SNP detection have been performed as described in *Chapter 2* and Fu *et al*. (2016). SNPs were identified as specific SNPs (only polymorphic in one set of crossing parents, i.e. a single population) and common SNPs (polymorphic in both populations). The origin of SNP markers is indicated in the name. For example, marker WGC10601_843_S1F2 means that this marker is developed from contig10601 of the gerbera EST dataset (*Chapter 2*; Fu *et al*. 2016). The number after the first underscore is the SNP position in the contig. At the end of each markers' name the source of polymorphism is indicated by population (and parent). If it is a specific SNP, it will be followed only with either S or F (S after the second underscore means polymorphic in both S population parents and S1 means only polymorphic in SP1, *et cetera*). Common SNPs are indicated with both an S and F in the name. In this case, S1F2 means this marker is a common marker which is polymorphic in parents S1 and F2 and can be found under an identical name in both maps.

Genotyping of selected reliable SNP markers in parents and all individuals of the two populations were performed by KBioscience (current name LGC genomics) using KASP technology. The genotyping data were visualized in SNPviewer (LGC genomics) to check the segregation type in each population, and SNP markers with segregation type 1:1 and 1:2:1 were included for genetic mapping. SNP markers segregating in a non-Mendelian inheritance pattern were analyzed by hypothesizing one or more null-allele present. After checking the goodness of fit to possible segregation types, these markers were rescored and included.

Genetic linkage map construction

The genotyping data of two gerbera populations were coded following the population type CP (cross pollinating) in JoinMap® 4.1 (van Ooijen 2006). After created maternal and paternal population nodes,

grouping of markers was based upon the test for independence LOD score with a threshold of 4. Genetic map construction used regression mapping and the Kosambi's mapping function. Integrated linkage maps of the parental maps were constructed based on the bridge markers (<hkxhk> type marker). Consensus linkage groups of the two populations were constructed with identical common markers segregating in both populations and numbering was named consistently between linkage maps.

QTL analysis

The means of disease score for each individual on *whole inflorescence*, *bottom* and *ray floret* test in the two populations were used independently as phenotypic data for QTL analysis. QTLs analysis for Botrytis resistance was performed in separate parental linkage maps using MapQTL® 6 (van Ooijen 2009). First, interval mapping was used to find QTL regions associated to each of the traits tested. Based on the result of interval mapping, MQM (multiple-QTL models) mapping was performed with the maximum likelihood mixture model using the closest markers as cofactors. Significance LOD thresholds were determined by 1000 permutations corresponding to a genome-wide confidence level of $P<0.05$.

Results

SNP selection and genotyping results

Gerbera cDNA reads were clustered and assembled into 36,770 EST contigs within which a large number of specific and common SNPs were detected in the parents from the two populations (*Chapter 2*; Fu *et al.* 2016). For genotyping in population S, a set of 677 polymorphic SNPs markers, including 477 SNPs common to both two populations and 200 specific SNPs, were selected. Similarly, there were 675 SNPs markers selected for population F, including 477 common markers and 198 specific SNPs.

A summary of segregation type for all SNP markers in both populations is shown in Table 3-1. Of all selected SNP markers, 68% were successfully genotyped by KASP in population S and 72% were successful in population F (Suppl. Fig. S3-1a, b, c illustrate the three visualized segregation results in SNPviewer). A number of markers showing a single group call are considered as non-polymorphic (Suppl. Fig. S3-1d); these include 166 SNPs in population S and 147 in population F. Markers showing scattered segregation without clear grouping were noted as not-fitting segregation (Suppl. Fig. S3-1e). The percentages of markers showing a not-fitting pattern were 7% in population S and 6% in population F.

Table 3-1 Overview of the genotyping results of selected SNPs marker

Population	Markers segregating in both parents	Markers segregating in P1 (seed parent) only	Markers segregating in P2 (pollen parent) only	Markers with a null-allele	no polymorphism	segregation not fitting
S	135/20%	159/23%	126/19%	41/6%	166/25%	50/7%
F	107/16%	230/34%	116/17%	34/5%	147/22%	41/6%

In a number of cases in both the S and F population (Table 3-1, null-allele), parental genotype scores do not seem to fit the found offspring genotypes. These segregating SNP markers could be further analyzed assuming the presence of null-alleles. For example, in marker WGC19112, the genotype of two parents are

A:G (P1) and G:G (P2) respectively, and the expected segregation in progeny should be [A:G]:[G:G] = 1:1, but the visualized genotyping result in SNPviewer (Suppl. Fig. S3-1f) showed three genotype cluster plots [A:A]:[A:G]:[G:G] ≈ 1:1:2 (74:69:133). The possible explanation is the presence of a null-allele in P2 (G:Ø), and the actual progeny segregation is [A:G]:[A:Ø]:[G:G]:[G:Ø] ≈ 1:1:1:1, because the genotyping technology cannot distinguish the genotype G:G and G:Ø (they are in the same cluster), also the P2 genotype G:Ø is recognized as G:G.

To use the marker information, we rescored these markers, like WGC19112, with the consideration that both parents are heterozygous. However information content differed between parents for such a marker. P1 is heterozygous and WGC19112 is used as fully informative <lmxll> marker (a). Both A:G and A:Ø offspring clusters are rescored as 'lm' and G:G (in fact containing [G:G] and [G:Ø]) as 'll'. P2 is also heterozygous and WGC19112 is here regarded as <nnxnp> marker (b). However, only offspring within groups A:A and A:G are informative for this parent (group A:A scored as 'nn' and group A:G as 'np'), the mixed group G:G (containing [G:G] and [G:Ø]) is discarded. To distinguish the two ways of scoring, we added a letter 'a' or 'b' in the end of the marker. Markers in which null-alleles were demonstrated with an 'a' and 'b' in the end were mapped at almost the same position on the integrated maps, but in eight markers sufficient linkage was only found in linkage groups of the most informative parent and not in the other parent.

Linkage map construction

Both maternal and paternal maps of the two populations were constructed, as well as integrated maps per population and a consensus map of the two populations. There were 30, 29, 27 and 28 linkage groups constructed in SP1, SP2, FP1 and FP2, respectively (Suppl. Table S3-1). Total marker number ranged from 259 in parent FP2 to 350 in parent FP1. The observed parental map lengths varied from 1103 cM to 1498 cM and the average marker distance varied from 3.50 cM to 4.41 cM per parental map (Suppl. Table S3-1).

Parental linkage maps could be aligned via the presence of bridge markers (<hkxhk> type markers) that are segregating from both parents (Suppl. Table S3-2). Based on the position of the bridge markers, markers order on parental maps showed good consistency, but distance between markers on parental linkage maps varied as can be expected. For instance, on maternal linkage group FP1_08, the distance of between marker WGC9125 and WGC18021 is 7.6 cM, while the distance on the paternal linkage group (FP2_08) is 12.9 cM (Suppl. Fig. S3-2a). By using these bridge markers, the two parental linkage maps could be combined into one integrated linkage map with the same linkage group number code (see Suppl. Fig. S3-2a).

Similarly, with the help of common markers, identical parental linkage maps of both crosses could be also be identified and aligned. For instance, there are around 35 markers in linkage group SP1_01, of which some markers are also found in two linkage groups in parents of the F population (i.e. common markers) indicating that these linkage groups are homologs of SP1_01. So these fragments are named as FP1_01.1, FP1_01.2 and FP2_01.1, FP2_01.2. Same situation happens on SP1_03, SP2_03, SP1_12, SP2_12 *etc*. (Suppl. Table S3-1). Few maternal and paternal linkage groups (e.g. SP1_21, SP2_23 and FP1_17, FP1_24, FP2_16, FP2_20) could not be aligned to a linkage group of another parent because just a single bridge

marker was present or there was a lack of informative common markers (Suppl. Table S3-1 and Suppl. Table S3-2).

These parental linkage maps, with in total 285 common markers present, can be integrated into a consensus map (see Suppl. Fig. S3-2b). In total 24 consensus linkage groups were merged (Fig. 3-1 Suppl. Table S3-3). As is described in Suppl. Table S3-3, the consensus linkage map of 687 SNPs covered 1601 cM. The marker density on the consensus map varied from 1.32 cM on LG09 to 5.16 cM on LG17, with an average density 2.57 cM. There were 14 gaps larger than 15 cM observed in the consensus linkage map.

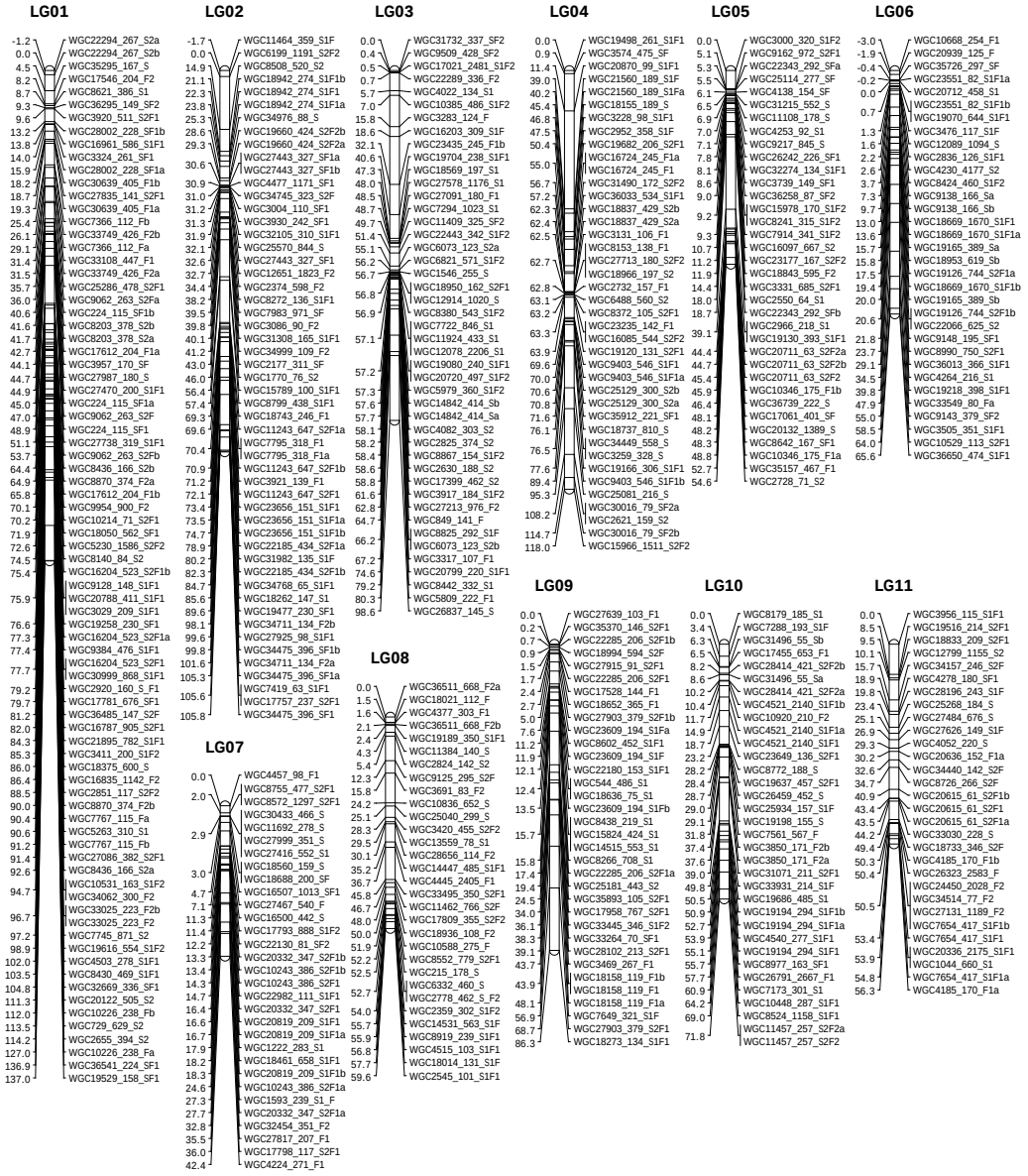

Fig. 3-1 Consensus linkage map of two gerbera populations.

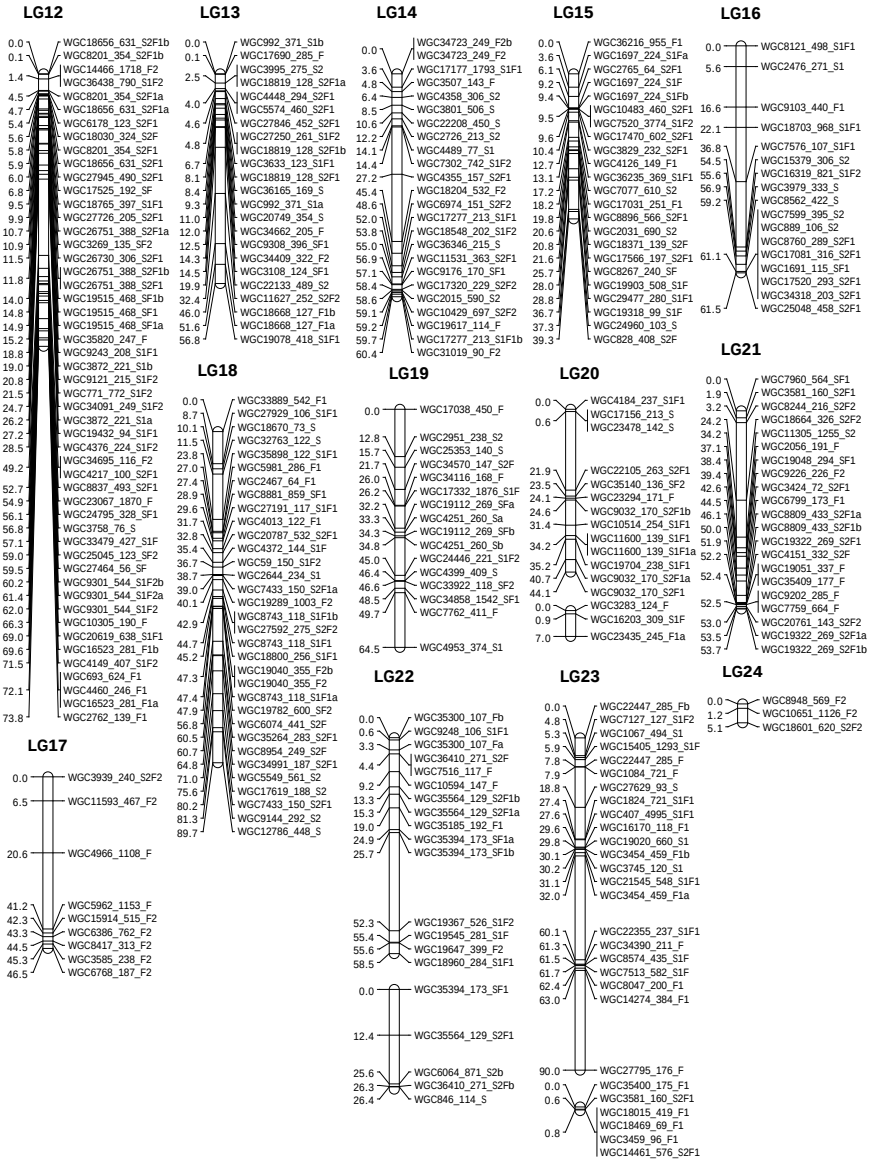

Fig. 3-1 (continued)

Phenotypic traits evaluation for Botrytis resistance

Phenotypic data of resistance to *B. cinerea* were assessed in three tests (*whole inflorescence*, *bottom* and *ray floret*). Histograms of disease testing resulting from these three traits in the mapping population S and F population indicating transgressive segregation are shown in Fig. 3-2. The mean of the phenotyping data in population S for *whole inflorescence*, *bottom* and *ray floret* were 2.42±0.55, 2.96±0.63 and 2.98±0.79. Means in population F were 3.64±0.40, 3.80±0.40 and 3.14±0.80, respectively. Based on the skewedness

and kurtosis scale of the distribution curves, all three tests in the two populations were considered as approximately normal distributed and no transformation of data was performed for QTL analysis.

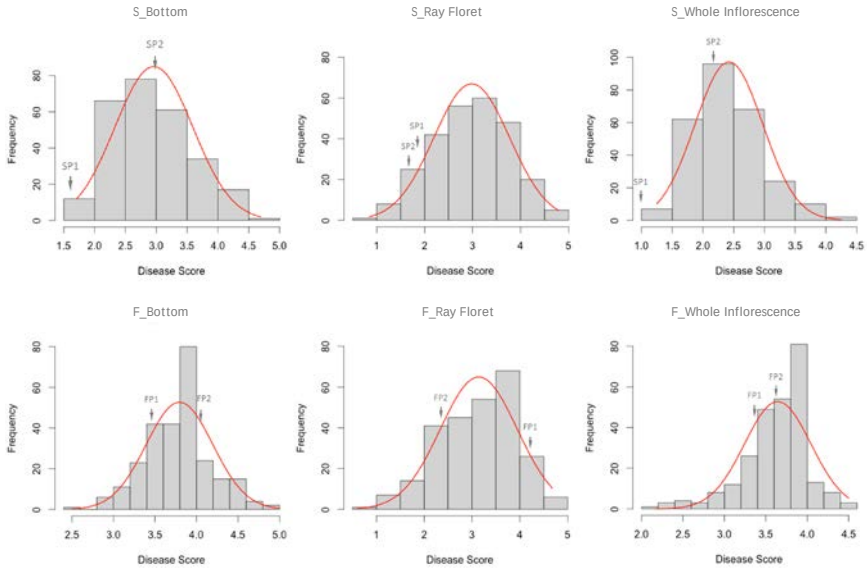

Fig. 3-2 Disease index distribution of S and F populations in *bottom*, *ray floret*, and *whole inflorescence*, respectively. Normal distribution curves are shown above the histograms in red. Arrows indicate the disease score of parents.

Disease index of the three disease tests in both populations were analyzed by Pearson correlation (Suppl. Table S3-4). The coefficients of *whole inflorescence* and *bottom* tests in both populations showed a moderately high correlations (R=0.83 in population S, R=0.67 in population F), but no significant correlation was found to the *ray floret* tests.

QTL analysis

QTL analysis was first performed on the four parental linkage maps individually. The genome-wide (GW) LOD significance thresholds (P<0.05) for *whole inflorescence*, *bottom* and *ray floret* were obtained using a Permutation Test (Table 3-2). Markers, with LOD scores above the GW threshold in every QTL after interval mapping (IM), were chose as co-factor for multiple-QTL models mapping (MQM mapping). Significant QTLs detected from the four parents are shown in Table 3-2 and Suppl. Fig. S3-3a, b, c.

Table 3-2 QTLs found for *bottom*, *ray floret,* and *whole inflorescence* test in the parental maps of both populations

QTL	Parents	Flanking Markers	LG	MQM	
				LOD (GW)	% expl
RBQB1	SP1	WGC11243_647_S2F1a	2	4.4 (4.0)	6.3
RBQB2	SP1	WGC2476_271_S1	16	4.6 (4.0)	6.6
RBQB3	SP2	WGC18733_346_S2F	11	4.6 (4.1)	7.6
RBQB4	FP1	WGC16204_523_S2F1	1	6.8 (4.0)	10.3
RBQB5	FP1	WGC28102_213_S2F1	9	4.5 (4.0)	7.5
RBQB6	FP2	WGC18158_119_F1b	9	4.8 (3.9)	8
RBQRF1	SP2	WGC17798_117_S2F1	7	5.3 (4.0)	8.9
RBQRF2	FP1	WGC22343_292_SFa	5	6.5 (4.1)	8.6
RBQRF3	FP1	WGC35370_146_S2F1	9	4.8 (4.1)	6.2
RBQRF4	FP1	WGC828_408_S2F	15	6.1 (4.1)	8
RBQRF5	FP1	WGC35264_283_S2F1	18	5.9 (4.1)	7.6
RBQRF6	FP2	WGC7520_3774_S1F2	15	4.9 (4.0)	7
RBQRF7	FP2	WGC6074_441_S2F	18	4.0 (4.0)	5.7
RBQRF8	FP2	WGC9226_226_F2	21	4.75 (4.0)	8.0
RBQWI1	SP1	WGC1044_660_S1, WGC33030_228_S	11	4.8 (4.0)	7.3
RBQWI2	SP1	WGC407_4995_S1F1	23	5.3 (4.0)	8.2
RBQWI3	SP2	WGC18733_346_S2F	11	5.2 (4.1)	8.6
RBQWI4	FP1	WGC1084_721_F	23	6.8 (4.1)	11.1
RBQWI5	FP2	WGC5962_1153_F	17	5.6 (4.0)	8.3
RBQWI6	FP2	WGC22447_285_Fb	23	7.6 (4.0)	11.4

Note: Name of QTLs are RBQ (as Resistance Botrytis QTL) followed by the initials of disease tests used: B=*Bottom*; RF=*Ray Floret*; WI=*Whole Inflorescence* test. LG indicates linkage group and the LG number in the two populations ; Null-alleles are marked with a letter 'a' or 'b' in the end; GW indicates genome wide significant threshold level P<0.05; %Expl. is the percentage of total variance explained by the QTL.

In S population, 7 significant QTLs for Botrytis resistance were detected by MQM mapping, and 13 QTLs in F population. The difference in numbers of QTLs found between the two populations is defined by the number of QTLs associated with Botrytis resistance in *ray floret*. There is only one *ray floret* QTL found in S population but 7 in F population (Table 3-2). Phenotypic variance explained by single QTLs ranged between 5.7 and 11.4%, with three QTL (RBQB4, RBQWI4 and RBQWI6) higher than 10%. Three QTLs, RBQWI2, RBQWI4 and RBQWI6 from SP1, FP1 and FP2 respectively, were found on LG23 at similar positions in the consensus map (see Suppl. Fig. S3-4) indicating this may be a single QTL locus. Interestingly, a QTL for *whole inflorescence* and *bottom* (RBQB3 and RBQWI3) shared an identical position with marker WGC18733_346_S2F on LG11 of population S.

Several QTLs were detected on both parental linkage groups separately and showed overlapping positions on the integrated linkage group, like RBQWI1 from SP1 and RBQWI3 from SP2 on LG11; RBQB5 from FP1 and RBQB6 from FP2 on LG 9. In these cases, alleles from both parents contributed to the resistance in progeny. We identified the favorable and unfavorable alleles from the parents of these QTLs.. Progeny can then be divided into four groups: progeny with the presence of two favorable alleles (+/+), with one favor allele from one of the parents (+/- or -/+) and no favorable allele present (-/-). The mean disease score of each progeny group for each QTLs are shown in Table 3-3. The mean disease scores of individuals with two favorable alleles (+/+) were all significantly lower than for individuals with no favorable allele present (-/-) and also show advantage over individuals with one favorable allele only.

Table 3-3 Difference between the mean score of individuals with presence of two, one or no favorable allele from the parents

	Bottom	*Ray Floret*		*Whole Inflorescence*	
QTLs	RBQB5+RBQB6	RBQRF4+RBQRF6	RBQRF5+RBQRF7	RBQWI1+RBQWI3	RBQWI4+RBQWI6
Flanking Markers	WGC28102_213_S2F1	WGC828_408_S2F	WGC35264_283_S2F1	WGC33030_228_S	WGC1084_721_F
	WGC18158_119_F1b	WGC7520_3774_S1F2	WGC6074_441_S2F	WGC18733_346_S2F	WGC22447_285_Fb
Genotype[1]	Mean±S.E.	Mean±S.E.	Mean±S.E.	Mean±S.E.	Mean±S.E.
+/+	3.621±0.044^{a2}	2.840±0.099^{a}	2.732±0.126^{a}	2.244±0.060^{a}	3.436±0.057^{a}
+/-	3.847±0.041^{bc}	3.328±0.090^{b}	3.152±0.071^{b}	2.440±0.067^{b}	3.631±0.043^{b}
-/+	3.764±0.058^{b}	3.299±0.096^{b}	3.152±0.071^{b}	2.316±0.057^{ab}	3.739±0.050^{bc}
-/-	3.938±0.052^{c}	3.414±0.121^{b}	3.430±0.115^{b}	2.635±0.074^{c}	3.776±0.041^{c}

1, +/+ represent individuals with presence of two favorable alleles from both parents; +/- represent individuals carrying one favorable from P1 and one unfavorable allele from P2; -/+ represent individuals carrying one unfavorable allele from P1 and favorable from P2; -/- represent individuals with presence of the two unfavorable alleles from both parents.
2, mean of each groups with letter a, b and c shows significant difference ($P < 0.05$)

Discussion

Genetic linkage mapping and integration

In this study, we constructed the first gerbera genetic linkage maps from two F1 populations using newly generated SNP markers. Genetic linkage maps construction for cultivated ornamental crops often use F1 populations (Debener and Mattiesch 1999; Han *et al.* 2002; Rajapakse *et al.* 2001; Shahin *et al.* 2011; Zhang *et al.* 2010). Because many ornamental plants, including gerbera, are outcrossing species with complex genetic background and high heterozygosity that cannot be easily selfed due to serious inbreeding depression effects.

Four parental genetic linkage maps were constructed by using SNPs markers from EST data (*Chapter 2*; Fu *et al.* 2016). Most selected SNP markers showed a Mendelian segregation in the populations. For some loci, the allele segregation and allele ratios indicated the presence of null-alleles. Three flanking markers of a QTL contained null alleles. These markers with null-alleles probably come from mutations in the marker region which in the RNAseq data analysis of Fu *et al.* (2016) may have led to assembly of these sequences in alternative contigs and thus stayed unnoticed during SNP identification. We scored markers with null

alleles for each parent separately in order to use the marker data as much as possible and found these two-way scored markers are mapped on almost the same positions in the integrated map and the consensus map. The fact that the four alleles of these markers are all different reflects the complex genetic background of gerbera.

Based on the location of bridge (<hkxhk> type) markers on maternal and paternal linkage maps, we found the markers order on parental maps shows good consistency, but distance between markers on parental linkage maps varies. This is caused by independent meiotic events occurring in the two heterozygous parents and the different frequency of recombination determines the location of markers in each parent (Gebhardt 2007). This also explains the difference in linkage group length between parents of a cross. Markers common to both populations could be used to merge maps between the two populations and to arrive at a consensus map which was helpful for comparisons between the two populations in QTL mapping. From the integrated and consensus maps, we notice that some parental chromosomes appeared as separate (fragmented) linkage groups in one genotype whereas they were in one LG in another genotype (eg FP1_01.1 and FP1_01.2 vs SP1_01). Fragmentation also occurs in integrated maps of single populations. Generally, this occurs more often in the F population (9 out of 20 integrated LGs) than the S population (6 out of 21 integrated LGs). This might be due to a lower number of markers in FP2. Given the offspring numbers in both populations a theoretical minimum marker distance of 0.4 cM is possible. So by introducing more markers, map quality may be further improved.

For gerbera, we expected 25 linkage groups (2n=50). However, a total of 24 consensus linkage groups could be established. There are no additional linkage groups left in any of the four parental maps which could be assigned to LG25. This could be related to the size of this particular chromosome and the number of markers used in our study. Introducing higher numbers of markers might result in retrieving LG25. Also a lack of polymorphism between alleles of this chromosome could cause the inability to find this linkage group.

Gerbera gray mold phenotyping

Gerbera gray mold occurs mainly on gerbera capitulum in the production and post-harvest process. Different symptoms in infected gerbera cultivars were found, either necrotic spots on ray and trans florets or rot on disc florets. The mechanism underlying plant resistance against *B. cinerea* is not well understood, but it is generally accepted that plant resistance to this necrotrophic pathogen is quantitative and polygenic (Poland *et al.* 2009; Rowe and Kliebenstein 2008; St.Clair 2010). In a structured mapping population, quantified disease indexes after inoculation can be used to analyze plant responses to this pathogen and perform QTL mapping. However, there is no standard bioassay approach for evaluating plant resistance to *B. cinerea* available.

Previous studies on Arabidopsis and tomato (AbuQamar *et al.* 2006; Denby *et al.* 2004; Ferrari *et al.* 2007; Finkers *et al.* 2008; Finkers *et al.* 2007b; Hu *et al.* 2013; Rowe and Kliebenstein 2008; ten Have *et al.* 2007; Zhang 2013) are mainly based on infection assays using drop-inoculation or spray-inoculation with conidia suspension on leaves or stems, then measuring the lesion expansion rate, lesion size or camalexin accumulation. In gerbera, leaf and stem infections are of little importance and mainly flower infections

lead to losses. To thoroughly assess the disease severity on gerbera flowers, we developed spray-inoculation tests on whole inflorescences (*whole inflorescence* and *bottom*), as well as a droplet-inoculation test for single ray florets. The tests were devised as simple tests in which a large number of flowers and ray florets could be tested in a relatively short period of time to avoid season influences during the testing period.

As a necrotrophic pathogen, Botrytis relies primarily on its abilities to kill host plant cell and subsequently decompose the plant tissue and consume it for its own growth (van Kan 2006). The fungus can use different infection paths in the complex organs that capitulum are and from experiments with cultivar panels, different responses between cultivars were observed and these led to the three different tests used. Among the three tests, there is a high correlation in both populations between the infection data of *whole inflorescence* and *bottom* disease symptoms. Apparently, the mechanism of defense within the chosen parents of the two crosses is more similar with regards to these two traits compared to the wider set of cultivars used in the development of the tests. The test on ray florets is clearly different from the other two tests. Therefore, for the *ray floret* test versus *whole inflorescence* and *bottom*, it can be envisaged that different genes are involved in resistance to Botrytis. Similarly, ten Have *et al.* (2007) also observed that resistance to Botrytis on tomato leaves and stems were distinct from each other.

QTLs mapping and analysis

QTLs detected varied between the two populations and also between the three tests. The reason for lower number of QTLs found in S population for ray floret resistance might be the small difference in *ray floret* disease score between the two parents of this population. The two F population parents, by contrast, showed a large difference in disease score for *ray floret*. Three QTL regions for *whole inflorescence* test, which were detected on different parental maps separately, co-localised on linkage group 23 for both populations. Although the QTL region still spans 20 cM on the consensus map between the most significant loci, the flanking bridge markers indicated possible overlapping of parental linkage groups and the existence of favorable alleles.

A relative high correlation between *bottom* and *whole inflorescence* was found in both populations, yet there is only one identical locus in both populations showing a significant QTL in both tests. More common QTLs for *whole inflorescence* and *bottom* might be expected given the correlation between the two tests. Apparently not all QTLs underlying the high correlation of the two tests can be detected which could be due to a lack of resolving power to detect minor QTLs for both disease indexes at the same time in a population. Environmental variance between test weeks may influence both tests in a similar way, however numbers of repetitions per week were too low to be able to study this.

In this study, several QTL with minor-effect for Botrytis resistance on gerbera inflorescences were detected. The results showed that, similar to Botrytis resistance in other plants, defense against *B. cinerea* on gerbera is quantitative and genetically complex, with probably the involvement of different infection mechanisms (Denby *et al.* 2004; Finkers *et al.* 2007a; Rowe and Kliebenstein 2008). QTLs found in our study may seem minor-effect QTLs, which are more difficult to use in breeding programs than major-effect QTLs or single resistance genes. However, several QTLs detected from separate parental linkage groups were found in

overlapping locations on the integrated map and we assume that these correlative QTLs are probably from a common gene with positive and negative alleles which can be defined as quantitative trait alleles (QTAs, Schäfer-Pregl *et al*. 1998). With the present of two positive QTAs, gerbera resistance to Botrytis increased significantly.

For two reasons we think there is potential for introgression of favorable QTL alleles in breeding to increase resistance to Botrytis in gerbera; a) phenotyping Botrytis disease is difficult and the large environmental component in testing has an downsizing effect on the total explained variance found in QTLs i.e. contributions of QTLs to genetic variance explained will be higher, b) compared to the disease tests (conditions chosen to avoid effects of incidence), the disease pressure in commercial greenhouses will be much lower and environmental conditions are less favorable for Botrytis infection (Finkers *et al*. 2007b). Under such conditions the effect of the QTLs may be much stronger.

This mapping study provides the first genetic map of gerbera and by using SNP markers derived from EST sequences (*Chapter 2*; Fu *et al*. 2016) a generally useable framework is provided which can be used for other studies as well and provides a first step in unraveling the complexity of the genetic background of resistance to botrytis in gerbera.

Acknowledgments

We are thankful for the support from the Foundation Technological Top Institute Green Genetics (3CFL030RP) and Jan van Kan for providing us with the Botrytis strain and useful advice in the start-up phase of disease testing.

Supplementary Tables and Figures

Table S3-1 Overview of parental genetic linkage maps

	SP1	Markers no.	Length (cM)	SP2	Markers no.	Length (cM)	FP1	Markers no.	Length (cM)	FP2	Markers no.	Length (cM)
LG01*	SP1_01	35	126.07	SP2_01.1	12	45.96	FP1_01.1	26	130.58	FP2_01.1	15	88.04
				SP2_01.2	22	112.34	FP1_01.2	11	45.11	FP2_01.2	9	37.91
LG02	SP1_02	26	102.53	SP2_02	20	108.2	FP1_02	30	95.65	FP2_02.1	4	11.9
										FP2_02.2	10	37.05
LG03	SP1_03.1	25	50.58	SP2_03.1	10	23.41	FP1_03.1	6	46.41	FP2_03	18	89.63
	SP1_03.2	5	7.74	SP2_03.2	3	-	FP1_03.2	3	6.95			
LG04	SP1_04	18	107.72	SP2_04.1	19	65.21	FP1_04	19	80.33	FP2_04.1	2	3.2
				SP2_04.2	3	5.83				FP2_04.2	2	3.08
										FP2_04.3	6	40.36
										FP2_04.4	5	53.58
LG05	SP1_05	23	42.07	SP2_05	20	61.36	FP1_05	12	64.66	FP2_05.1	12	45.8
										FP2_05.2	3	0.78
LG06	SP1_06	19	62.53	SP2_06	12	22.95	FP1_06	18	77.31	FP2_06	3	5.55
LG07	SP1_07	19	24.74	SP2_07	17	34.17	FP1_07	15	53.08	FP2_07	11	35.27
LG08	SP1_08	15	59.67	SP2_08	13	61.37	FP1_08	18	53.03	FP2_08	18	60.05
LG09	SP1_09.1	14	29.47	SP2_09	10	56.58	FP1_09	17	92.44	FP2_09	7	30.94
	SP1_09.2	2	18.29									
LG10	SP1_10	17	81.09	SP2_10	7	26.05	FP1_10.1	9	29.45	FP2_10	12	43.82
							FP1_10.2	9	33.05			
LG11	SP1_11	11	39.28	SP2_11	12	47.78	FP1_11	16	57.19	FP2_11	13	26.8
LG12	SP1_12.1	10	14.94	SP2_12.1	13	13.13	FP1_12	26	50.92	FP2_12	26	83
	SP1_12.2	8	15.78	SP2_12.2	6	8.79						
LG13	SP1_13	8	9.87	SP2_13	13	51.7	FP1_13	9	51.6	FP2_13	7	48.91
LG14	SP1_14.1	5	10.97	SP2_14	12	52.11	FP1_14.1	4	3.87	FP2_14	10	66.21
	SP1_14.2	5	8.3				FP1_14.2	3	22.15			
LG15	SP1_15.1	5	19.53	SP2_15	14	43.39	FP1_15	17	54.54	FP2_15	6	39.75
	SP1_15.2	3	9.69									
LG16	SP1_16	7	54.2	SP2_16	12	7.3	FP1_16	10	63.41	FP2_16	-	-
LG17	SP1_17	2	10.06	SP2_17.1	2	32.48	FP1_17	-	-	FP2_17	9	46.45
				SP2_17.2	3	-						
LG18	SP1_18	10	31	SP2_18.1	8	57.07	FP1_18	17	98.34	FP2_18	14	95.26
				SP2_18.2	3	0.82						
LG19	SP1_19	9	46.05	SP2_19	5	24.56	FP1_19	6	73.13	FP2_19	5	19.55
LG20	SP1_20	7	37.26	SP2_20	4	23.02	FP1_20	8	49.71	FP2_20	-	-
LG21	SP1_21	-	-	SP2_21	11	55.46	FP1_21.1	10	18.24	FP2_21	13	45.72
							FP1_21.2	6	0.84			
LG22	SP1_22.1	2	0.67	SP2_22	5	26.42	FP1_22	11	62.62	FP2_22	10	53.36
	SP1_22.2	2	12.59									
	SP1_22.3	2	2.3									
LG23	SP1_23	12	61.14	SP2_23	-	-	FP1_23	14	83.36	FP2_23	6	24.74
LG24	SP1_24	3	56.34	SP2_24	2	36.15	FP1_24	-	-	FP2_24	3	5.06
LG25	SP1_25	-	-	SP2_25	-	-	FP1_25	-	-	FP2_25	-	-
Total		329	1152.5		293	1103.6		350	1498		259	1141.8
Total number of LGs	30			29			27			28		
Map density (average cM/marker)	3.5			3.77			4.28			4.41		
Number of unlinked/ungro-uped markers	6			4			5			15		

*. LG is Linkage Group.

Table S3-2 Overview of integrated map of the populations

	S_LG	Markers no.	Length (cM)	F_LG	Markers no.	Length (cM)
LG01*	S_LG01	53	102.3	F_LG01.1	40	154.9
				F_LG01.2	11	45.1
				F_LG01.3	8	37.9
LG02	S_LG02	34	106.8	F_LG02.1	32	91.9
				F_LG02.2	7	37.4
LG03	S_LG03.1	27	50.5	F_LG03.1	18	89.5
	S_LG03.2	5	7.5	F_LG03.2	4	33.8
LG04	S_LG04	33	49.1	F_LG04.1	18	80.3
				F_LG04.2	5	53.6
				F_LG04.3	3	40.2
LG05	S_LG05	30	54.0	F_LG05.1	12	45.5
				F_LG05.2	8	65.2
				F_LG05.3	3	0.8
LG06	S_LG06	28	64.5	F_LG06	18	77.3
LG07	S_LG07	20	37.6	F_LG07	21	50.3
LG08	S_LG08	21	63.1	F_LG08	24	61.3
LG09	S_LG09	20	59.8	F_LG09	22	92.0
LG10	S_LG10	19	80.9	F_LG10.1	15	42.2
				F_LG10.2	9	29.5
LG11	S_LG11	19	46.9	F_LG11	21	50.7
LG12	S_LG12.1	19	17.3	F_LG12	42	78.7
	S_LG12.2	10	20.8			
LG13	S_LG13	16	52.9	F_LG13	11	69.4
LG14	S_LG14	17	55.3	F_LG14.1	10	66.2
				F_LG14.2	3	3.9
				F_LG14.3	2	22.9
LG15	S_LG15	14	43.1	F_LG15	19	60.4
LG16	S_LG16.1	11	7.3	F_LG16	(10)	-
	S_LG16.2	5	54.5			
LG17	S_LG17	(6)	-	F_LG17	(9)	-
LG18	S_LG18.1	10	30.2	F_LG18	23	46.5
	S_LG18.2	8	57.1			
LG19	S_LG19.1	9	46.0	F_LG19	8	39.6
	S_LG19.2	4	24.9			
LG20	S_LG20	8	36.8	F_LG20	(8)	-
LG21	S_LG21	(11)	-	F_LG21.1	10	46.5
				F_LG21.2	7	59.0
LG22	S_LG22.1	5	24.4	F_LG22	15	18.3
	S_LG22.2	2	2.2			
	S_LG22.3	2	2.3			
LG23	S_LG23	(12)	-	F_LG23.1	17	84.3
				F_LG23.2	6	0.8
LG24	S_LG24	3	56.3	F_LG24	(3)	-
LG25	S_LG25	-	-	F_LG25	-	-

*. LG is Linkage Group.

Table S3-3 Overview of the genetic consensus map of gerbera based on the integrated maps of both populations

Linkage Group (LG)	Length (cM)	Markers no.	Density	Gaps (>15cM)
LG01	134.44	78	1.72	0
LG02	108.38	48	2.26	0
LG03	97.35	45	2.16	1
LG04	117.93	38	3.1	1
LG05	58.52	34	1.72	1
LG06	68.48	32	2.14	0
LG07	42.88	28	1.53	0
LG08	59.58	31	1.92	0
LG09	40.96	31	1.32	0
LG10	74.78	32	2.34	0
LG11	59.33	28	2.12	0
LG12	73.11	46	1.59	1
LG13	57.97	22	2.64	0
LG14	59.68	25	2.39	1
LG15	42.83	22	1.95	0
LG16	61.54	17	3.62	1
LG17	46.45	9	5.16	1
LG18	90.4	31	2.92	0
LG19	60.53	16	3.78	2
LG20	41	12	3.42	1
LG21	53.44	20	2.67	1
LG22	56.6	17	3.33	1
LG23	90	22	4.09	2
LG24	5.06	3	1.69	0
Average	1601.24	687	2.57	

Table S3-4 The correlation of disease indexes in both populations

Population S		S_*B*	S_*WI*	S_*RF*	Population F		F_*B*	F_*WI*	F_*RF*
S_*B*	Pearson Correlation	1	0.83**	0.11	F_*B*	Pearson Correlation	1	0.67**	-0.01
	Sig. (2-tailed)		0	0.09		Sig. (2-tailed)		0	0.93
S_*WI*	Pearson Correlation	0.83**	1	0.08	F_*WI*	Pearson Correlation	0.67**	1	0.05
	Sig. (2-tailed)	0		0.18		Sig. (2-tailed)	0		0.46
S_*RF*	Pearson Correlation	0.11	0.08	1	F_*RF*	Pearson Correlation	-0.01	0.05	1
	Sig. (2-tailed)	0.09	0.18			Sig. (2-tailed)	0.93	0.46	

Table S3-5 The board-sense heritability of the three disease tests from two populations

H^2	*Whole inflorescence*	*Bottom*	Ray Floret
S population	28.24%*	32.60%	38.76%
F population	38.83%	46.41%	48.70%

*one-way ANOVA (analysis of variance) test by SPSS (version 21) was used to measure the between group variation and the within group variation. Between group variation (variation among individuals within a population) is considered as genetic, and within group variation (variation between clones of offspring) is due to the environmental factors. The board-sense heritability (H^2) = between group variation/total variation×100%

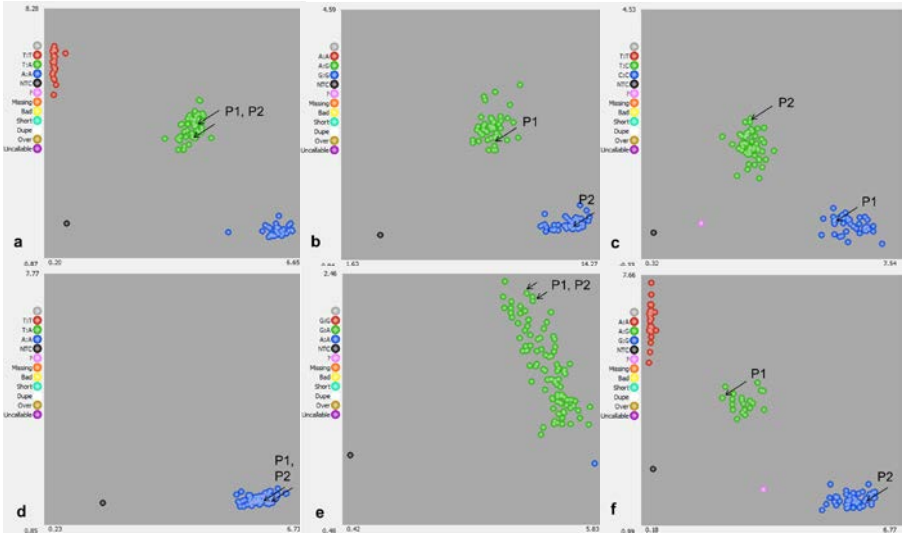

Fig. S3-1. Examples of different segregation types for SNP markers visualized by SNPviewer. a: the parental genotypes are T:A indicating a <hkxhk> marker, and the progeny genotypes are T:T (n=66), T:A (n=140) and A:A (n=69) segregating in the ratio 1:2:1. b: the first parent genotype is A:G, and the second is are G:G indicating a <lmxll> marker. The progeny genotypes are A:G (n=135) and G:G (n=141) segregating in the ratio 1:1. c: the first parent genotype is C:C, and the second is are T:C indicating a <nnxnp> marker. The progeny genotypes are C:C (n=133) and T:C (n=134) segregating in the ratio 1:1. d: not polymorphic marker. Two parents and all progeny are in the same A:A cluster without segregation. e: not-fitting segregation. The parental genotypes are G:A, and the progeny should be segregating in 1:2:1 ratio but in fact they are scattered and no clear clusters can be defined. f: non-Mendelian segregating marker with putative null-allele. Parents seem to be A:G and G:G, whereas progeny show clusters A:A, A:G and G:G with 74, 69 and 133 individuals respectively indicating that the true genotype of parent P2 is likely G: Ø.

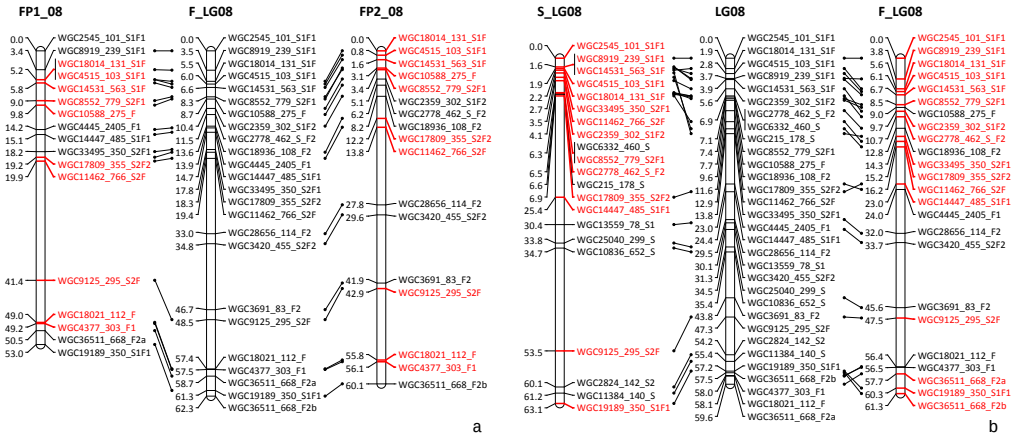

Fig. S3-2. a: Parental and integrated map of LG08 from F population. Identical markers are linked. The anchor markers (<hkxhk> type loci) are highlighted in red. b: Integrated and consensus map (in the middle) of LG08 of the two populations. Bridge (common) markers of S and F population are indicated in red.

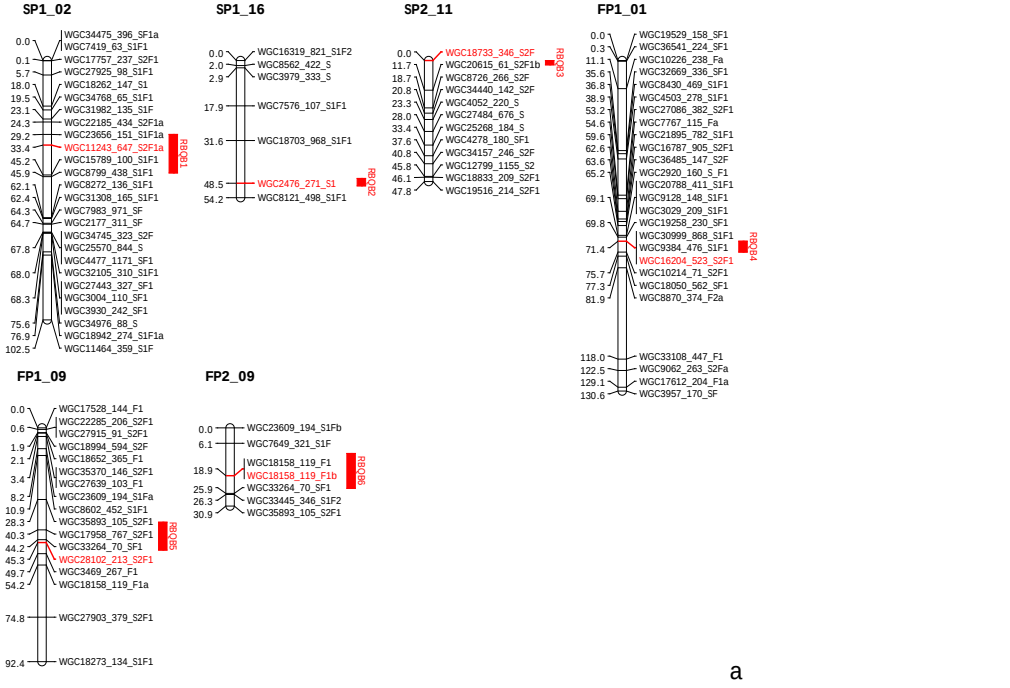

Fig. S3-3 The location of all QTLs. a: QTLs locations in parental linkage maps for *bottom* test. b: QTLs locations in parental linkage maps for *ray florets* test. c: QTLs locations in parental linkage maps for *whole inflorescence* test. Red bars represent the LOD 95% confidence intervals for QTL peaks and red markers represent the QTL peak location.

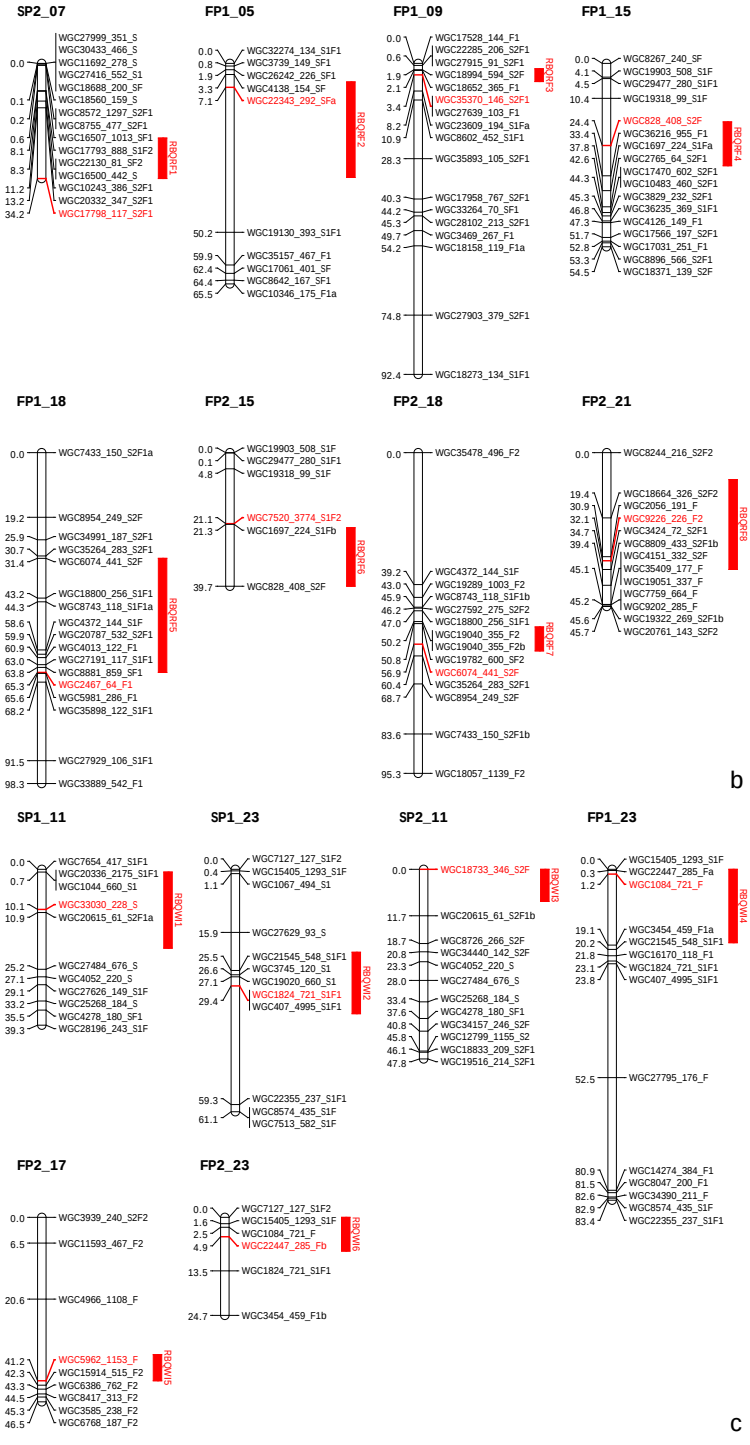

Fig. S3-3 (continued)

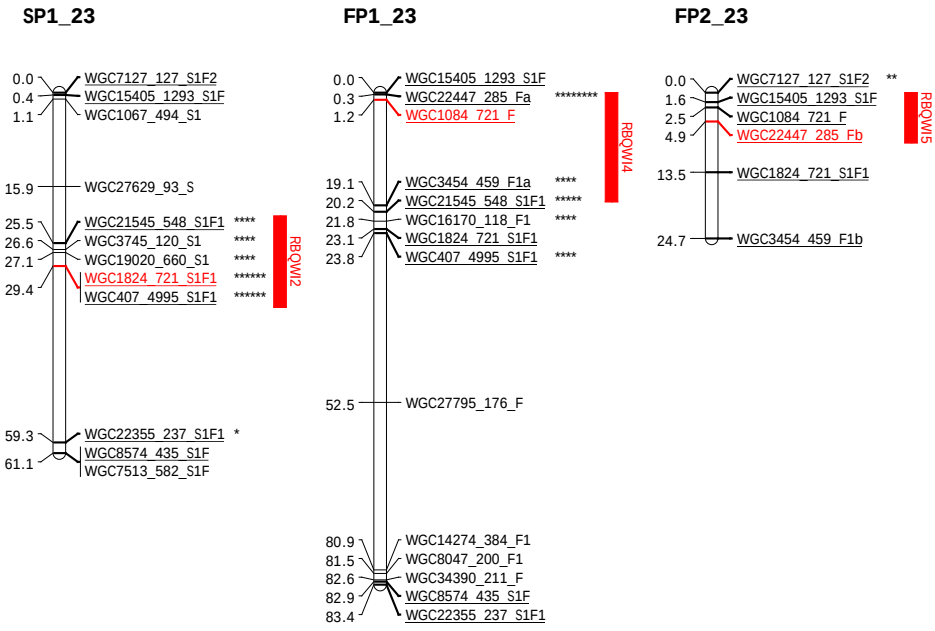

Fig. S3-4 The location of QTLs (RBQWI2, RBQWI4 and RBQWI6) on parental linkage maps. The bars in red color show the position of the QTL for *whole inflorescence in* three different parental linkage maps and **, ****, ****** indicated significant at P=0.05, 0.005, 0.0005 of Kruskal-Wallis test. The highest marker QTL is in red marker and common markers are underlined.

Chapter 4 The use of a candidate gene approach to arrive at Botrytis resistance in *Gerbera hybrida*

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This chapter has been published partly in Acta Horticulturae 1087, 461-466.

Abstract

Candidate genes (CG) described in the literature that were found to affect Botrytis resistance were mapped on gerbera linkage maps using a rapid, low-cost and high-throughput platform for SNP genotyping. With this method we could use high-resolution melting analysis to screen and map potential functional genes in a highly heterozygous ornamental plant species without genome information. In total, 29 candidate genes were mapped on previously constructed linkage maps in which several QTLs for botrytis resistance have been found. Four CGs were mapped in the previous identified QTLs intervals and three co-localized with QTLs. The analysis of allelic diversity on CGs which are involved in phenotypic variation demonstrated the heterozygosity of gerbera. Involved CGs were up-regulated after Botrytis inoculation. The candidate gene approach in this study is a useful tool to identify possible causal genes and can make a contribution to gerbera improvement and also for understanding the molecular mechanisms of Botrytis resistance in gerbera.

Keywords

QTLs, gerbera gray mold, MAS, allele

Introduction

Gerbera is an economically important ornamental plant which is mainly used as cut flower. In pre-harvest and post-harvest processes, high relative humidity regularly occurs often leading to gray mold infections which cause major losses in gerbera production. Gerbera gray mold may be suppressed by cultural management and fungicide application during production in greenhouses, yet prevention and control of this disease in transportation or after sales is difficult since fungicide cannot be applied in the post-harvest process. Moreover, flowers are subjected to the active process of senescence once harvested and thus vulnerable to pathogen attack. Infected gerbera will be thrown away or have a shortened vase life. Breeding companies will receive complaints from growers when cultivars are very susceptible and customers may buy other cut flowers as a substitute when experience with vase-life is disappointing. Therefore, breeding gerberas that are resistant to gerbera gray mold should be an important goal of all breeding companies.

Traditionally, breeding in gerbera is conducted using F1 crossing and selection on generations after generations in several breeding programs. Although this traditional method is considered time and labour consuming, it is still widely in use for ornamental plant breeding since lots of horticultural characteristics of ornamental plants, like flower color, flower pattern and shape, as well as agronomical traits as multiplication rate and flower production are segregating due to the high variation in parental genotypes and can be easily selected as it are partly visible traits (color, pattern, shape) and partly easily quantifiable traits (multiplication, production). However, selection for genetic complex quantitative traits, which are often also influenced by environment and present themselves in continuous distributions (Paterson *et al*. 1988), is very difficult using only visual selection schemes. Thus, more and more molecular DNA markers have been exploited for trait selection due to their abundance and independence of environmental influences (Collard *et al*. 2005). DNA markers which are linked to a trait of interest enable marker assisted selection (MAS) in breeding and MAS has been widely accepted as a potential tool for crop improvement.

Resistance to *B. cinerea*, the cause of gerbera gray mold, is a quantitative trait which means that the resistance requires contributions of multiple loci to reduce disease severity (Mengiste *et al*. 2003; Poland *et al*. 2009). QTL mapping of quantitative resistance to Botrytis in Arabidopsis and tomato has revealed numerous small-effect QTLs affecting the disease (Finkers *et al*. 2008; Finkers *et al*. 2007a; Finkers *et al*. 2007b; Rowe and Kliebenstein 2008; ten Have *et al*. 2007). Similarly, also in gerbera (*Chapter 3*; Fu *et al*. 2017), we detected 20 QTLs and explained variance of most detected QTLs were below 10%. The results reflected the complex response of plants to Botrytis infection.

Selection using several small-effect QTLs in breeding programs might limit the efficiency of MAS. Genetic linkage between random DNA markers and a QTL allele with the trait of interest, especially when the QTL region is wide, leaves the possibility that linkage can be broken by recombination (Andersen and Lübberstedt 2003). Furthermore, in cross-pollinating highly diverse ornamental species, QTL alleles are likely more random associated with marker alleles in the germplasm (Kumar *et al*. 2000). The observed marker alleles which were detected in a created single F1 outbred population might not be directly applied to predict the association with the allele of a QTL in another population for practical breeding programs

(Liu *et al.* 2011; Williams 1997). Thus, developing an alternative strategy to increase selection efficiency in gerbera breeding is required.

For ornamental crops, with increasing EST sequence data availability the use of a candidate gene (CG) approach was recently recommended (Arens *et al.* 2012; Collard *et al.* 2005; Debener 2009; Pflieger *et al.* 2001; Smulders *et al.* 2011). A CG approach is based on the hypothesis that homologs of known functional genes from other species could control similar traits of interest in the focus species (Collard *et al.* 2005; Pflieger *et al.* 2001). Through co-localization of CGs with QTLs, promising genes underlying the QTLs could be quickly pinpointed (Decroocq *et al.* 2005; Gardner *et al.* 2016; Kawamura *et al.* 2011; Norelli *et al.* 2009; Pflieger *et al.* 2001) and used for understanding molecular mechanisms of interested traits (Smulders *et al.* 2011). Developing molecular markers which are derived directly from polymorphic loci in functional candidate genes themselves will be in complete linkage with the causal genes (Andersen and Lübberstedt 2003) and would be more efficient than using random DNA markers in marker-assisted selection.

To develop candidate gene markers, understanding the resistance mechanism for this necrotrophic pathogen is essential. The infection process of *B. cinerea* is widely studied and usually described by the following stages: penetration of the host epidermal cell surface, primary lesion formation, lesion expansion/tissue maceration and sporulation (Choquer *et al.* 2007; Jarvis 1962; van Kan 2006). During the infection, a series of genes are involved in plant disease resistance against the necrotrophic pathogen. The first category of genes are those involved in cell wall biosynthesis or affecting cell wall composition. For a host plant, the cell wall is the first barrier to pathogen invasion. As a necrotrophic pathogen, *B. cinerea* can secrete an arsenal of enzymes to decompose plant cell wall polysaccharides in order to facilitate penetration (Zhang 2013). The pathogen prefers to colonize plant species whose cell walls are rich in pectin while plants with low pectin contents are considered poor hosts for Botrytis (van Kan 2006).

The second category of genes is those involved in regulation of signaling pathways. To modulate induced defense responses, plants have developed a complex system of signals to activate defense response. Functional deficient plants in the JA (coi1, jar1, and iop1), ET (ein2, ein3) and SA (nahG) signal transduction pathways have an altered Botrytis interaction in Arabidopsis (AbuQamar *et al.* 2006). A large number of regulatory genes encoding JA, ET and SA response transcription factors, e.g. R2R3MYB, WRKY33, MYC and ORA59, also influence the resistance to Botrytis (Lorenzo *et al.* 2004; Mengiste *et al.* 2003; Pre *et al.* 2008; Zheng *et al.* 2006). An abscisic acid deficient mutant of tomato sitiens (sit) has increased resistance to *B. cinerea* (Asselbergh *et al.* 2007; Audenaert *et al.* 2002) and indicated that ABA is also involved in Botrytis resistance.

Meanwhile, to resist against microbial attack and detoxify the low-molecular-weight phytotoxic compounds produced by *B. cinerea*, plants can produce a remarkably diverse array of secondary metabolites and the genes involved in the often complex pathways of plant natural product biosynthesis (Dixon 2001) form a third category of genes. Inoculating multiple camalexin-deficient Arabidopsis mutants with several *B. cinerea* isolates confirmed that camalexin plays a major role in resistance (Kliebenstein *et al.* 2005). *B. cinerea* lesion outgrowth has been shown to be inhibited by accumulation of camalexin in Arabidopsis (Rowe and Kliebenstein 2008). Asteraceae are renowned for their ability to

produce a wide range of unique secondary metabolites including: monoterpenes, diterpenes, triterpenes, polyacetylenes, flavonoids, phenolic acids, coumarins and pyrrolizidine alkaloids (Calabria *et al*. 2009). Enzymes or regulatory genes involved in those phenylpropanoid compound synthesis pathways can affect the synthesis of antimicrobial plant natural products that are considered potential weapons against botrytis.

In this study, we have developed SNP markers targeted on candidate genes which may affect plant resistance to Botrytis and mapped them using two gerbera populations that were previously used to perform Botrytis resistance QTL mapping. The use of a candidate gene approach in gerbera aims to turn markers which are linked with QTLs to markers in potential CGs and eventually leading to the possibility for selection of functional gene alleles. In this research, we obtain a preliminary understanding of the allele diversity in gerbera through the allele distinction from several CGs and provide a number of loci that may be further studied for resistance to botrytis in gerbera under glasshouse production situations.

Materials and Methods

Mapping populations and Botrytis disease test

The plant materials used were two gerbera populations already used for QTL mapping previously (*Chapter 3*; Fu *et al*. 2017). In short, population Schreurs (abbreviated as S, with 276 progeny) and Florist (abbreviated as F, with 270 progeny) were produced by crosses between heterozygous hybrids with different resistance levels for *B. cinerea* susceptibility. Phenotypic data available for these two F1 progenies were based on three tests, *whole inflorescence*, *bottom* (of disc floret) and *ray floret* respectively with scores ranging from 0 (no symptom) to 5 (very serious) as described by Fu *et al*. (2017) and in *Chapter 3*.

Genome DNA extraction

Young leaf material from all progeny of the two populations and four parents was sampled by leaf punches. After dehydrating with silica gel at room temperature over 48 hours, three 6mm leaf discs per sample were grounded to powder using a TissueLyser II (Qiagen). Genomic DNA was isolated following the DNA isolation protocol of Fulton *et al*. (1995) with some adaptations. In short: 750µl of fresh-prepared microprep buffer with RNAse 100µg/ml was added to 2ml-eppendorf tubes with ground leaf disc powder and after mixing this was incubated at 60°C for at least 60 min. After that, 800µl chloroform:isoamyl (24:1) was added and mixed well. tubes were centrifuged at 13,000 rpm for 5 min and 500µl of the upper aqueous phase was pipetted into a new Eppendorf tube to which an equal volume cold isopropanol was added. Mix by inverting tubes repeatedly until DNA precipitates and centrifuge tubes at 13,000 rpm for 5 min. Wash the DNA pellet with 500µl 70% ethanol twice and dry DNA pellet by leaving tube upside down on paper towel for approximately 1h. Re-suspend DNA pellet in TE-4. DNA quality and quantity of all samples were checked on 1% TBE agarose gel and also by Nanodrop.

Candidate gene identification and marker development

Candidate genes from Arabidopsis or other crops that are considered involved in Botrytis resistance were selected based on published literature and sequences were obtained from NCBI. A Gerbera EST database

containing 36,770 assembled contigs which were used for detecting SNPs for linkage mapping in the two described populations was available from *Chapter 2* and Fu *et al.* (2016). Candidate genes from literature were run against this gerbera ESTs database using tBLASTn in the blast-2.2.28+ program (ftp://ftp.ncbi.nih.gov/blast/executables/blast+/).

From the alignments between the candidate genes and contigs from the gerbera EST database, the contig with the highest similarity and sequence homology was selected (minimum threshold E-value < 1e-15, bits-score > 50 and identity > 30%). SNP identification was performed as described in *Chapter 2* and Fu *et al.* (2016) using QualitySNPng (Nijveen *et al.* 2013). To avoid SNP markers in exon/intron boundaries, Open Reading Frames (ORFs) were identified in the selected best-hit sequences using the ORF data of the query genes from literature.

To readily group the offspring, we mainly focused on SNPs heterozygous in just one parent. Primers were selected to generate fragments between 80 to 120bp using Primer3 online (Koressaar and Remm. 2007; Untergasser *et al.* 2012). Intron spanning primers were avoided if possible.

Genotyping using HRM analysis

Primers were first tested on the four parents and randomly selected four offspring to filter out the primers with no amplification or multiple bands on gel. PCR reactions were conducted using a DNA Engine Thermal Cycler PCR machine (Bio-RAD, USA). The 10-µl reaction mixtures used for Lightscanner PCR reaction included: 2µl genomic DNA (5ng/µl), 1µl forward and reverse primer mix (10µM), 0.4µl dNTP mix (5mM), 0.1µl Phire Hot Start II DNA Polymerase (5U), 2µl 5× Phire Reaction Buffer (containing 7.5 mM MgCl2), 1µl LCGreen Plus + and 3.5µl Milli-Q water. PCR reaction mixes were overlaid with 10µl of mineral oil in 96-well plates covered with sealing film. PCR was initiated at 98°C for 30s, then 40 cycles of 5s at 98°C, 5s at 60°C and 10s at 72°C, and one hold of 60s at 72°C, 30s at 94°C, 30s at 25°C, stored (hold) at 10°C at last.

After PCR reaction, PCR products from each genotype were screened by analysing melting curve in the LightScanner (Idaho Technology) to check SNP marker polymorphisms. Samples with initial fluorescence signals below 800 were discarded. Raw melting data were normalized to have the same start and end fluorescence baseline and shift curve using the default setting. Only when the melting curve of the two parents and selected individual could be clearly distinguished, the marker will be used for whole population genotyping.

Statistical analysis and candidate genes mapping

Offspring could be separated based on the shapes of the melting curves due to the allelic variation of the amplified CGs fragment. The segregation ratios were tested by χ^2 (1:1) statistics. The mean of disease scores (from *bottom*, *ray floret*, and *whole inflorescence* test) between the two allelic groups were tested for significance ($P<0.05$) by *t*-test using SPSS software (Version 21). Mapping was performed using JoinMap®4 (van Ooijen 2006). Candidate gene map positions were mapped using the linkage maps that were previously constructed for Botrytis resistance QTL mapping (*Chapter 3*; Fu *et al.* 2017). Similarly, QTL

analyzes using the candidate gene markers added to the maps were repeated as described earlier (*Chapter 3*; Fu *et al*. 2017).

Allele identification of candidate genes

For promising candidate genes, which are co-located with previous detected QTLs and showing statistical difference in Botrytis scores between groups, allelic diversity was assessed among the four parents. To assess haplotype information for CGs in the parents, the existing transcriptome data (*Chapter 2*; Fu *et al*. 2016) were supplemented by Sanger sequencing to obtain longer and if possible full length gene sequence information. Primers for amplifying candidate gene fragments for Sanger sequencing were designed by Primer3 online. Fragments were amplified from genomic DNA. PCR conditions were 95°C for 30s, then 35 cycles of 30s at 95°C, 30s at annealing temperature (annealing temperature based on Tm of primer pair) and 1min at 72°C, followed by 10min at 72°C.

PCR products showing single bands and expected sizes were purified using the QIAquick PCR Purification Kit (Qiagen). Purified PCR products were checked on 1% agarose gels again to guarantee the quality and cloned into the pGEM®-T Easy Vector System I following the recommended instruction (Promega). Transformation of recombinant vector into E. coli DH5α™ competent cells (Invitrogen) was performed by 45 second heat-shock in a water bath at 42°C and chilling on ice for 5 min. To cells 1ml LB broth was added after which cell were incubated for 1h at 37°C. Of each transformation culture, 100uL were plated onto LB plates containing ampicillin (100 mg/mL) with 100μL IPTG (100mM) and 20μL X-Gal (50 mg/mL) on the surface. Plates were incubated overnight at 37°C and seven white single colonies (and one blue colony on each plate as control) were selected for colony PCR to quickly screen for recombinant clones based on fragment size. Confirmed white colonies were cultured overnight at 37°C with shaking in LB liquid medium including ampicillin (100 mg/mL). Plasmid DNA was isolated by QIAprep® Spin Miniprep Kit (Qiagen) for Sanger sequencing.

B. cinerea inoculation

B. cinerea (strain B05.10 provided by Dr. Jan van Kan, Wageningen University & Research) was grown for one week at 25°C in the dark on potato dextrose agar (PDA) medium after which conidia were transferred onto fresh PDA medium and grown until mycelia covered the whole Petri dish surface and sporulation started. Spores were collected by pouring 5 ml of 0.1% Tween 80 solution on each plate detaching all aerial mycelium and conidiophores from the agar by a glass rod. The spore suspension was filtered through three thin layers of cheesecloth and other 5 ml 0.1% Tween 80 solution was added to rinse the surface of the agar and remove any remaining spores. Spore concentration was calculated by Fuchs-Rosenthal Counting Chamber (0.2mm depth).

A concentration of 1×10^7/ml spore suspension was prepared as stock and diluted with 3% potato dextrose to a working solution 5×10^5/ml. Ray floret s from the four gerbera parents were sampled when the first circle of disk florets shows pollen development (stage 9 according to Helariutta *et al*. 1993 and Laitinen *et al*. 2007). The candidate genes expression analysis was only performed on ray florets due to the limited availability of flowers. To induce Botrytis defense-related gene expression ray florets were placed in plastic

containers with wet filter paper, three 2µl-droplets spore suspension (one droplet for FP2 due to its relative small ray floret size) were inoculated on the upper face of each single ray floret after which containers were put in plastic bags to maintain a high humidity at room temperature. Inoculated ray florets were collected at different time points (0 hpi, 6 hpi, 12 hpi, 24 hpi also 36 hpi, 48 hpi, 72 hpi at initial experimental setup) whereas mock samples were taken at 24hpi only. Single ray florets (for FP2 three ray florets) were put in an Eppendorf tube with two metal balls frozen in liquid nitrogen and stored at -80 0C upon further gene expression analysis.

Candidate gene expression analysis on ray florets

For RNA isolation, infected ray florets were grinded to powder using a TissueLyser II (Qiagen) and RNA was extracted using Trizol (Life Technologies) according to the manufacturer's protocol with modification (chloroform washing twice). After RNA extraction, the quality and quantity of RNA samples were checked on 1% agarose gel and by NanoDrop. After DNase treatment, 1µg RNA was used for cDNA synthesis according to the iScript™ cDNA Synthesis Kit (Bio-Rad). For Real time quantitative PCR, 10µl reaction mix was used, containing 2µl cDNA template (~50ng/µl), 5µl 2x iQ™ SYBR® Green supermix (Bio-Rad), 0.3µl forward and reverse primers (10µM) and 2.7µl Milli-Q water. Optimized RT-qPCR was initiated at 95°C for 3min, followed by 40 cycles of 15s at 95°C, 1min at 60°C, and one hold of 10s at 95°C, 5s at 65°C and increased to 95°C by 0.5°C for generating a final melting curve. The reference gene (GAPDH) was used in quantitative real time PCR (qRT-PCR) using the primer sequences as described by Deng *et al.* (2014). Recommended fragment size for SYBR green qRT-PCR is around 100bp, thus the primers for candidate genes expression analysis were the same used for LightScanner HRM analysis. Reference and candidate genes were always run together on the same 96-well plates with three biological replicates and three technical replicates.

Relative quantification method (Livak and Schmittgen 2001) was used to analyze data from real-time quantities PCR. The change in expression of candidate (target) gene was normalized to reference gene expression relative to reference group (sample at time point 0 in this experiment) and presented as fold change. The equation of this method is *fold change* = $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT=(C_{T,Target}- C_{T,GAPDH})_{Time\_X}-(C_{T,Target}- C_{T,GAPDH})_{Time\_00}$.

Results

Selection of candidate genes and homologues of gerbera

A list of 71 genes was identified from literature as potential candidate genes for Botrytis resistance in gerbera (Suppl. Table S4-1). All these candidate genes are belonging to genes affecting cell wall composition, signal transduction or secondary metabolism. Most of the candidate genes with confirmed involvement in Botrytis resistance were derived from the well-studied model plant Arabidopsis, and also from two major host crops for Botrytis, grapevine and tomato. Some of the genes from the phenylpropanoid and flavonoid biosynthetic pathway from the Asteraceae family, like sunflower, Artemisia and gerbera itself, are also included in the CGs list as they are important for flavonoid phytoalexin accumulation during Botrytis infection.

The corresponding homologous sequences of gerbera for all potential CGs sequences were searched in the Gerbera ESTs database (*Chapter 2*; Fu *et al.* 2016). At least one hit could be found for all CGs in the Gerbera ESTs, the contigs with the highest hit score, lowest e-value and highest identity percentage were identified as the homologous CG sequence (Suppl. Table S4-1). Over half of the identified homologs (42/71) were with an e-value < 1E-180 and the identities of most of the contigs (64/71) are above 50%. Frames showing the longest ORF were used for re-BLAST analysis. Identified gerbera CG homologs re-BLAST (blastp) against the NCBI database, showed best hits to coding sequences with even higher hit scores and identical annotations to the original query CG (Suppl. Table S4-1). In general, almost full length gerbera ORFs were identified based on the coding regions of other species.

Choice of primers for HRM

Primers were designed to flank a targeted SNP in the CG ORF region with expected PCR products sizes of 80-150 bp and only primers amplifying single band amplicons were chosen for further analysis (Suppl. Table S4-2). Parents as well as a number of randomly selected offspring were scanned on the Lightscanner to check the polymorphism by analysing the melting curve.

The four possible outcomes of HRM output in primer polymorphism detection in parental samples are illustrated in Fig. 4-1. In Fig. 4-1a (contig12863/*ghPER21* gene); the PCR product size of CGs fragment was exactly as expected and the two parents as well as selected offspring can be clearly grouped via the HRM showing the presence of a single SNP marker. The PCR product size of *ghPgD* (contig28711, Fig. 4-1b) was as expected but the two parental and selected offspring melting curves overlaid indicating the absence of polymorphism. For contig28693/*ghLOX* gene (Fig. 4-1c), a single band was visible on the gel yet the size was higher than expected and the melting curve showed a swing line which indicated the presence of more than one SNP in this amplicon. In Fig. 4-1d (contig29198/*ghDELLA* gene), multiple bands (and also potential multiple polymorphic loci) existed that will not result in a clear grouping result for HRM scanning analysis. Only primer pairs resulting in clear grouping as is the situation in Fig. 4-1a were used for further HRM analysis.

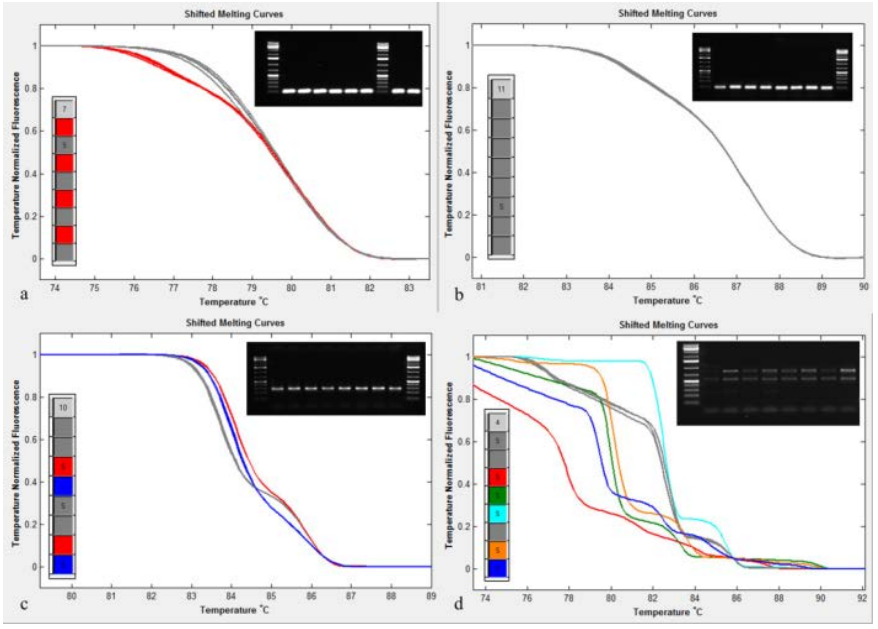


Fig. 4-1. Four possible cases of amplicon HRM results in parent samples. a: (contig12863/*ghPER21* gene): the two parents can be clearly grouped via the HRM result; b: (contig28711/*ghPgD* gene): the PCR product size was as expected between 75-200bp on the agarose gel, but the overlapping parental melting curves cannot be used for offspring genotyping; c: (contig28693/*ghLOX* gene): Single band on the gel and the size was higher than expected, but the melting curve showed a swing line which means at least two SNPs in this region.d: (contig29198/*ghDELLA* gene): Multiple bands and also possible multiple polymorphic loci existed are insufficient for HRM analysis. And the top right of each plot is the PCR product of the parental samples on the agarose gel.

Genotyping by HRM analysis, mapping and co-localization

Primers designed on 29 CGs with clear grouping results in the parents test (Fig. 4-1a) were used for genotyping the whole population. PCR products of homoduplex samples (from homozygous offspring) and heteroduplex samples (from heterozygous offspring) can be easily distinguished by the change in the normalized melting curve shape (Suppl. Fig. S4-2). All tested CG markers could divide the mapping populations into two clear groups. After χ^2 statistic test, all the χ^2 values are less than 3.84 (95% confidence level with 1 degree of freedom) and indicated a 1:1 segregation ratio (Table 4-1). The phenotypic means of Botrytis infection disease score on *whole inflorescence*, *bottom* and ray floret, respectively, between the separated two groups with different alleles from these CG markers were tested by *t*-test. Seventeen CG markers showed a significant difference in at least one test at the level of ≤ 0.05, of which nine showed an even higher threshold value; ≤ 0.01 (*gh2-PS*, *ghCHI*, *ghERF*, *ghFDH*, *ghPER62*), ≤ 0.001 (*ghPG1*, *ghPG9*, *ghsit*, *ghSS*). Interestingly, the CG marker for CHI showed a highly significant association (P≤ 0.01) on both populations with variation in Botrytis disease score for *whole inflorescence* (Table 4-1).

Table 4-1 Overview of mapped candidate genes, x^2 test and *t*-test results

Candidate gene	hit	CGs marker	x^2(1:1)	mapped on	*t*-test of the two genotypic groups on		
					Bottom	*Ray Floret*	*Whole inflorescence*
gh2-PS	contig11873	2_PS_11873_814	0.73	FP1_23	0.0052	0.7792	0.0528
ghACS	contig17688	ACS_17688_842	2.09	SP2_24	0.5025	0.2891	0.7043
ghAS	contig1926	AS_1926_I2	0.39	SP1_08	0.4974	0.6818	0.4380
ghCHI	contig22447	CHI_22447_421	0.08	FP2_23	0.1338	0.9597	0.0043
ghCHI	contig22447	CHI_22447_421	0.49	SP1_23	0.0506	0.3098	0.0090
ghCHS1	contig29143	CHS_29143_1582	0.04	SP1_17	0.0723	0.9820	0.0389
ghcutin	contig4918	cutin_4918_3081	3.06	SP2_02	0.0141	0.3986	0.2436
ghDND	contig29068	DND_29068_911	2.10	FP2_04	0.4132	0.7622	0.4412
ghDND	contig29068	DND_29068_911	0.62	SP2_04	0.2133	0.2612	0.0611
ghELP2	contig2398	ELP2_2398_1249	0.02	SP1_06	0.0174	0.3721	0.2071
ghERF	contig11093	ERF_11093_229	0.93	FP2_09	0.0075	0.2194	0.0583
ghET	contig14926	ET_14926_1910	0.00	FP1_16	0.3624	0.6401	0.2525
ghFDH	contig12215	FDH_12215_1158	0.00	SP1_02	0.0041	0.9751	0.0491
ghGSL5	contig30332	GSL5_30332_2672	2.79	FP1_20	0.4063	0.7067	0.2516
ghLOX	contig13375	LOX_13375_1313	0.10	SP1_12	0.1136	0.0120	0.0258
ghMPP3	contig15525	MPP3_15525_1055	0.82	SP2_01	0.7767	0.2504	0.7008
ghMYB	contig11297	MYB_11297_481	0.17	FP2_22	0.1389	0.6366	0.4696
ghP450	contig25584	P450_25584_2562	2.43	SP2_02	0.4991	0.0699	0.7834
ghPER21	contig12863	PER21_12863_774	3.28	FP2_05	0.9363	0.6885	0.2686
ghPER21	contig12863	PER21_12863_774	0.26	SP2_05	0.8169	0.0114	0.6455
ghPER62	contig31925	PER62_31925_540	1.71	FP2_18	0.9971	0.0016	0.4303
ghPG1	contig15001	PG1_15001_1052	0.81	FP2_21	0.6880	0.0002	0.7800
ghPG1	contig15001	PG1_15001_1052	0.00	SP1_21	0.2836	0.6503	0.1400
ghPG2	contig1158	PG2_1158_857	0.08	FP2_18	0.1045	0.0214	0.5778
ghPG7	contig35500	PG7_35500_1285	0.07	SP1_06	0.2745	0.4141	0.8631
ghPG9	contig25150	PG9_25150_888	2.32	SP2_02	0.0003	0.7148	0.0236
ghPG10	contig11147	PG10_11147_3429	0.01	FP2_06	0.3194	0.1243	0.4550
ghPG10	contig11147	PG10_11147_5246	0.04	SP1_06	0.0473	0.2727	0.2633
ghPGIP1	contig17627	PGIP1_17627_1064	0.33	SP2_22	0.3155	0.3981	0.0322
ghPGIP2	contig20957	PGIP2_20957_910	0.13	FP2_08	0.8248	0.9677	0.7393
ghRD21	contig20206	RD21_20206_4286	0.47	FP1_10	0.9234	0.4332	0.6737
ghRD21	contig20206	RD21_20206_I2	0.04	SP1_10	0.0679	0.2106	0.1072
ghsit	contig19807	sit_19807_6510	1.76	FP1_05	0.6503	0.0003	0.2911
ghsit	contig19807	sit_19807_I2	0.08	FP1_05	0.2024	0.0017	0.3657
ghsit	contig19807	sit_19807_6510	1.60	SP1_05	0.0134	0.0967	0.0835
ghSR1	contig1903	SR1_1903_2688	0.16	FP1_11	0.1517	0.2194	0.5784
ghSS	contig5198	SS_5198_1668	0.80	FP2_16	0.9438	0.8962	0.6704
ghSS	contig5198	SS_5198_1668	0.10	SP1_16	0.0009	0.3052	0.0119

* CG markers are named as: gene name _hit contig no._SNP position in the contig.

All 29 candidate genes could be mapped to at least one of the linkage maps (Table 4-1), and seven candidate genes (*ghCHI, ghDND*, *ghPER21*, *ghPG1, ghPG10, ghsit,* and *ghSS*) could be mapped to linkage maps of both populations. Three CGs (*ghsit, ghPG1* and *ghCHI*) mapped in previously detected QTLs regions (RBQRF2, RBQRF8 and RBQWI6; Suppl. Fig. S4-3a, b, c). CGs *ghPG9* and *ghcutin* were mapped on the paternal linkage map SP2_02 close to markers WGC23656_151_S1F1 and WGC11243_647_S2F1. On maternal linkage map (SP1_01), QTL RBQB1 was located between these two markers (Suppl. Fig. S4-3d). Two CGs (*ghPER62* and *ghSS*) co-localized with QTLs, RBQRF7, RBQB2 (Table 4-2 and Suppl. Fig. S4-3f, g).

Table 4-2 CGs mapped and co-localized in QTLs regions

CGs	CGs marker	Mapped inside QTL region	Maps near to QTL region
ghCHI	CHI_22447_421	RBQWI4/RBQWI6	
ghPG1	PG1_15001_1052	RBQRF8	
ghsit	sit_19807_6510	RBQRF2	
ghPG9	PG9_25150_888	RBQB1/RBQB8	
ghcutin	cutin_4918_3081		RBQB1 (2.6 cM)
ghPER62	PER62_31925_540		RBQRF7 (4.9 cM)
ghSS	SS_5198_1668		RBQB2 (4.98 cM)

With the newly mapped CGs, we have conducted QTL mapping again (Suppl. Table S4-3). Compared with the previous QTL analysis (*Chapter 3*; Fu *et al*. 2017), a number of small differences were detected. Two new QTLs were detected, RBQB7 from SP1_06 (LOD = 5.33) with the two CGs (*ghPG7* and *ghPG10*) mapped (Suppl. Fig. S4-3e); and RBQB8 in SP2_02 was just above the GW threshold (LOD = 4.0) with the newly added CG marker (PG9_25150_888) from *ghPG9*, and it could be correlated with the previous QTL RBQB1 which was detected on the maternal map (SP1_02) at a similar position (Suppl. Fig. S4-3d); CG marker (PG1_15001_1052) from *ghPG1* was mapped on linkage group FP2_21 in a 13 cM marker interval and re-analyzes of the QTL lead to a shift of the location of the maximum LOD score around 10cM to this locus. Variance explained by this QTL (RBQRF8) increased from 8.0% to 10.7%. Adding CG marker (from CG *ghPER62*) showed that the LOD score of the previous detected QTL RBQRF7 (4.09) was just a fraction below the GW threshold (4.1).

Allelic diversity of candidate gene

Using a single SNP marker, alleles having a positive contribution on the QTLs could only be identified from the parent in which the marker is polymorphic. Allelic diversity was assessed for the four parents of candidate genes showing significance below the 0.01 threshold level in the *t*-test between mean disease score of *whole inflorescence*, *bottom* and/or *ray floret* of the two allelic groups. As a diploid heterozygous crop, at most 8 haplotypes/alleles per gene can be expected in these four parents. In Fig. 4-2 (and see also Suppl. Fig. S4-4), specific haplotypes for each gene in each parent are represented by only indicating SNP positions. Allelic diversity ranged from at least 3 haplotypes (*ghSS* and *ghERF*) in the CGs loci of the four parents to at most 7 haplotypes (*ghPG9*). In all analyzed CGs, unique haplotype(s) which were not shared with other parents existed. Each of the parents had an unique haplotype combination at each CG locus. In three CGs (*ghFDH*, *ghPER62*, *ghPG9*), the unique haplotype contributed to the resistance effect. Two

alleles from all ten analyzed CGs loci from parent FP1 are heterozygous, SP1 has nine heterozygous loci (except *ghcutin*) and FP2 eight heterozygous loci (except *ghsit* and *ghcutin*), while in SP2, only four loci contained two distinctive alleles in the CGs loci (*ghERF*, *ghPER62*, *ghcutin, ghPG9*).

on ORF	240	249	261	262	300	303	312	339	354	376	384	401	485	494	496	528
SP1	A	A	A	C	G	T	G	T	G	A	G	A	C	A	G	C
	G	C	G	A	T	G	A	C	A	G	A	G	T	T	A	A
SP2	A	A	A	C	G	T	G	T	G	A	G	A	C	A	G	C
	G	C	G	A	T	G	A	C	A	G	A	G	T	T	A	A
FP1	G	C	G	A	T	G	A	C	A	G	A	G	T	T	A	A
	A	A	A	C	G	T	G	T	G	A	G	A	C	A	G	A
FP2	A	A	A	C	G	T	G	T	G	A	G	A	C	A	G	C
	G	C	G	A	T	G	A	C	A	G	A	G	T	T	A	A

+

Fig. 4-2 The alignment of alleles of *ghERF* (ethylene-responsive transcription factor, from contig11093). SNPs in the ORF of the gene are shown and 528 is the SNP used for the HRM.

More than half of the SNPs were present in the third base of the codon. Overall, 57% of the SNPs were synonymous SNPs whereas the other 43% were non-synonymous SNPs (nsSNP). Two alleles from *ghERF*, *ghPER62*, *ghSS,* and three alleles from *ghcutin, ghPG9* encoded the same protein (Suppl. Fig. S4-4). Interestingly, a 36bp insertion–deletion (InDel) was found in the first intron of *ghsit* (Suppl. Fig. S4-4g), while a 1-bp InDel in the second exon region was found for an *ghCHI* allele which would lead to an early stop codon (also Suppl. Fig. S4-4f).

Expression of candidate genes

Expression levels of seven candidate genes which showed association with Botrytis disease score variation were analyzed on ray florets. In the initial experimental setup, inoculated ray florets were sampled for expression analysis at 0 hpi, 6 hpi, 12 hpi, 24 hpi, 36 hpi, 48 hpi and 72 hpi. Spores of Botrytis already germinated on ray florets 6 hpi (Suppl. Fig. S4-5). The initial stages of Botrytis infection resulted in necrotic lesions which were clearly visible on ray florets and then lesions expanded quickly from the initial necrotic lesions to the whole ray floret (Suppl. Fig. S4-5). Expression of gerbera house-keeping gene at later stages of infection (36 h, 48 h and 72 h after inoculation) was already absent because florets had become necrotic. So final expression analyzes were performed on ray florets at 0, 6, 12 and 24 hrs after inoculation, with a control sample with mock inoculation sampled after 24 hrs.

The expression levels of candidate genes are shown in Table 4-3, all seven CGs were expressed in SP1 and FP2. Expression of *ghCHS1* and *ghPER62* was not detected in SP2 and no expression of *ghCHI*, *ghPG1* and *ghPER62* was found in FP1. The expression pattern of studied CGs showed variation at different time points but with significant up-regulation after Botrytis infection. In general, gene expression levels reached their peak at 12h or 24 h after Botrytis inoculation. All CGs expression levels from the resistant parent FP2 (except *ghPER21*) were reached with a significant increase before or at 12hpi. There was no significant difference in gene expression between start time point 0h and 24h mock except for CG *ghPER21* in SP2 and FP2. The expression of *ghPER21* increased even without Botrytis inoculation.

Table 4-3 The expression levels of CGs on ray florets of gerbera

CGs	Parents	00hpi	06hpi	12hpi	24hpi	24hpi_mock
ghCHI	SP1	0.97±0.26c	0.75±0.31bc	2.39±0.78**a**	1.48±0.34b	1.04±0.25bc
	SP2	1.3±0.59b	2.61±1.75b	2.59±1.28b	4.69±2.33**a**	1.38±0.63b
	FP1	-	-	-	-	-
	FP2	1.54±1.3b	1.68±1.13b	11.89±6.3**a**	1.49±0.83b	0.89±0.4b
ghCHS1	SP1	1.08±0.39b	1.6±0.35b	4.55±1.41a	3.77±2.51**a**	0.56±0.07b
	SP2	-	-	-	-	-
	FP1	1.08±0.47b	1.05±0.32b	20.07±4.38**a**	-	1.49±0.89b
	FP2	1.07±0.42b	0.46±0.17c	4.26±0.73**a**	0.89±0.17b	0.8±0.25bc
ghLOX	SP1	1.02±0.27c	1.25±0.11bc	2.15±0.17b	11.14±2.14**a**	0.48±0.1c
	SP2	1.07±0.45b	1.23±0.26b	1.92±0.4b	7.72±6.94**a**	0.65±0.14b
	FP1	1±0.11c	4.83±1.35b	15.38±3.55**a**	-	-
	FP2	1.05±0.41c	2.45±0.32b	7.2±2.39**a**	1.38±0.65c	0.47±0.21c
ghPER21	SP1	1.03±0.26b	1.86±1.27**a**	1.26±0.13ab	0.82±0.58b	1.53±0.42ab
	SP2	1.06±0.38c	0.32±0.19d	0.39±0.22d	5.01±0.82**a**	3.95±0.75b
	FP1	1.12±0.6bc	0.75±0.23c	3.36±2.14**a**	-	1.93±0.63b
	FP2	1.24±0.85bc	1.04±0.79c	1.85±1.43bc	13.44±7.96**a**	5.2±3.41b
ghPER62	SP1	1.15±0.59b	1.25±1.13b	1.59±0.71b	25.49±12.72**a**	0.24±0.06b
	SP2	-	-	-	-	-
	FP1	-	-	-	-	-
	FP2	1.82±2.15b	2.24±1.66b	20.59±15.26**a**	2.3±1.99b	0.58±0.34b
ghsit	SP1	0.97±0.22b	0.73±0.21b	1.32±0.36b	2.8±1.64**a**	0.52±0.04b
	SP2	1.15±0.68c	2.17±0.85b	1.88±0.57bc	4.03±1.68**a**	0.97±0.23c
	FP1	1.02±0.24b	2.51±0.32b	9.64±4.12**a**	-	1.42±0.2b
	FP2	1.02±0.22c	4.92±2.06b	17.44±5.63**a**	2.46±1.14c	0.93±0.22c
ghPG1	SP1	1.63±0.5c	3.98±2.1b	13.85±0.95**a**	4.3±0.22b	1.96±0.78bc
	SP2	1.12±0.49b	1.92±1.41b	2.19±2.31b	5.6±2.69**a**	3.16±3.22b
	FP1	-	-	-	-	-
	FP2	1.24±0.51c	4.61±4.23b	6.24±7.56**a**	3.12±1.61b	1.31±0.52c

Note: The expression levels of different timepoints were performed by pairwise comparisons, and the letters a, b, c, *etc* indicate the LSD test performance (P<0.05).

Discussion

Candidate gene genotyping by HRM

In this study, we selected putative candidate genes involved in plant resistance against Botrytis from literature and screened our gerbera ESTs database (*Chapter 2*; Fu *et al.* 2016) for homologous genes. After alignment of the homologous gene sequences found among the parents and selection of suitable SNP markers, CGs were mapped on the genetic maps of two populations used for QTL mapping previously (*Chapter 3*; Fu *et al.* 2017).

For good HRM results several criteria need to be satisfied in order to generate amplicons with just single SNPs producing easy to distinguish single-base differences on the melting curve. In such cases, all homozygotes melt in a single transition and heterozygotes produce a deviating melting curve arising from the integrated melting curves of two homoduplexes and two heteroduplexes (Gundry *et al*. 2003; Reed and Wittwer 2004). First, PCR conditions must be optimized and primers checked to amplify a single PCR fragment, as non-specific bands can significantly reduce HRM performance (Lehmensiek *et al*. 2008). For 14 candidate gene primers (12 in both populations and 2 in one population) multiple bands after amplification have been found. It might be mainly because these genes, such as DELLA and CHS, belong to gene families with homologues genes existing in the genome. In such cases the melting curve represents a combination of individual melting curves of homologous genes which cannot be used for genotyping.

Another criterion is the amplicon size. Product size should be below 300 bp and preferably much smaller to keep a high sensitivity and specificity to detect the possible heterozygotes by HRM without error (Reed and Wittwer 2004). De Koeyer *et al*. (2010) suggested that produced amplicons within the size range from 50 to 400 bp are suitable for HRM analyzes in potato. However, when product length increases, the difference between homozygote and heterozygote curves will become smaller making SNP calling more difficult (Reed and Wittwer 2004). Moreover, gerbera is a highly heterozygous ornamental crop and if the target sequence is larger the potential for inclusion of other unexpected SNPs is increasing. Amplicons with several SNPs always result in a complex situation with several melting curves for analysis that do not render clear groups. Except only the CG marker (CHI_22447_421) from *ghCHI*, multiple SNPs all located in one fragment of the parental allele and the amplified allelic sequence variation were separated by HRM analysis. According to our previous study (*Chapter 2*; Fu *et al*. 2016), the average number of polymorphic SNP sites within each of the four parental ESTs varied from 3.7 to 4.8 SNPs per kb. That means there is at least one SNP in every 200~250 bp in each parental genotype. Thus, we produced small fragments to avoid additional SNPs. Furthermore, our primer design was based on cDNA sequence, if longer size PCR amplicons are selected without having information on gene structure, there will be a higher chance that amplicons span an intron region where more SNPs exist. Most primers were designed taking into account intron exon structure from the homologous gene coding sequence aiming to flank a single SNP as detected in the Gerbera EST database. We also successfully mapped three CGs which primers (AS_1926_I2, sit_19807_I2, RD21_20206_I2) were designed flanking a ~100bp-size intron region that was known to contain a SNP from whole genome Sanger sequencing. Sometimes, amplicons with two nearby SNPs can be distinguished in groups, but in most cases this leads to unclear grouping.

Mapping of candidate genes and the co-localization with QTLs

In total, we designed 89 primer pairs for 71 CGs, and 29 CGs with a clear 1:1 segregation in the offspring were mapped in at least one population. The percentage of genes successful mapped is 41%, yet other primers dropped out mainly because of additional SNPs and multiple bands. Additional SNPs from included intron regions can only be excluded if more genomic information from the focal or other species is obtained to avoid designing primers spanning an intron region. Primers yielding multiple bands due to the gene belonging to a gene family might focus on the 5'- or 3'- ends of the gene, since these regions might be more specific. Through the recently developed amplicon sequencing in combination with NGS

sequencing (e.g. Ion AmpliSeq™) problems with additional SNPs and gene family membership may be overcome without the relative high costs for conventional sequencing.

Out of the 29 mapped CGs, several genes are from the same gene family, for example, *ghCHS1* (chalcone synthase gene), *ghSS* (stilbene synthase gene), *gh2-PS* (2-pyrone synthase gene); *ghPER21* (peroxidase gene) and *ghPER62*; and several polygalacturonase genes (*ghPG1*, *ghPG2*, *ghPG7*, *ghPG9*, *ghPG10*). Those genes were mapped on different linkage groups, except PG7 and PG10 which were mapped at the same linkage group close to each other (1cM apart).

Using the candidate gene approach for identifying Botrytis resistance genes in gerbera is effective. Several mapped candidate gene alleles are showing significantly difference in resistance performance of *whole inflorescence*, *bottom* and *ray floret* in the two populations, and several CGs were found mapped in a QTL region or co-localized with the identified QTLs. Few CGs showed allelic variation that gave a high significance level ($P<0.01$) in the *t*-test, but couldn't be detected as a QTL. The underestimated QTLs might be the environmental conditions which could influence the power of detecting QTLs or interaction of alleles from different genes and parental alleles.

Possible mechanisms for Botrytis resistance in gerbera

Several candidate genes with statistical associations with the *whole inflorescence*, *bottom* and *ray floret* tests might be involved in Botrytis resistance under multiple mechanisms. Plant cuticle and cell wall are constituted as the first protective barriers to defense Botrytis invasion (Curvers *et al*. 2010). The function of the cuticle against Botrytis has been studied showing that genes involved in cuticle synthesis have a strong effect on botrytis susceptibility (Chassot *et al.* 2007; Bessire *et al.*2007). Lignin is crucial for structural integrity of the cell wall and peroxidase is a key enzyme in biosynthesis of lignin (Boerjan *et al*. 2003). Botrytis resistance might be associated with increases in peroxidase activity (Lurie *et al*. 1997). *B. cinerea* secretes polygalacturonases (PGs) to decompose plant cell walls (Ferrari *et al*. 2003) and there are at least 6 genes in Botrytis encoding PGs (van Kan 2006). However, fungal PGs can be inhibited by plant polygalacturonase-inhibiting proteins (PGIPs) whereas these proteins may not inhibit a plant's own endo-PGs. Blanco-Ulate *et al*. (2014) suggested that *B. cinerea* might be able to manipulate plants to produce endo-PGs in order to degrade plant cell walls as they will not be inhibited by PGIP. From our studies, two of the gerbera endo-PGs (*ghPG1* and *ghPG9*) were found associated with Botrytis resistance on gerbera *ray floret* and *bottom* test respectively showing high statistical significance in the disease tests. After QTL mapping, the two candidate loci were detected as QTLs and explained 10.7% and 6.1% of the phenotypic variation. However, no statistical association was found between the two *ghPGIP* genes with phenotypic variation or detected QTL yet. We assume *B. cinerea* might indeed be manipulating endo-PGs in gerbera plants to take the advantage of this in the infection process.

Plant hormones are considered to play an essential role in defense against Botrytis, especially the ET and JA pathways. The ethylene responsive transcription factor (ERF) family encode proteins in disease resistance regulation pathways (Gutterson and Reuber 2004) and their binding target sequence is the GCC box which is found in several promoters of pathogen related and ET- or JA-induced genes. Overexpression of ERF1 in Arabidopsis is sufficient to enhance tolerance to *B. cinerea* (Berrocal-Lobo *et al*.

2002). Also in our study, the *ghERF* is related to the phenotypic variation in the *bottom* test (P = 0.0075) and could be a promising candidate locus. ABA signalling is also believed to play an important role to *B. cinerea* resistance from the studies on the interaction between ABA-deficient tomato mutant sitiens and *B. cinerea* (Asselbergh *et al.* 2007; Curvers *et al.* 2010). ABA signalling regulated the cuticle and pectin composition which affect Botrytis resistance. The last step of ABA biosynthesis (ABA-aldehyde oxidation) in sitiens is blocked and leads to accumulation of trans-ABA instead of ABA (Rock *et al.* 1991) and sitiens is identified as a member of the ABA-aldehyde oxidase genes. The difference of sitiens wild type allele (sit+) and mutant allele (sit) in tomato is in the deletion of intron 1 and division of exon 2 (Harrison *et al.* 2011). Furthermore, considering that gerbera gray mold infection mainly happens in post-harvest when plant are experiencing senescence whereas ABA level is strongly related to senescence, it is likely that the reduced ABA level contributes to Botrytis resistance in gerbera. In our study, the two groups sorted by gerbera sit homologous gene allelic variation showed significant difference in the *ray floret* test (at $p<0.001$ level) and mapped in the QTL interval of RBQRF2. Interestedly, like the tomato sit allele, we also found a 36 bp InDel in intron 1 of the four gerbera parental sit alleles as well as quite a lot of these similar alleles in other gerbera genotypes (date not shown). Different structures in the first intron have been identified to have an effect on human genes (Bornstein *et al.* 1987) or having a promoter-like function in maize (Salgueiro *et al.* 2000). Therefore, we assume the polymorphic sequence in the intron 1 may affect the gene function, but this needs further confirmation.

Phenylpropanoid compounds are natural secondary products which are derived from the general phenylpropanoid pathway and the consecutive flavonoid pathway. These derivatives, like anthocyanins, are known for the origin of flower pigmentation (Winkel-Shirley 2001) but other derivates like isoflavonoid phytoalexins are active in plant defense (Dixon *et al.* 2002). Enzymes in the phenylpropanoid/flavonoid pathway have been well studied (Ainasoja 2008; Deng *et al.* 2014; Elomaa *et al.* 1993; Helariutta *et al.* 1995) in some crops and include phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), dihydroflavonol reductase (DFR), flavanone 3-hydroxylase (F3H) on the main phenylpropanoid pathway, and 2-pyrone synthase (2-PS) and stilbene synthase (SS) on the branch for flavonoid production. Several of these metabolites were confirmed to be involved in Botrytis resistance (Dixon *et al.* 2002; Koskela *et al.* 2011; Laquitaine *et al.* 2006) or their expression was enhanced by Botrytis infestation (Blanco-Ulate *et al.* 2015). Tomato with increased anthocyanin accumulation lead to an extended shelf life and is less susceptible to *B. cinerea* (Bassolino *et al.* 2013). Our study confirms that for a number of genes they might play a similar role in botrytis defense in gerbera.

The variation of inflorescence colors in commercial gerbera cultivars is a result of the interaction of carotenoid and flavonoid pigments which are all under genetic control (Tyrach and Horn 1997). In gerbera, several CHS have been confirmed to be involved with flavonoid and anthocyanin synthesis (Ainasoja 2008; Deng *et al.* 2014; Elomaa *et al.* 1993; Helariutta *et al.* 1995). In our study, CG marker SS_5198_1668, the homologous sequence of stilbene synthase from grapevine, is found to be co-localized with the *bottom* test QTL RBQB2. Alignment of the ORF region of this contig5198 with the GCHS4 (AM906210.1), a gerbera chalcone synthase gene showed that the two sequences are identical. Deng *et al.* (2014) found that GCHS4 is highly expressed in carpels. The *bottom* test is in accordance with 'heart rot',

which is describing the disc florets infection by Botrytis. Interestingly, the disk florets color of SP1 which contributes to the QTL is black, while the other three parents have green/yellowish disk florets. Therefore, we assume that differences in disc floret color might be connected with the 'heart rot' in gerbera and is probably related to the expression of the *ghSS* gene.

Another key enzyme in flavonoid biosynthesis pathway, chalcone isomerase (CHI) catalyzes chalcones into their corresponding flavonones (Mehdy and Lamb 1987) and is considered the rate-limiting enzyme of flavonoid biosynthesis during tomato fruit development (Muir *et al*. 2001). A China aster (Callistephus chinensis) mutant with two recessive alleles deficient in chalcone isomerase activity caused an accumulation of chalcone and a completely blocked anthocyanin synthesis, but in wild-type plants, no chalcone was detected and high isomerase activity increased the amounts of downstream products, like flavones, flavonols, and anthocyanins (Kuhn *et al*. 1978). Besides the differences caused by genetic variation, chalcone isomerase is regulated by several biotic and abiotic stresses and CHI expression was strongly induced during Colletotrichum lindemuthianum (a fungal bean pathogen) infection (Mehdy and Lamb 1987). The polymorphisms of *ghCHI* in the two gerbera populations are associated with *whole inflorescence* test and the *ghCHI* gene might be the causal gene underlying the QTLs which were identified in the F population (RBQWI4, RBQWI6). After the Botrytis inoculation, *ghCHI* expression in SP1, SP2 and FP2 increased but no *ghCHI* expression was detected in FP1. Yellow-pigmented carnation, cyclamen and antirrhinum were all identified as due to the absence or reduction of CHI activity (Forkmann and Dangelmayr 1980; Ono *et al*. 2006; Takamura *et al*. 1995). Considering that parent FP1 is yellow colored, it might also have lost its CHI activity. Interestingly, we found a SNP deletion in the two *ghCHI* alleles of FP1. The loss-of-function allele might decrease the accumulation of flavonoid secondary metabolites which contribute to Botrytis resistance in gerbera.

Allelic variation and expression of the candidate genes

Candidate genes allelic diversity was conducted mainly on genes co-localized with detected QTLs and statistically correlated with phenotypic variation. The sequence polymorphisms of these selected CGs offer a glimpse of the heterozygosity of gerbera. Although only two species are considered to be involved in the origin of modern gerbera cultivars with a possible bottleneck at the moment of hybridisation (Hansen 1999), genetic diversity is rather high in gerbera germplasm. The SNP density in the specific genes involved in Botrytis resistance varies from 5.7 to 27.2 SNPs/kb which is higher than the average SNP density identified within the four parental overall ESTs (from 3.7 ~ 4.8 SNPs/kb) (*Chapter 2*; Fu *et al*. 2016); or in eleven safflower (Carthamus tinctorius) individuals in which SNP density is 10.5 SNP/kb in the exons and introns of 7 genes (Chapman and Burke 2007). We mainly looked at the SNPs in exon regions and more variation would be expected if the upstream and downstream region of the gene (e.g. 3'-UTR, 5'-UTR) and also intron sequences are all taken into consideration.

Multiple alleles existed at the candidate loci of the (four) parental genotypes and all genotypes are unique. Acquaah (2012) implied that for the improvement of cross-pollinated species breeding has to focus on increasing the frequency of favorable alleles. QTL analysis from bi-parental populations of gerbera in our previous study (*Chapter 3*; Fu *et al*. 2017) only indicates the favorable segregating alleles present in our

populations. Considering the heterozygous and heterogeneous in each genotype, it could be a practicable start to screen possible alleles focusing on these CGs in a broader gene pool and linking these to botrytis resistance.

The upregulation of expression levels of the CGs upon Botrytis infection, given the function of these genes in other species, is a clear implication that these CGs are related with Botrytis resistance in gerbera. Tracking the Botrytis infection process on gerbera ray florets of parents till 72 hpi, we found all the ray florets were infected eventually while the speed of disease development varied. Few candidate gene expressions were not detected in SP2 and FP1 and that might be because of the genetic variation resulting in no expression or other genes playing a role in these two genotypes. All the studied CGs expressed in FP2 and the highest level of gene expression were at or before 12 hpi which was in line with this genotype FP2 having a relative high resistance to Botrytis in ray florets. A quick response of disease-related genes reaching the highest expression level as early as possible seems important to resist the attack of Botrytis.

The candidate gene approach as used in this study in gerbera, for which crop no genome sequence is available, can efficiently pinpoint a number of potential causal genes. Whereas using QTL regions in outcrossing crops possess practical problems in the implementation for MAS, finding causal genes involved in a trait would be a major step and can also help in understanding the molecular interactions between Botrytis and gerbera.

Acknowledgments

We are thankful for the support from the Foundation Technological Top Institute Green Genetics (3CFL030RP).

Table S4-1 The information of candidate genes for Botrytis resistance and blasted homologues in gerbera

Candidate gene	Function	Accession	from	amino acids	hit	Score (Bit)	E-value	Identities	ORF size in contig	BLAST back				
										Description	Score (Bit)	Query cover	E-value	Identities	Accession
2-PS	2-pyrone synthase	CAA86219.2	*Gerbera hybrida*	**402**	contig11873	817	0	99%	**402**	2-pyrone synthase, 2-PS,	817	100%	0	99%	P48391.2
ACS	ACC synthase	AEE82992.1	*Arabidopsis thaliana*	**495**	contig17688	658	0	68%	**481**	putative ACC synthase 1	855	99%	0	84%	AAP14019.1
AF	plant defensin	NP_199255.1	*Arabidopsis thaliana*	**80**	contig29000	56.6	2.00E-10	52%	**183**	DNA/RNA helicase, ATP-d	342	98%	2.00E-107	90%	KVI04017.1
AIR12	auxin-induced in root cultures	NP_566306.3	*Arabidopsis thaliana*	**273**	contig1278	169	1.00E-47	53%	**387**	PREDICTED: cytochrome	544	94%	0	68%	XP_011089631.1
AOS	allene oxide synthase	AED94842.1	*Arabidopsis thaliana*	**518**	contig28753	602	0	62%	**479**	allene oxide synthase [Ar	746	97%	0	76%	ADZ24000.1
AS	anthranilate synthase compone	AED90916.1	*Arabidopsis thaliana*	**624**	contig1926	806	0	71%	**597**	anthranilate synthase alp	1022	97%	0	85%	OIT06829.1
BIK1	Botrytis-induced kinase	NP_181496.1	*Arabidopsis thaliana*	**395**	contig14337	496	1.00E-170	66%	**402**	protein kinase apk1b, ch	660	100%	0	80%	OIT01161.1
BOS	putative transcription factor I	NP_187301.1	*Arabidopsis thaliana*	**323**	contig3562	267	1.00E-85	56%	**308**	PREDICTED: transcription	396	97%	5.00E-136	66%	XP_011027944.1
bre1	botrytis-resistant 1	NP_182022.2	*Arabidopsis thaliana*	**878**	contig15621	821	0	52%	**878**	PREDICTED: E3 ubiquitin-	1177	99%	0	67%	XP_010661359.1
cel1	endo-1,4-beta-glucanase	NP_001234862.1	*Solanum lycopersicum*	**489**	contig13541	782	0	83%	**501**	Endoglucanase 8 [Morus r	828	93%	0	85%	XP_010101981.1
cel2	endo-1,4-beta-glucanase	NP_001234867.1	*Solanum lycopersicum*	**501**	contig5926	650	0	68%	**505**	endoglucanase [Nicotiana	700	98%	0	67%	OIT33211.1
CHI	Chalcone-flavonone isomerase	Q42663.1	*Callistephus chinensis*	**237**	contig4923	597	0	75%	**222**	Chalcone isomerase [Saus	369	99%	6.00E-128	78%	Q8LKP9.1
CHI	Chalcone isomerase	NP_001234421.1	*Solanum lycopersicum*	**262**	contig22447	270	1.00E-57	64%	**220**	chalcone isomerase [Cam	249	87%	2.00E-80	65%	AHB32112.1
CHS3	chalcone synthase	CAA86218.1	*Gerbera hybrida*	**398**	contig1332	729	0	89%	**402**	Chalcone synthase 3 [Ger	832	100%	0	99%	P48392.1
CHS1	chalcone synthase	CAA86218.1	*Gerbera hybrida*	**398**	contig29143	517	0	100%	**259**	Chalcone synthase 1 [Ger	531	100%	0	100%	P48390.1
cutin	cutin biosynthesis; long-chain a	NP_175368.2	*Arabidopsis thaliana*	**665**	contig4918	936	0	68%	**660**	long chain acyl-coa synth	1110	99%	0	79%	OIT07312.1
DELLA	DELLA protein RGA	AEC05469.1	*Arabidopsis thaliana*	**587**	contig29198	687	0	64%	**562**	DELLA 1 [Lactuca sativa]	1014	99%	0	90%	BAG71200.1
DND	cyclic nucleotide-gated ion cha	NP_197045.1	*Arabidopsis thaliana*	**726**	contig29068	952	0	74%	**699**	cyclic nucleotide gated i	1250	97%	0	90%	AIT39758.1
EDR3	dynamin-related protein 1E	NP_567094.1	*Arabidopsis thaliana*	**623**	contig24013	1013	0	80%	**644**	dynamin-related protein	1095	99%	0	83%	OIT39247.1
EIN2	ethylene signal transduction co	NP_195948.1	*Arabidopsis thaliana*	**1294**	contig25557	932	0	45%	**1184**	ethylene signaling protei	1171	97%	0	50%	NP_001234518.1
ELP2	elongator subunit 2	NP_175377.2	*Arabidopsis thaliana*	**838**	contig2398	1092	0	65%	**835**	elongator complex protei	1266	99%	0	72%	OIS97280.1
ERF	ethylene-responsive transcript	AED95487.1	*Arabidopsis thaliana*	**243**	contig18430	176	8.00E-53	55%	**229**	AP2/ERF domain-containi	370	99%	3.00E-128	79%	KVH91971.1
ERF	ethylene-responsive transcript	AEE76738.1	*Arabidopsis thaliana*	**218**	contig11093	186	2.00E-57	57%	**196**	AP2-ERF transcription fac	241	94%	1.00E-78	70%	AFA36063.1
ET	ethylene-insensitive protein	AAR08678.1	*Petunia x hybrida*	**1310**	contig14926	777	0	46%	**865**	Ethylene-insensitive 2 [C	1255	98%	0	71%	KVH93917.1
EXLA2	expansin-like A2	NP_195553.1	*Arabidopsis thaliana*	**265**	contig14980	313	2.00E-105	67%	**260**	PREDICTED: expansin-like	404	95%	6.00E-141	74%	XP_002534094.1
EXP1	expansin 1 protein	AAQ12264.1	*Solanum lycopersicum*	**261**	contig12313	423	2.00E-146	80%	**284**	PREDICTED: expansin-A4	456	91%	5.00E-161	84%	XP_008384848.1
FDH	fiddlehead protein; 3-ketoacyl-	AAF73973.1	*Arabidopsis thaliana*	**550**	contig12215	878	0	76%	**554**	hypothetical protein Ccr	1059	100%	0	92%	KVH88528.1
GDSL	GDSL-like Lipase/Acylhydrolase	NP_194743.1	*Arabidopsis thaliana*	**348**	contig11795	265	3.00E-83	45%	**360**	Lipase, GDSL [Cynara car	479	98%	6.00E-167	65%	KVI00927.1
GLK1	transcription activator Golden	NP_565476.1	*Arabidopsis thaliana*	**420**	contig15145	237	3.00E-71	46%	**411**	transcription activator g	429	100%	5.00E-145	58%	OIT00132.1
GSL5	callose synthase 12	NP_192264.1	*Arabidopsis thaliana*	**1780**	contig30332	2565	0	70%	**1756**	callose synthase 12 [Nico	2806	99%	0	75%	OIT39115.1
GST1	glutathione S-transferase	AEE27498.1	*Arabidopsis thaliana*	**208**	contig17531	149	9.00E-44	53%	**160**	Glutathione S-transferas	535	100%	2.00E-69	68%	KVI12466.1
LOX	lipoxygenase 2	AGO03786.1	*Tanacetum cinerariifoli*	**899**	contig28693	1318	0	72%	**901**	Lipase/lipooxygenase, PL	2575	100%	0	78%	KVH90900.1
LOX	lipoxygenase	AGO03785.1	*Tanacetum cinerariifoli*	**907**	contig13375	1484	0	82%	**920**	lipoxygenase 1 [Tanacetu	1550	100%	0	82%	AGO03785.1
LOX	lipoxygenase 2	AAA32749.1	*Arabidopsis thaliana*	**896**	contig4808	1011	0	59%	**829**	Lipase/lipooxygenase, PL	1369	100%	0	78%	KVI00262.1
MKP2	MAP kinase phosphate 2	NP_850522.1	*Arabidopsis thaliana*	**167**	contig427	181	4.00E-55	59%	**179**	Dual specificity phosphat	306	92%	1.00E-104	88%	KVH88866.1
MYB46	transcription factor MYB46	AED91824.1	*Arabidopsis thaliana*	**280**	contig11297	**211**	2.00E-63	90%	**330**	MYB transcriptional facto	332	100%	5.00E-110	54%	BAR45570.1
OPR	12-oxophytodienoate reductas	AEE35875.1	*Arabidopsis thaliana*	**372**	contig196	608	0	79%	**378**	PREDICTED: putative 12-(	651	95%	0	84%	XP_017237794.1

Table S4-1 (continued)

Candidate gene	Function	Accession	from	amino acids	hit	Score (Bit)	E-value	Identities	ORF size in contig	BLAST back				
										Description	Score (Bit)	Query cover	E-value	Identities	Accession
OPR3	12-Oxophytodienoate reducta	NP_001233873.1	*Solanum lycopersicum*	**396**	contig12803	639	0	76%	**394**	artemisinic aldehyde redi	720	99%	0	87%	BAU61367.1
P450	cytochrome P450 reductase	AFO64618.1	*Artemisia annua*	**704**	contig15117	1117	0	85%	**709**	cytochrome P450 reduct	1243	100%	0	86%	AFO64618.1
P450	cytochrome P450 mono-oxyger	ADO16182.1	*Artemisia annua*	**515**	contig8091	674	0	67%	**378**	cytochrome P450 mono-c	512	98%	1.00E-177	67%	ADO16182.1
PAD	auxin signaling F-box 2	NP_566800.1	*Arabidopsis thaliana*	**575**	contig12282	685	0	59%	**574**	PREDICTED: protein TRAN	981	99%	0	81%	XP_011082496.1
PAL	cytochrome P450 reductase	AFO64618.1	*Artemisia annua*	**704**	contig25584	1122	0	83%	**711**	cytochrome P450 reduct	1225	100%	0	83%	AFO64618.1
PAL	phenylalanine ammonia-lyase 1	NP_181241.1	*Arabidopsis thaliana*	**725**	contig10623	1135	0	85%	**619**	phenylalanine ammonia-ly	1205	99%	0	93%	AAL55242.1
PDF1.2	ethylene- and jasmonate-respo	NP_199255	*Arabidopsis thaliana*	**80**	contig13068	56.2	8.00E-11	52%	**123**	defensin [Helianthus annu	143	90%	4.00E-42	74%	AAM27914.1
PER21	Peroxidase 21	Q42580	*Arabidopsis thaliana*	**327**	contig12863	449	1.00E-153	66%	**324**	PREDICTED: peroxidase 2	503	99%	1.00E-177	72%	XP_007027286.1
PER62	Peroxidase 62	Q9FKA4	*Arabidopsis thaliana*	**319**	contig31925	321	1.00E-106	51%	**315**	PREDICTED: peroxidase 2	472	95%	9.00E-166	74%	XP_012078787.1
PFT1	polymerase II transcription sul	NP_173925.3	*Arabidopsis thaliana*	**836**	contig12433	714	0	59%	**768**	Phytochrome and floweri	948	89%	0	71%	EOY02123.1
PG1	polygalacturonase 2	NP_001185361.1	*Arabidopsis thaliana*	**626**	contig15001	642	0	58%	**622**	polygalacturonase 1 beta	812	99%	0	65%	OIT26928.1
PG10	polygalacturonase 1	AEE33681.1	*Arabidopsis thaliana*	**624**	contig11147	704	0	63%	**631**	polygalacturonase-1 non-	872	96%	0	68%	OIS96021.1
PG2	polygalacturonase	BAA89476.1	*Salix gilgiana*	**393**	contig1158	380	4.00E-126	49%	**410**	Polygalacturonase [Theot	429	96%	5.00E-146	53%	EOX93416.1
PG7	polygalacturonase	NP_001234256.1	*Solanum lycopersicum*	**452**	contig35500	375	5.00E-124	50%	**450**	PREDICTED: probable poly	678	100%	0	69%	XP_012085412.1
PG9	polygalacturonase 3	XP_002884668.1	*Arabidopsis lyrata* sub	**391**	contig25150	323	2.00E-106	50%	**347**	PREDICTED: exopolygalac	372	93%	1.00E-124	58%	XP_011003515.1
PgD	defensin	AAR84643.1	*Picea glauca*	**83**	contig28711	56.2	5.00E-11	53%	**63**	Defensin-like protein [Gly	103	100%	9.00E-28	76%	KHN06998.1
PGIP1	polygalacturonase-inhibiting pr	AIF27225.0	*Helianthus annuus*	**325**	contig17627	500	9.00E-176	80%	**329**	polygalacturonase-inhibit	536	96%	0	80%	AIF27224.1
PGIP2	polygalacturonase-inhibiting pr	AIF27225.1	*Helianthus annuus*	**330**	contig20957	440	2.00E-146	70%	**329**	polygalacturonase-inhibit	521	99%	0	78%	AIF27227.1
PLDß1	phospholipase D beta 1	NP_565963.2	*Arabidopsis thaliana*	**1083**	contig14327	1344	0	79%	**1091**	phospholipase d beta 1 [N	1408	99%	0	65%	OIT39456.1
PLP2	phospholipase A 2A	NP_180224.1	*Arabidopsis thaliana*	**407**	contig6394	575	0	69%	**444**	Phospholipase A 2A, IIA,P	617	90%	0	74%	EOY14528.1
PME3	pectin methylesterase 3	NP_188048.1	*Arabidopsis thaliana*	**592**	contig28400	772	0	72%	**585**	pectinesterasepectinest	941	100%	0	75%	OIT26967.1
RAP	ethylene-responsive transcript	NP_850582.1	*Arabidopsis thaliana*	**375**	contig25598	214	7.00E-64	46%	**332**	AP2/ERF domain-containi	519	100%	0	75%	KVI05829.1
RD21	cysteine proteinase	NP_564497.1	*Arabidopsis thaliana*	**462**	contig20206	607	0	69%	**455**	cysteine protease-1 [Heli	757	99%	0	79%	BAC75923.1
rwa2	Reduced wall acetylation2	NP_001078116.1	*Arabidopsis thaliana*	**568**	contig29675	843	0	74%	**545**	PREDICTED: protein REDL	929	100%	0	80%	XP_004229023.1
sitiens / sit	ABA-deficient mutant / ABA alde	ADR31354.1	*Solanum lycopersicum*	**1361**	contig19807	1727	0	67%	**1341**	Lactuca sativa aldehyde	2033	99%	0	75%	BAE72098.1
SR1	calmodulin-binding transcriptio	NP_850023.1	*Arabidopsis thaliana*	**1032**	contig1903	962	0	51%	**1080**	PREDICTED: calmodulin-bi	1427	99%	0	66%	XP_010645223.1
SS	stilbene synthase 1	NP_001267939.1	*Vitis vinifera*	**392**	contig5198	597	0	75%	**389**	chalcone synthase [Gerbe	801	100%	0	99%	CAP20328.1
SSI2	acyl-[acyl-carrier-protein] des	NP_181899.1	*Arabidopsis thaliana*	**401**	contig11878	661	0	87%	**426**	stearoyl-acyl-carrier pro	790	92%	0	94%	ABF66638.1
STP13	sugar transport proteins 13	NP_198006.1	*Arabidopsis thaliana*	**526**	contig6824	813	0	85%	**519**	PREDICTED: sugar transp	914	98%	0	86%	XP_011090738.1
TAGL1	TAGL1 transcription factor	AAM33101.2	*Solanum lycopersicum*	**269**	contig30005	281	4.00E-92	66%	**247**	MADS-box protein, GAGA1	505	100%	0	97%	CAA08800.1
WRKY33	WRKY transcription factor 33	AAM34736.1	*Arabidopsis thaliana*	**512**	contig16847	347	2.00E-111	56%	**498**	WRKY33 [Chrysanthemun	751	100%	0	76%	AJF11718.1
ZF	zinc finger CCCH domain-conta	AEC09788.1	*Arabidopsis thaliana*	**597**	contig30219	529	4.00E-180	52%	**610**	Zinc finger family protein	671	99%	0	53%	EOY17761.1
ZF	zinc ion binding protein	NP_565200	*Arabidopsis thaliana*	**358**	contig1325	172	5.00E-49	44%	**331**	Zinc finger, RING-type [C	478	100%	8.00E-168	79%	KVI10234.1
ZF	zinc finger protein	AEE30870.1	*Arabidopsis thaliana*	**277**	contig30683	172	2.00E-51	50%	**238**	Cys2/His2-type zinc fing	315	100%	5.00E-106	66%	AFF57513.1

Table S4-2 Primers list for candidate genes amplicon HRM analysis in parents and qRT-PCR analysis

CGs markers	Primer	HRM results in parents		note
		S	F	
2-PS_11873_814_F	GGCCTGAACCCACAATGAGT	b	a	*
2-PS_11873_814_R	GGACCCAATGAGAACCACCTT			
ACS_17688_842_F	ACCCTTTGGGCACTTTTCTT	a	a	
ACS_17688_842_R	CGCAAATGAGGTGGATGTTT			
AF_29000_1070_F	CCGAGAAGTTGGCTCAAGAC	d	d	
AF_29000_1070_R	TTTCTCGGCTTCGACTTTGT			
AIR12_1278_1722_F	ATCTAACCTCCCTTTCGCCC	d	d	
AIR12_1278_1722_R	GGAATTCCGGCGAAACATGA			
AOS_28753_1931_F	CTGATCTCCGACGCCAAT	c	c	
AOS_28753_1931_R	TCCCCAATACAATCAAATGGA			
AOS_28753_2837_F	GTCGCTTGGAACTGTGGAAT	b	d	
AOS_28753_2837_R	CCTCCTCTTCTTCATGCTCAA			
AS_1926_1135_F	TCTGCACATTGTTTTTCATCA	a	a	
AS_1926_1135_R	CAGGAGAGGGAAGACACCAA			
AS_1926_I2_F	CCAGGTTTAAAGGCTTCCAGT	a	b	
AS_1926_I2_R	AGCTCCAATAACACTGTACCG			
BOS_3562_1071_F	TGCATACATTATTGGATTAGGAAGA	b	b	
BOS_3562_1071_R	TCACAAATTAGAACATGTATCTCACAA			
bre1_15621_850_F	GCTTCTGAACTTGATCCGAACA	c	c	
bre1_15621_850_R	AGAGGAAGATTCAACAGGGCA			
cel2_5926_809_F	ATTCTCCGGGAACTGAGGTG	c	c	
cel2_5926_809_R	AAGCTTTCTCGAGTACTTTGAATCC			
CHI_22447_421_F	GACGGTGAAAATGGTGATTGT	a	a	
CHI_22447_421_R	TTGTGAGGTGGTCGTGTTTT			
CHI_22447_175_F	GAAACGATAACCGGAAATGC			**
CHI_22447_175_R	CCGTCGCTTAGCTTAACCAG			
CHI_4923_1704_F	CCATAACTGCCGGAGTGAAC	b	b	
CHI_4923_1704_R	GAGGTATCTAACTTTTCCTGTTCTCC			
CHS_1332_1045_F	ACCGGTGGTGGTCTTTCC	d	d	
CHS_1332_1045_R	TGGTAATATGTCCAGCGCCT			
CHS_29143_1582_F	GGAATCAAGGTGGGTGTCAT	a	b	**
CHS_29143_1582_R	CTCGCGGAGAACAATAAAGG			
cutin_11795_2336_F	GGACTTGAGGAGCACCATCC	a	a	
cutin_11795_2336_R	TGGGTTTCGATATTAAGGTTTTG			
cutin_11795_2266_F	CCCATCGGGAAAGTCAATC	c	c	
cutin_11795_2266_R	TTACTGAGGCAAAAGCCAAT			
cutin_11795_2066_F	AACAAGGGACTTGAGGAGCA	d	b	
cutin_11795_2066_R	GGTTTCGATATTAAGGTTTTGATTT			
cutin_4918_2779_F	GTGACATGTTGCACCGTTCT	d	d	
cutin_4918_2779_R	TGAAACATCCACCACAACTTTC			
cutin_4918_3081_F	GTGGCGAAATTTGCCTGAG	a	a	
cutin_4918_3081_R	TCACGCTTGTGATACCCAGA			
cutin_6590_1039_F	GCTGTTCAAGCTGTTGCAGT	b	a	
cutin_6590_1039_R	TACTCTGAGCCCAGCAATCA			
DELLA_29198_2133_F	TTATTTGCAGGAAGTGGGTTG	d	d	
DELLA_29198_2133_R	TGCAACAAAACCCCTGTATTC			
DELLA_29198_2293_F	ACAGGGGTTTTGTTGCAGAG	d	d	
DELLA_29198_2293_R	TGCAACAACTTCACCTTCTCTT			
DND_29068_911_F	GGTCGGTTCCACACATGTAA	a	a	
DND_29068_911_R	TCGAGCCAGGAGGGTATTTA			
ELP2_2398_1249_F	ATTCAGTCGAGTGGCAACCA	a	a	
ELP2_2398_1249_R	GCTTTCGGGTTGATAACACGGG			
ERF_11093_229_F	CAAATAGTCACTACCCAAATCCTC	a	a	
ERF_11093_229_R	GAGAAGGAGGCTAAGTTGGAAA			
ERF_18430_480_F	GCCGGACACCTCTGTAATGT	b	c	
ERF_18430_480_R	TGAGATTTCAAGCGGAATCG			
ET_14926_1910_F	CCGATATCTCCGGGCTTT	c	a	
ET_14926_1910_R	CTGGACTGGGAAGACTGACC			
EXLA2_14980_495_F	GGCCGGAACCCTACTTGT	d	d	
EXLA2_14980_495_R	AAGCCCTAATTATCTTGCCATCA			
FDH_12215_1158_F	GCCGGTTCCATCACTTTCTC	a	a	
FDH_12215_1158_R	AACACCAAAACCAGCACCG			
GSL5_30332_2672_F	CGAGTGTCACGAAGCTCATT	c	a	
GSL5_30332_2672_R	AACCGACGATCTTAGGAGTTC			
GST1_17531_312_F	TTATCAGATCCGTCCCCTTG	c	b	
GST1_17531_312_R	ATTCGAATCAAGGGCGATTA			

Table S4-2 (continued)

CGs markers	Primer	HRM results in parents		note
		S	F	
LOX_13375_1313_F	GATGGCTTCCCAGATCAAGA	a	a	**
LOX_13375_1313_R	TCCTGACCCGAGTCAAAGAC			
LOX_13375_3067_F	TTGCAAGGGAAATGAACAGG	a	c	
LOX_13375_3067_R	GGAGCAATCACCGTCACTAA			
LOX_28693_4515_F	TGGTCTCCGTCAATGGATCT	c	c	
LOX_28693_4515_R	GGCGATGAAAATCCTTACCC			
MMP3_15525_1055_F	ACCGTACAACACCTGAATGC	a	a	
MMP3_15525_1055_R	GGAGTACGGAAAGTCGAGCT			
MYB_11297_481_F	GGCAACAGGTGGTCTCAAAT	b	a	
MYB_11297_481_R	CCAGAAGTTCTTGATTTCGTTATCT			
OPR_196_1138_F	AGGCTTTGGGTCTATACAT	d	d	
OPR_196_1138_R	TTTTCATCCTCGGTTC			
OPR_196_514_F	CCAGGTATATGGACAAAAGAACAA	c	a	
OPR_196_514_R	CGTGAACCGCATCTACAATG			
OPR3_12803_809_F	CGGTGGCTTTCTCATCACTG	c	c	
OPR3_12803_809_R	TCCAAGCCTCGACTTGTTCT			
P450_15117_2118_F	TTCCATGCTGCGAAATCAT	a	a	
P450_15117_2118_R	AAAACGCCTTGTTCCTGTTG			
P450_8091_1686_F	TGGGCCGTAGGATTTAGAGA	b	c	
P450_8091_1686_R	TTGCACCAGATCAACAAATCA			
PAD_12282_675_F	ATGAAAGGCGACGAAGCTCT	a	a	
PAD_12282_675_R	GAACCACAAGCCCCAGACTA			
PAD_12282_1133_F	TGAAGTGAGCCTAGAACAAATGG	a	b	
PAD_12282_1133_R	CCTATCAGGGTTCTGGGATG			
PAL_10623_2338_F	GATCGAATCACCTCAATTTGC	a	b	
PAL_10623_2338_R	CCAAAACAAGACCGTTACGC			
PAL_25584_2562_F	GTCTGCTTTGATTGCCTTGG	a	a	
PAL_25584_2562_R	AGCAGGAGATGCGAGGAAT			
PAL_28938_2050_F	CCTGAAAATTCCCACCGTGA	a	a	
PAL_28938_2050_R	GGCTAGGTCCGCTTATCGAGG			
PER21_12863_774_F	GCGAGATACAACTGAATTACTACTCA	a	a	**
PER21_12863_774_R	AACGATTTCTTCGGCATTTG			
PER62_31925_540_F	ACGGACCAAATGCTGAGAAA	c	a	**
PER62_31925_540_R	CTTCGCTTTTGCATTCTCTACT			
PFT1_12433_1433_F	TGCCGTCAAATCAGAGTCCT	c	c	
PFT1_12433_1433_R	TTTACAGTAGCGGGAGGAATG			
PG1_15001_1052_F	GTGGGGTTTTCTGGCTACG	a	a	**
PG1_15001_1052_R	AGTATCATTCGCGCCGTTAC			
PG10_11147_5246_F	GTCGGATCGGTTGAAGACAT	a	b	
PG10_11147_5246_R	CGTTTGTGTTGTCGGTTGTC			
PG10_11147_2266_F	GCCGGAAACTCAGAAGACC	a	b	
PG10_11147_2266_R	ACGAGGGTTTCGGTAGATCA			
PG10_11147_3429_F	GCAGGGACCAGACGTTCAC	b	a	
PG10_11147_3429_R	GTTTCCCTTTTTGCCGTAGC			
PG2_1158_857_F	TTGACCAGAAGGGCAATATTCT	b	a	
PG2_1158_857_R	AATGCATTTCGAAGACATCGT			
PG3_10267_1039_F	CAATAGGGTTCCTTGAATTTCG	b	c	
PG3_10267_1039_R	ACTACACCGGCCAAGCTTCT			
PG3_10267_143_F	AATTTTTGCCCGTGAATGTT	b	b	
PG3_10267_143_R	TTAGTGGCCAATTTGTGCAT			
PG4_21627_1257_F	TCAAACAAGGCATGTTCAAA	d	d	
PG4_21627_1257_R	TGAAGTTCCCTTAATCTTCACGA			
PG5_8410_454_F	TGGTTTGCCGTTGTTTCTTA	c	c	
PG5_8410_454_R	CAAAAACTCAAACCGCAATG			
PG6_17218_1141_F	ATGGTGTCCGCATCAAAACT	b	c	
PG6_17218_1141_R	GGTTCGTCACATTCTGCATC			
PG6_17218_605_F	GACCGTGTTGACAACCTCCT	b	c	
PG6_17218_605_R	GCAGGAATTTTTCCACCAAA			
PG7_35500_1285_F	ACAAAGGCTGGACGGTAAAA	a	c	
PG7_35500_1285_R	TCTGCAGACGGTTGAACTAA			
PG8_10107_508_F	TGAGGCCGAAGTACCTTTGA	c	b	
PG8_10107_508_R	GCCCTTTCCAGAAATGTGAC			
PG9_25150_888_F	CAGAGGAACTTCAGGGACGA	a	c	
PG9_25150_888_R	TCAAGTCCACTTCAGAAATCTCC			
PgD_28711_481_F	TTTGGTGCAGAAACAACGAC	c	c	
PgD_28711_481_R	AAGACTGAAGGCTTTCAAGGTG			

Table S4-2 (continued)

CGs markers	Primer	HRM results in parents		note
		S	F	
PGIP1_17627_1064_F	GACGACAAACCGTATCACTGAC	a	c	
PGIP1_17627_1064_R	CAAATCACCAATGGCATCAG			
PGIP1_17627_575_F	TGTCACCAGTGTGTTTTCCA	d	d	
PGIP1_17627_575_R	CGATGTTATTTGGTGCATCG			
PGIP1_17627_928_F	TAGACTCAAATACGTCACGTT	a	c	
PGIP1_17627_928_R	GCCCGATAGGTTGTTGAATGA			
PGIP2_20957_2153_F	TTGTACCATTGGCAGCAATC	d	d	
PGIP2_20957_2153_R	CTCTCTCCCAAACCTGCAAC			
PGIP2_20957_910_F	GGATCAAACAAGACGGTTCAA	b	a	
PGIP2_20957_910_R	GACTCCCGGAAATCGTGTTA			
PLDB1_14327_2622_F	TGATGCTGGATATGGTAAAAGAAA	c	c	
PLDB1_14327_2622_R	ACCGACAAGATTACCCGTGA			
RAP_25598_2336_F	TTGAAGGAAACTGGGATGCT	c	c	
RAP_25598_2336_R	GAAGGTCATCGAAGGTCCAA			
RAP_25598_2431_F	TCTTTGGACCTTCGATGACC	a	c	
RAP_25598_2431_R	CCTTATTTACAAACATGAGCGAAA			
RAP_25598_705_F	CAGCCTCCAAAGACTCTGCT	c	c	
RAP_25598_705_R	TTCTCACCTTGATTATCAGACTTTACA			
RD21_20206_4283_F	TGGAAACGAGTGCGATTAAG	a	a	
RD21_20206_4283_R	TCTCAATCCGTTCCACCTCT			
RD21_20206_I2_F	GATGGAAAATGCGACAGTGCC	a	c	
RD21_20206_I2_R	CAATCGAAACAACCTTCGCGTT			
RWA_29675_2498_F	TCCGGCAATCCTGATCTTAG	d	d	
RWA_29675_2498_R	CATGGCTGGGAAAAATCACT			
sit_19807_6510_F	TTCAACCACCTGTTTTGCAG	a	a	**
sit_19807_6510_R	AATTCTGGGATTTGTTCAAGATT			
sit_19807_I2_F	GACACGTTGAGTGTGGTTCAA	b	a	
sit_19807_I2_R	TCCACCAAAACCTCACAACA			
SR1_1903_2688_F	AAGCCTGTTGGAACATGTTATG	b	a	
SR1_1903_2688_R	TCAACAAGAATTGCAGCATTG			
SS_5198_1668_F	GACCCAACTATGATAGCAGCAG	a	a	
SS_5198_1668_R	GCCTGATGAAACCCATCTTG			
SS_5198_2832_F	CGCTTTTTGTAACACGGAAA	a	a	
SS_5198_2832_R	ATTATGGCCATCGGGACAG			
SSI2_11878_539_F	CTTCCACCCTTGGATCGTC	c	c	
SSI2_11878_539_R	ATATTCTGCTCAGCCCAACC			
STP13_6824_1336_F	CTGAAGAGTACAGGCGCGTA	c	c	
STP13_6824_1336_R	GTCGCATCGCTAAAGAAGTCA			
WRKY33_16847_1652_F	GGTTGCTTTGGACTTTCACC	b	a	
WRKY33_16847_1652_R	GAACCCAATTGGAGCTTCTTT			
ZF_1325_432_F	GCAACACGACTGTGCATCTT	a	a	
ZF_1325_432_R	ACGATTTCCGCCACCGTA			
ZF_30219_1665_F	CCCGTTGTCATCGGTTAAAT	a	a	
ZF_30219_1665_R	CTGTGAATTCCGTTGAAACC			
ZF_30219_1910_F	TCGATCCGTTTCTTCTTCCA	c	a	
ZF_30219_1910_R	ATGGGTTTGTATCGGAGGTG			
ZF_30683_646_F	CAAGAGACGCCACTATGAAGG	c	c	
ZF_30683_646_R	ACCCTCCGACGAGGTGAC			
GAPDH_F	CCAGGAACCCAGAGGAGATACC			**
GAPDH_R	GGAGCGGATATGATGACCTTCTTG			

* Four possible cases after PCR reaction and HRM analysis (a, b, c, d) see also Fig.4-1
** Primer used for qRT-PCR

Table S4-3 QTLs found for *bottom*, *whole inflorescence*,and *ray florets* test in the parental genetic maps of two populations (after adding the new CG markers)

QTL	Flanking Markers	Linkage Group	MQM	
			LOD (GW)	% Expl.
RBQB1	WGC11243_647_S2F1a	SP1_02	5.19 (4.2)	7.3
RBQB2	WGC2476_271_S1	SP1_16	4.48 (4.2)	6.3
RBQB3	WGC18733_346_S2F	SP2_11	5.25 (4.0)	8.6
RBQB4	WGC16204_523_S2F1	FP1_01	6.87 (4.2)	10.4
RBQB5	WGC28102_213_S2F1	FP1_09	5.28 (4.2)	7.8
RBQB6	WGC18158_119_F1b	FP2_09	4.74 (4.1)	7.9
RBQRF1	WGC17798_117_S2F1	SP2_07	5.21 (4.0)	8.7
RBQRF2	*WGC22343_292_SFa, sit_19807_6510*	*FP1_05*	*6.81 (4.1)*	*8.9*
RBQRF3	WGC35370_146_S2F1	FP1_09	5.27 (4.1)	6.8
RBQRF4	WGC828_408_S2F	FP1_15	6.83 (4.1)	8.9
RBQRF5	WGC35264_283_S2F1, WGC6074_441_S2F	FP1_18	5.5 (4.1)	7.1
RBQRF6	WGC7520_3774_S1F2, WGC828_408_S2F	FP2_15	4.31 (4.1)	6.5
RBQRF7	WGC6074_441_S2F	FP2_18	4.09 (4.1)	5.9
RBQRF8	*PG1_15001_1052*	*FP2_21*	*6.42 (4.1)*	*10.7*
RBQWI1	WGC33030_228_S	SP1_11	4.74 (4.0)	7.2
RBQWI2	WGC1824_721_S1F1	SP1_23	5.46 (4.0)	8.4
RBQWI3	WGC18733_346_S2F	SP2_11	5.25 (4.0)	8.6
RBQWI4	WGC22447_285_Fa	FP1_23	6.96 (4.2)	11.4
RBQWI5	WGC5962_1153_F	FP2_17	5.34 (4.1)	8
RBQWI6	WGC1084_721_F	FP2_23	7.25 (4.1)	11
*RBQB7**	*WGC19218_398_S1F1*	*SP1_06*	*5.33 (4.2)*	*7.6*
*RBQB8**	*PG9_25150_888*	*SP2_02*	*4.0 (4.0)*	*6.1*

Note: Name of QTLs are RBQ (as Resistance Botrytis QTL) followed by the initials of disease tests used: B=*Bottom*; RF=*Ray Floret*; WI=*Whole inflorescence* test. LG indicates linkage group and the LG number in the two populations ; Null-alleles are marked with a letter 'a' or 'b' in the end; GW indicates genome wide significant threshold level P<0.05; %Expl. is the percentage of total variance explained by the QTL. QTLs with * indication are the two new QTLs.

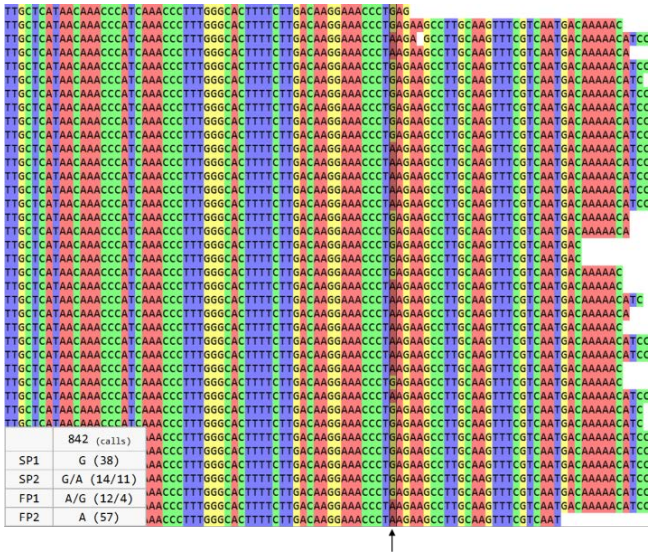


Fig. S4-1. Visualized output from QualitySNPng which is presented SNP in contig17688 of the parental reads. Arrow indicates the SNP at the position 842 for marker design and table on the left *bottom* shows the SNPs calls on different parental reads.

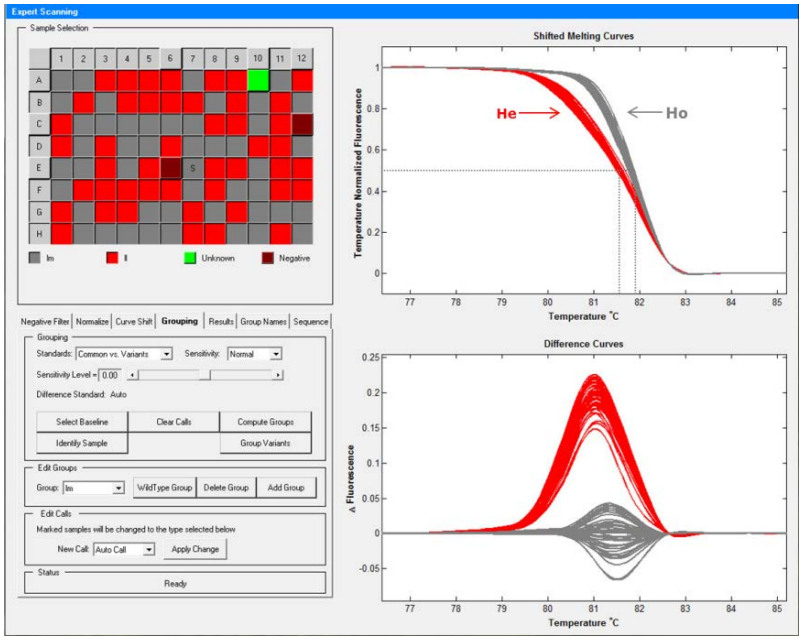
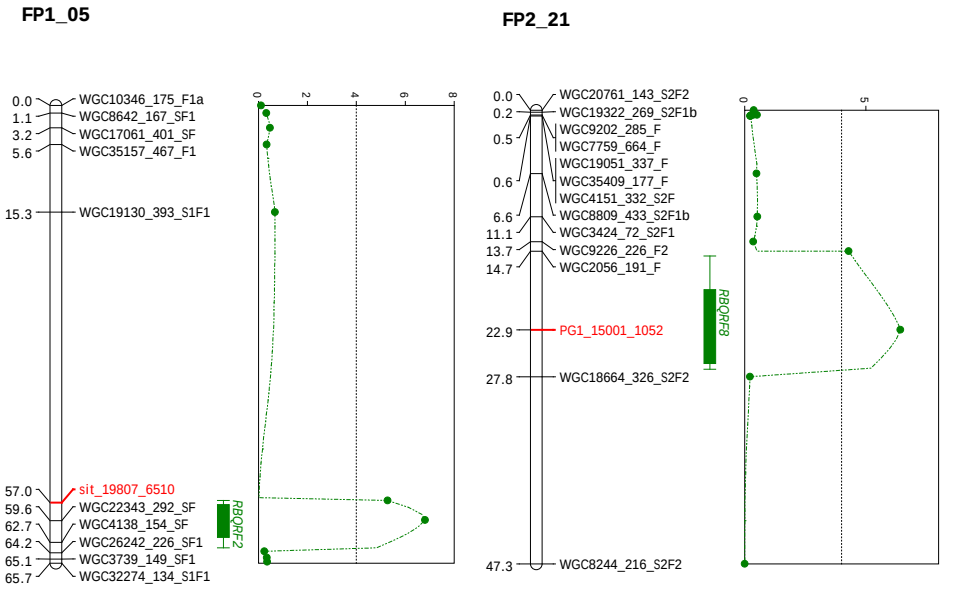


Fig. S4-2 HRM results of contig1926 (the homologue hit of anthranilate synthase/AS gene in Arabidopsis) on part offspring of the Schreurs population. Grey curve group represents homozygous alleles; red curve group represents heterozygous alleles. Note: H11 and H12 on the 96-well plate represent Parent 1 and Parent 2 (of population S), and the others represent individuals in the population. All individuals are in three plates. He. Heterozygote. Ho. Homozygote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5.6 WGC35157_467_F1
15.3 WGC19130_393_S1F1
57.0 sit_19807_6510
59.6 WGC22343_292_SF
62.7 WGC4138_154_SF
64.2 WGC26242_226_SF1
65.1 WGC3739_149_SF1
65.7 WGC32274_134_S1F1
RBQRF2
0 2 4 6 8
FP2_21
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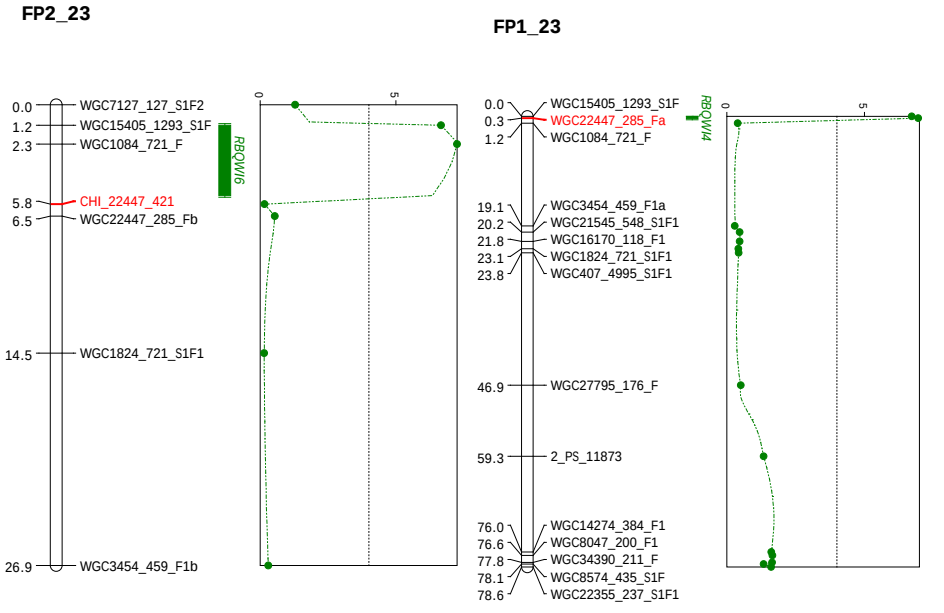
0.2 WGC19322_269_S2F1b
0.5 WGC9202_285_F
WGC7759_664_F
WGC19051_337_F
0.6 WGC35409_177_F
WGC4151_332_S2F
6.6 WGC8809_433_S2F1b
11.1 WGC3424_72_S2F1
13.7 WGC9226_226_F2
14.7 WGC2056_191_F
22.9 PG1_15001_1052
27.8 WGC18664_326_S2F2
47.3 WGC8244_216_S2F2
RBQRF8
0 5
a
b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2.3 WGC1084_721_F
5.8 CHI_22447_421
6.5 WGC22447_285_Fb
14.5 WGC1824_721_S1F1
26.9 WGC3454_459_F1b
RBQWI6
0 5
FP1_23
0.0 WGC15405_1293_S1F
0.3 WGC22447_285_Fa
1.2 WGC1084_721_F
19.1 WGC3454_459_F1a
20.2 WGC21545_548_S1F1
21.8 WGC16170_118_F1
23.1 WGC1824_721_S1F1
23.8 WGC407_4995_S1F1
46.9 WGC27795_176_F
59.3 2_PS_11873
76.0 WGC14274_384_F1
76.6 WGC8047_200_F1
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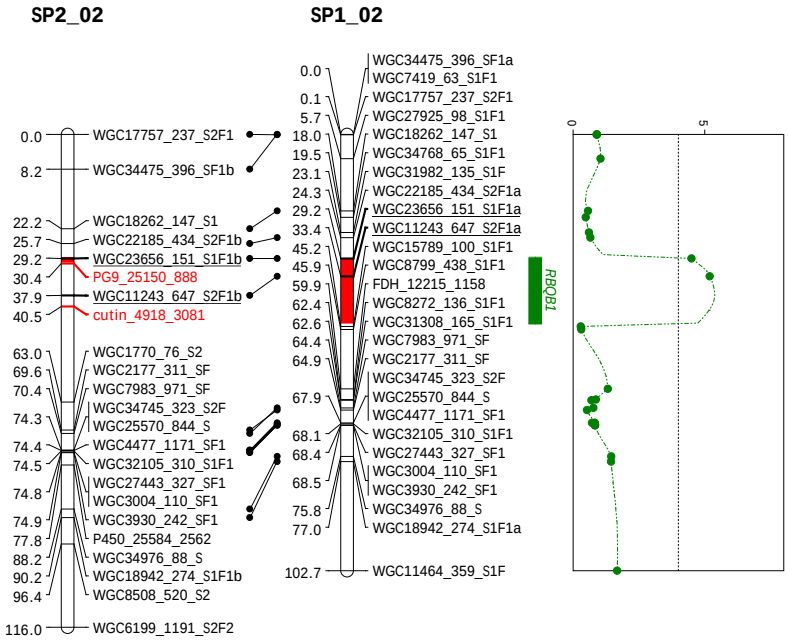
78.1 WGC8574_435_S1F
78.6 WGC22355_237_S1F1
RBQWI4
0 5
c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29.2 WGC23656_151_S1F1b
30.4 PG9_25150_888
37.9 WGC11243_647_S2F1b
40.5 cutin_4918_3081
63.0 WGC1770_76_S2
69.6 WGC2177_311_SF
70.4 WGC7983_971_SF
74.3 WGC34745_323_S2F
WGC25570_844_S
74.4 WGC4477_1171_SF1
74.5 WGC32105_310_S1F1
74.8 WGC27443_327_SF1
WGC3004_110_SF1
74.9 WGC3930_242_SF1
77.8 P450_25584_2562
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90.2 WGC18942_274_S1F1b
96.4 WGC8508_520_S2
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5.7 WGC17757_237_S2F1
18.0 WGC27925_98_S1F1
19.5 WGC18262_147_S1
23.1 WGC34768_65_S1F1
24.3 WGC31982_135_S1F
29.2 WGC22185_434_S2F1a
33.4 WGC23656_151_S1F1a
45.2 WGC11243_647_S2F1a
45.9 WGC15789_100_S1F1
59.9 WGC8799_438_S1F1
62.4 FDH_12215_1158
62.6 WGC8272_136_S1F1
64.4 WGC31308_165_S1F1
64.9 WGC7983_971_SF
67.9 WGC2177_311_SF
WGC34745_323_S2F
68.1 WGC25570_844_S
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WGC3930_242_SF1
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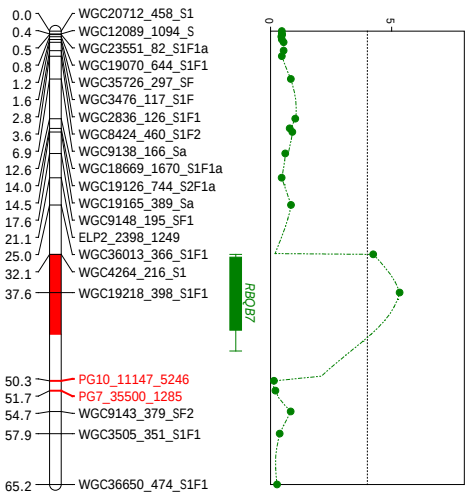
WGC18942_274_S1F1a
102.7 WGC11464_359_S1F
RBQB1
0
5

d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0.5 WGC23551_82_S1F1a
0.8 WGC19070_644_S1F1
1.2 WGC35726_297_SF
1.6 WGC3476_117_S1F
2.8 WGC2836_126_S1F1
3.6 WGC8424_460_S1F2
6.9 WGC9138_166_Sa
12.6 WGC18669_1670_S1F1a
14.0 WGC19126_744_S2F1a
14.5 WGC19165_389_Sa
17.6 WGC9148_195_SF1
21.1 ELP2_2398_1249
25.0 WGC36013_366_S1F1
32.1 WGC4264_216_S1
37.6 WGC19218_398_S1F1
50.3 PG10_11147_5246
51.7 PG7_35500_1285
54.7 WGC9143_379_SF2
57.9 WGC3505_351_S1F1
65.2 WGC36650_474_S1F1
RBQB7
0
5

e

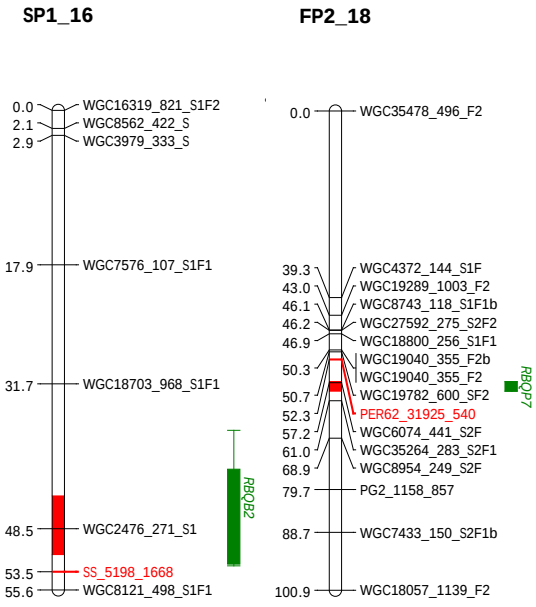
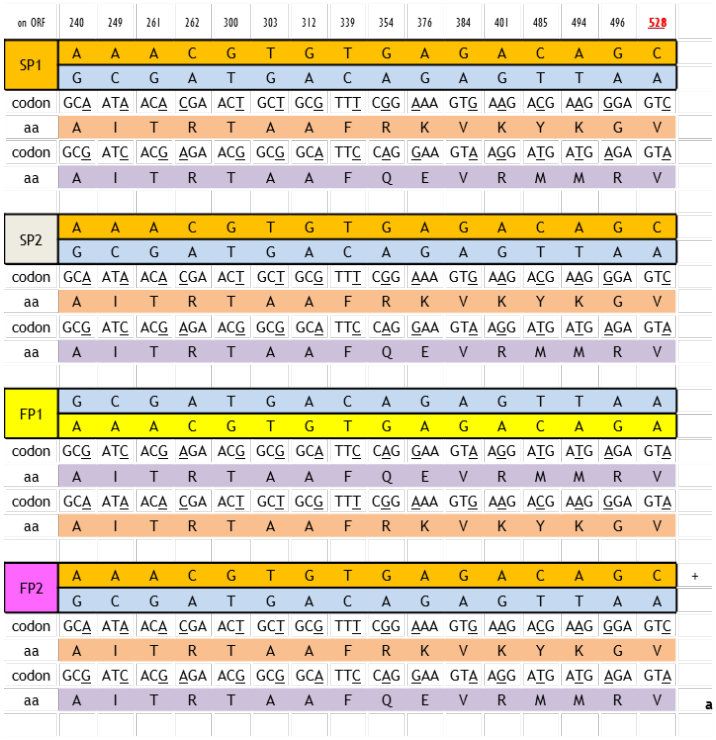


Fig. S4-3 Mapped CGs and QTL analysis after added CGs. a, the mapped *ghsit*on FP2_05 in the region of QTL RBQRF2, explained the 6.81% variance. b, the mapped *ghPG1* on FP2_21 in the region of QTL RBQRF8, explained the 10.7% variance. c The QTLs RBQWI4 and RBQWI6 in the maternal and paternal LG23 of F populationsd. The marker WGC22447_285_Fa is a KASP marker from contig22447 and CHI_22447_421 is a marker for HRM. d, *ghPG9* and *ghcutin* mapped on the SP2_02 around WGC23656_151_S1F1 and WGC11243_647_S2F1 and a QTLs is between the markers on SP1_02. e, new QTL RBQB7 from SP1_06 (LOD = 5.33) with the two CGs (*ghPG7* and *ghPG10*) mapped; f, the mapped *ghSS* on SP1_16 near the region of QTL RBQB2. g, the mapped *ghPER62* on FP2_18 near the region of QTL RBQRF7.



on ORF	240	249	261	262	300	303	312	339	354	376	384	401	485	494	496	528	
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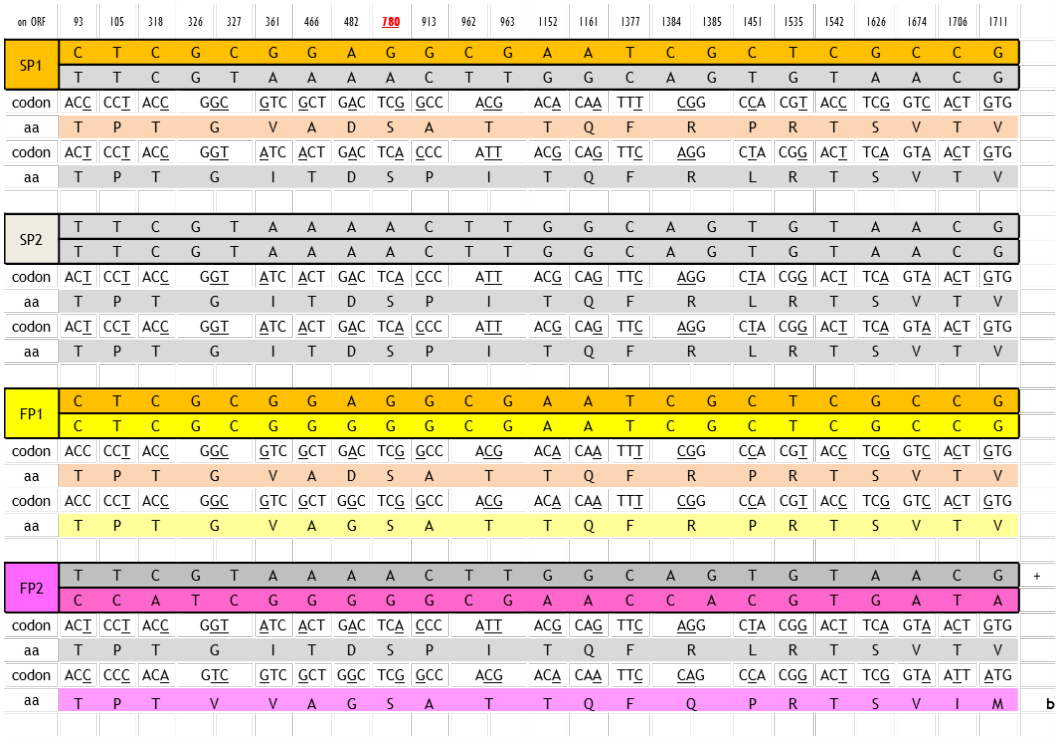
	G	C	G	A	T	G	A	C	A	G	A	G	T	T	A	A	
codon	GCA	ATA	ACA	CGA	ACT	GCT	GCG	TTT	CGG	AAA	GTG	AAG	ACG	AAG	GGA	GTC	
aa	A	I	T	R	T	A	A	F	R	K	V	K	Y	K	G	V	
codon	GCG	ATC	ACG	AGA	ACG	GCG	GCA	TTC	CAG	GAA	GTA	AGG	ATG	ATG	AGA	GTA	
aa	A	I	T	R	T	A	A	F	Q	E	V	R	M	M	R	V	
SP2	A	A	A	C	G	T	G	T	G	A	G	A	C	A	G	C	
	G	C	G	A	T	G	A	C	A	G	A	G	T	T	A	A	
codon	GCA	ATA	ACA	CGA	ACT	GCT	GCG	TTT	CGG	AAA	GTG	AAG	ACG	AAG	GGA	GTC	
aa	A	I	T	R	T	A	A	F	R	K	V	K	Y	K	G	V	
codon	GCG	ATC	ACG	AGA	ACG	GCG	GCA	TTC	CAG	GAA	GTA	AGG	ATG	ATG	AGA	GTA	
aa	A	I	T	R	T	A	A	F	Q	E	V	R	M	M	R	V	
FP1	G	C	G	A	T	G	A	C	A	G	A	G	T	T	A	A	
	A	A	A	C	G	T	G	T	G	A	G	A	C	A	G	A	
codon	GCG	ATC	ACG	AGA	ACG	GCG	GCA	TTC	CAG	GAA	GTA	AGG	ATG	ATG	AGA	GTA	
aa	A	I	T	R	T	A	A	F	Q	E	V	R	M	M	R	V	
codon	GCA	ATA	ACA	CGA	ACT	GCT	GCG	TTT	CGG	AAA	GTG	AAG	ACG	AAG	GGA	GTA	
aa	A	I	T	R	T	A	A	F	R	K	V	K	Y	K	G	V	
FP2	A	A	A	C	G	T	G	T	G	A	G	A	C	A	G	C	+
	G	C	G	A	T	G	A	C	A	G	A	G	T	T	A	A	
codon	GCA	ATA	ACA	CGA	ACT	GCT	GCG	TTT	CGG	AAA	GTG	AAG	ACG	AAG	GGA	GTC	
aa	A	I	T	R	T	A	A	F	R	K	V	K	Y	K	G	V	
codon	GCG	ATC	ACG	AGA	ACG	GCG	GCA	TTC	CAG	GAA	GTA	AGG	ATG	ATG	AGA	GTA	
aa	A	I	T	R	T	A	A	F	Q	E	V	R	M	M	R	V	

a



on ORF	93	105	318	326	327	361	466	482	780	913	962	963	1152	1161	1377	1384	1385	1451	1535	1542	1626	1674	1706	1711	
SP1	C	T	C	G	C	G	G	A	G	G	C	G	A	A	T	C	G	C	T	C	G	C	C	G	
	T	T	C	G	T	A	A	A	A	C	T	T	G	G	C	A	G	T	G	T	A	A	C	G	
codon	ACC	CCT	ACC	GGC		GTC	GCT	GAC	TCG	GCC	ACG		ACA	CAA	TTT	CGG		CCA	CGT	ACC	TCG	GTC	ACT	GTG		
aa	T	P	T	G		V	A	D	S	A	T		T	Q	F	R		P	R	T	S	V	T	V		
codon	ACT	CCT	ACC	GGT		ATC	ACT	GAC	TCA	CCC	ATT		ACG	CAG	TTC	AGG		CTA	CGG	ACT	TCA	GTA	ACT	GTG		
aa	T	P	T	G		I	T	D	S	P	I		T	Q	F	R		L	R	T	S	V	T	V		
SP2	T	T	C	G	T	A	A	A	A	C	T	T	G	G	C	A	G	T	G	T	A	A	C	G	
	T	T	C	G	T	A	A	A	A	C	T	T	G	G	C	A	G	T	G	T	A	A	C	G	
codon	ACT	CCT	ACC	GGT		ATC	ACT	GAC	TCA	CCC	ATT		ACG	CAG	TTC	AGG		CTA	CGG	ACT	TCA	GTA	ACT	GTG		
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codon	ACT	CCT	ACC	GGT		ATC	ACT	GAC	TCA	CCC	ATT		ACG	CAG	TTC	AGG		CTA	CGG	ACT	TCA	GTA	ACT	GTG		
aa	T	P	T	G		I	T	D	S	P	I		T	Q	F	R		L	R	T	S	V	T	V		
FP1	C	T	C	G	C	G	G	A	G	G	C	G	A	A	T	C	G	C	T	C	G	C	C	G	
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codon	ACC	CCT	ACC	GGC		GTC	GCT	GGC	TCG	GCC	ACG		ACA	CAA	TTT	CGG		CCA	CGT	ACC	TCG	GTC	ACT	GTG		
aa	T	P	T	G		V	A	G	S	A	T		T	Q	F	R		P	R	T	S	V	T	V		
FP2	T	T	C	G	T	A	A	A	A	C	T	T	G	G	C	A	G	T	G	T	A	A	C	G	+
	C	C	A	T	C	G	G	G	G	G	C	G	A	A	C	C	A	C	G	T	G	A	T	A	
codon	ACT	CCT	ACC	GGT		ATC	ACT	GAC	TCA	CCC	ATT		ACG	CAG	TTC	AGG		CTA	CGG	ACT	TCA	GTA	ACT	GTG		
aa	T	P	T	G		I	T	D	S	P	I		T	Q	F	R		L	R	T	S	V	T	V		
codon	ACC	CCC	ACA	GTC		GTC	GCT	GGC	TCG	GCC	ACG		ACA	CAA	TTC	CAG		CCA	CGG	ACT	TCG	GTA	ATT	ATG		
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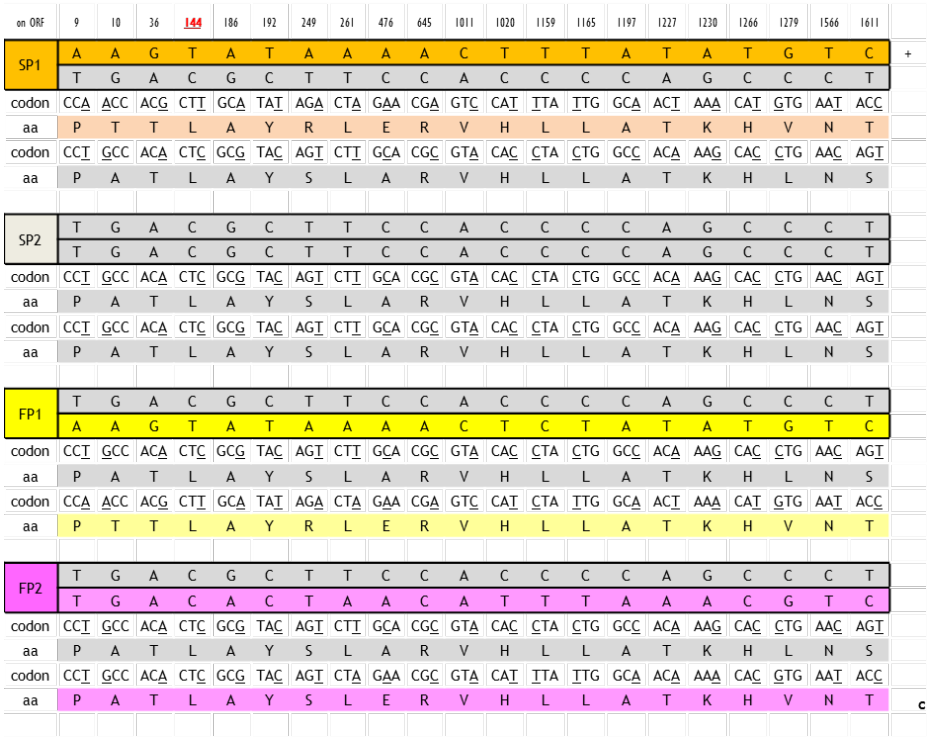
b



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	T	G	A	C	G	C	T	T	C	C	A	C	C	C	C	A	G	C	C	C	T	
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codon	CCT	GCC	ACA	CTC	GCG	TAC	AGT	CTT	GCA	CGC	GTA	CAC	CTA	CTG	GCC	ACA	AAG	CAC	CTG	AAC	AGT	
aa	P	A	T	L	A	Y	S	L	A	R	V	H	L	L	A	T	K	H	L	N	S	
SP2	T	G	A	C	G	C	T	T	C	C	A	C	C	C	C	A	G	C	C	C	T	
	T	G	A	C	G	C	T	T	C	C	A	C	C	C	C	A	G	C	C	C	T	
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FP1	T	G	A	C	G	C	T	T	C	C	A	C	C	C	C	A	G	C	C	C	T	
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FP2	T	G	A	C	G	C	T	T	C	C	A	C	C	C	C	A	G	C	C	C	T	
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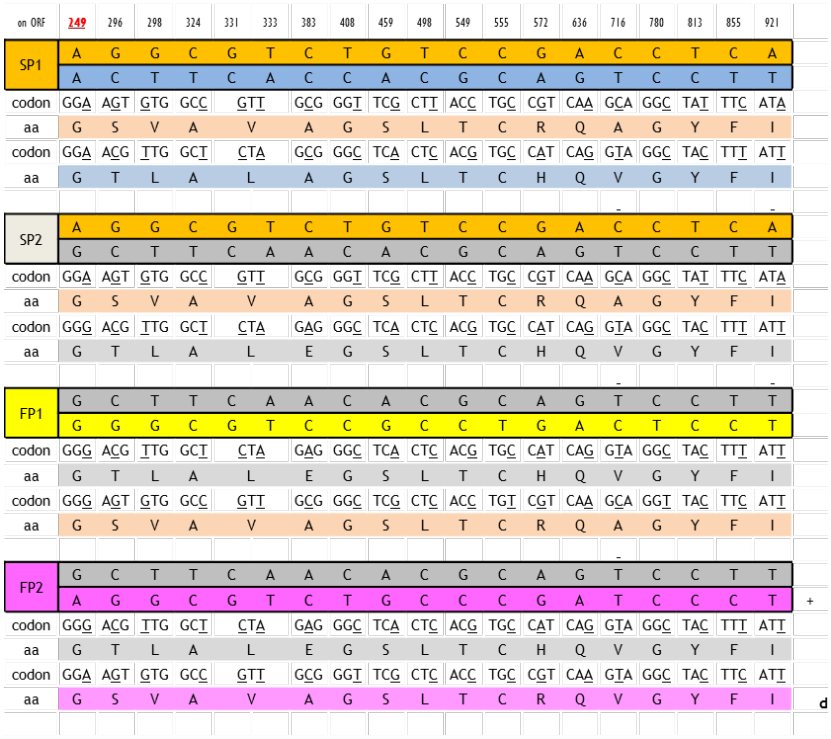

on ORF	249	296	298	324	331	333	383	408	459	498	549	555	572	636	716	780	813	855	921	
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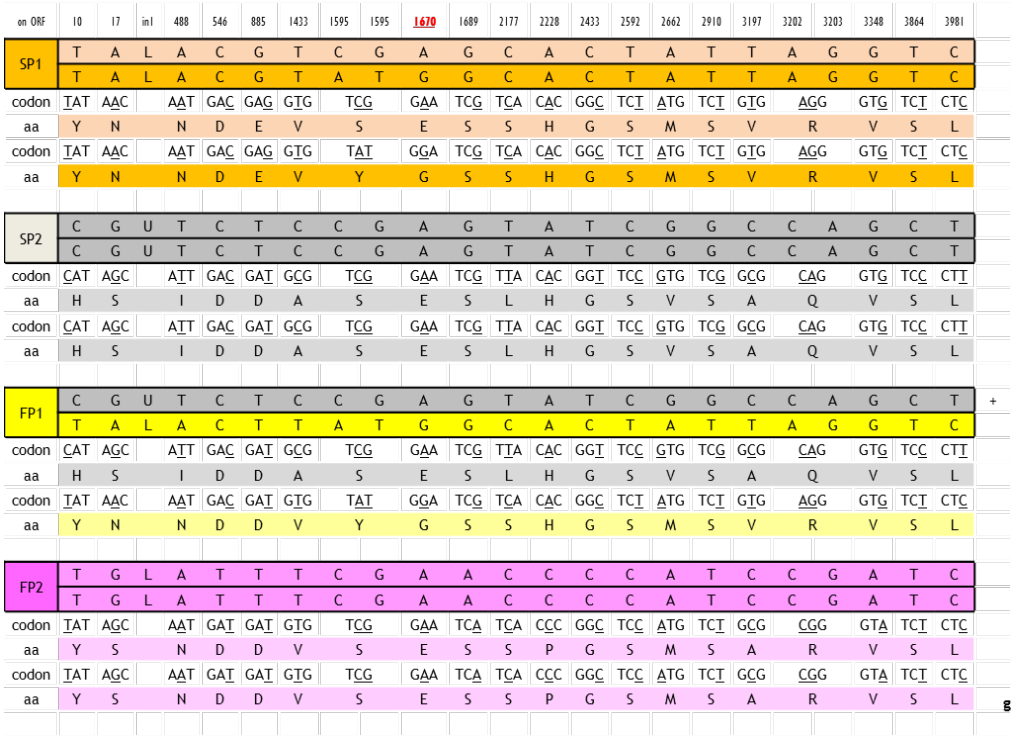
	A	C	T	T	C	A	C	C	A	C	G	C	A	G	T	C	C	T	T	
codon	GGA	AGT	GTG	GCC	GTT		GCG	GGT	TCG	CTT	ACC	TGC	CGT	CAA	GCA	GGC	TAT	TTC	ATA	
aa	G	S	V	A	V		A	G	S	L	T	C	R	Q	A	G	Y	F	I	
codon	GGA	ACG	TTG	GCT	CTA		GCG	GGC	TCA	CTC	ACG	TGC	CAT	CAG	GTA	GGC	TAC	TTT	ATT	
aa	G	T	L	A	L		A	G	S	L	T	C	H	Q	V	G	Y	F	I	
SP2	A	G	G	C	G	T	C	T	G	T	C	C	G	A	C	C	T	C	A	
	G	C	T	T	C	A	A	C	A	C	G	C	A	G	T	C	C	T	T	
codon	GGA	AGT	GTG	GCC	GTT		GCG	GGT	TCG	CTT	ACC	TGC	CGT	CAA	GCA	GGC	TAT	TTC	ATA	
aa	G	S	V	A	V		A	G	S	L	T	C	R	Q	A	G	Y	F	I	
codon	GGG	ACG	TTG	GCT	CTA		GAG	GGC	TCA	CTC	ACG	TGC	CAT	CAG	GTA	GGC	TAC	TTT	ATT	
aa	G	T	L	A	L		E	G	S	L	T	C	H	Q	V	G	Y	F	I	
FP1	G	C	T	T	C	A	A	C	A	C	G	C	A	G	T	C	C	T	T	
	G	G	G	C	G	T	C	C	G	C	C	T	G	A	C	T	C	C	T	
codon	GGG	ACG	TTG	GCT	CTA		GAG	GGC	TCA	CTC	ACG	TGC	CAT	CAG	GTA	GGC	TAC	TTT	ATT	
aa	G	T	L	A	L		E	G	S	L	T	C	H	Q	V	G	Y	F	I	
codon	GGG	AGT	GTG	GCC	GTT		GCG	GGC	TCG	CTC	ACC	TGT	CGT	CAA	GCA	GGT	TAC	TTC	ATT	
aa	G	S	V	A	V		A	G	S	L	T	C	R	Q	A	G	Y	F	I	
FP2	G	C	T	T	C	A	A	C	A	C	G	C	A	G	T	C	C	T	T	
	A	G	G	C	G	T	C	T	G	C	C	C	G	A	T	C	C	C	T	+
codon	GGG	ACG	TTG	GCT	CTA		GAG	GGC	TCA	CTC	ACG	TGC	CAT	CAG	GTA	GGC	TAC	TTT	ATT	
aa	G	T	L	A	L		E	G	S	L	T	C	H	Q	V	G	Y	F	I	
codon	GGA	AGT	GTG	GCC	GTT		GCG	GGT	TCG	CTC	ACC	TGC	CGT	CAA	GTA	GGC	TAC	TTC	ATT	
aa	G	S	V	A	V		A	G	S	L	T	C	R	Q	V	G	Y	F	I	d

on ORF	14	30	100	582	645	684	783	792	
SP1	A	G	G	A	C	A	C	G	+
	T	A	A	T	T	G	C	C	
codon	GAG	GCG	GAC	ACA	TTC	CCA	GTC	ACG	
aa	E	A	D	T	F	P	V	T	
codon	GTG	GCA	AAC	ACT	TTT	CCG	GTC	ACC	
aa	V	A	N	T	F	P	V	T	
SP2	T	A	A	T	T	G	C	C	
	T	A	A	T	T	G	C	C	
codon	GTG	GCA	AAC	ACT	TTT	CCG	GTC	ACC	
aa	V	A	N	T	F	P	V	T	
codon	GTG	GCA	AAC	ACT	TTT	CCG	GTC	ACC	
aa	V	A	N	T	F	P	V	T	
FP1	A	G	G	A	C	A	C	G	
	A	A	G	A	C	A	T	G	
codon	GAG	GCG	GAC	ACA	TTC	CCA	GTC	ACG	
aa	E	A	D	T	F	P	V	T	
codon	GAG	GCG	GAC	ACA	TTC	CCA	GTT	ACG	
aa	E	A	D	T	F	P	V	T	
FP2	A	G	G	A	C	A	C	G	
	T	A	A	T	T	G	C	C	
codon	GAG	GCG	GAC	ACA	TTC	CCA	GTC	ACG	
aa	E	A	D	T	F	P	V	T	
codon	GTG	GCA	AAC	ACT	TTT	CCG	GTC	ACC	
aa	V	A	N	T	F	P	V	T	

e

on ORF	70	113	145	180	199	317	320	356	366	380	395	414	479	520	665	
SP1	G	C	A	G	A	A	C	T	G	A	T	T	A	G	T	
	A	C	G	G	T	T	-	A	A	G	C	C	G	G	T	+
codon	GAG	CCG	ACC	TTG	ATC	AAG	ACG	ATG	GTG	GAT	GTG	ACT	CAA	GTT	ATA	
aa	E	P	T	L	I	K	T	M	V	D	V	T	Q	V	I	
codon	AAG	CCG	GCC	TTG	TTC	ATG		AAG	GTA	GGT	GCG	ACC	CGA	GTT	ATA	
aa	K	P	A	L	F	M		K	V	G	A	T	R	V	I	
SP2	A	T	G	C	T	T	-	A	A	G	C	C	A	G	C	
	A	T	G	C	T	T	-	A	A	G	C	C	A	G	C	
codon	AAG	CTG	GCC	TTC	TTC	ATG		AAG	GTA	GGT	GCG	ACC	CAA	GTT	ACA	
aa	K	L	A	F	F	M		K	V	G	A	T	Q	V	T	
codon	AAG	CTG	GCC	TTC	TTC	ATG		AAG	GTA	GGT	GCG	ACC	CAA	GTT	ACA	
aa	K	L	A	F	F	M		K	V	G	A	T	Q	V	T	
FP1	A	T	G	C	T	T	-	A	A	G	C	C	A	G	C	+
	A	C	G	G	T	T	-	A	A	G	C	C	G	G	T	
codon	AAG	CTG	GCC	TTC	TTC	ATG		AAG	GTA	GGT	GCG	ACC	CAA	GTT	ACA	
aa	K	L	A	F	F	M		K	V	G	A	T	Q	V	T	
codon	AAG	CCG	GCC	TTG	TTC	ATG		AAG	GTA	GGT	GCG	ACC	CGA	GTT	ATA	
aa	K	P	A	L	F	M		K	V	G	A	T	R	V	I	
FP2	G	C	A	G	A	A	C	T	G	A	T	T	A	G	T	+
	A	T	G	C	T	T	-	A	A	G	C	C	A	A	C	
codon	GAG	CCG	ACC	TTG	ATC	AAG	ACG	ATG	GTG	GAT	GTG	ACT	CAA	GTT	ATA	
aa	E	P	T	L	I	K	T	M	V	D	V	T	Q	V	I	
codon	AAG	CTG	GCC	TTC	TTC	ATG		AAG	GTA	GGT	GCG	ACC	CAA	GTT	ACA	
aa	K	L	A	F	F	M		K	V	G	A	T	Q	V	T	

f



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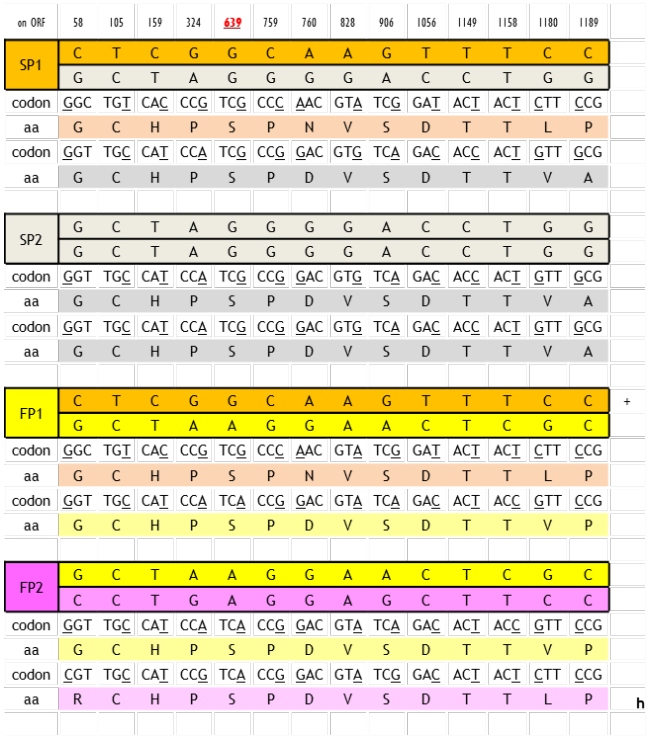
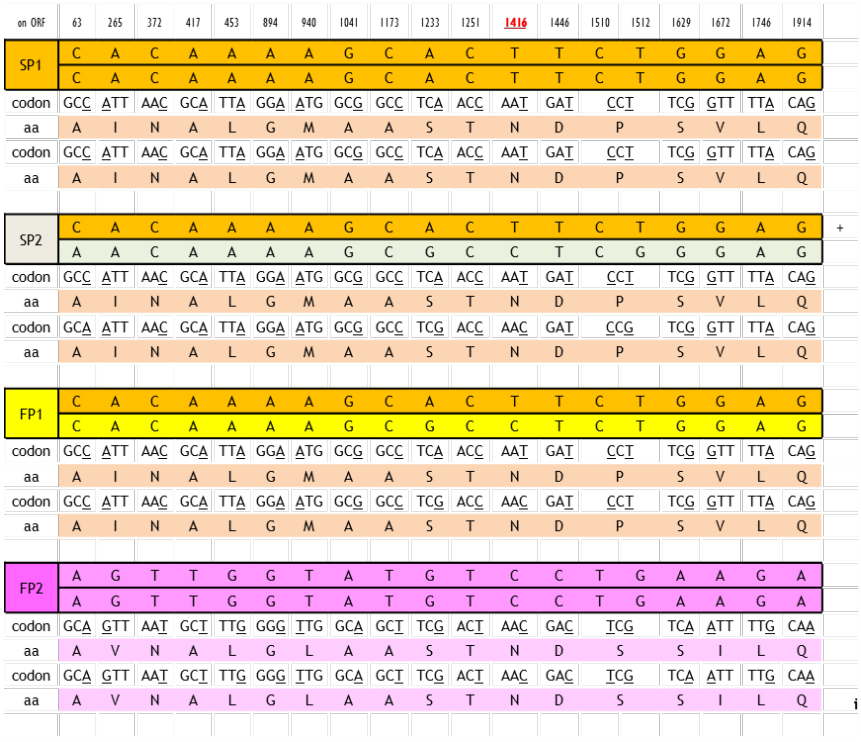
SP1	T	A	L	A	C	G	T	C	G	A	G	C	A	C	T	A	T	T	A	G	G	T	C	
	T	A	L	A	C	G	T	A	T	G	G	C	A	C	T	A	T	T	A	G	G	T	C	
codon	TAT	AAC		AAT	GAC	GAG	GTG	TCG		GAA	TCG	TCA	CAC	GGC	TCT	ATG	TCT	GTG	AGG		GTG	TCT	CTC	
aa	Y	N		N	D	E	V	S		E	S	S	H	G	S	M	S	V	R		V	S	L	
codon	TAT	AAC		AAT	GAC	GAG	GTG	TAT		GGA	TCG	TCA	CAC	GGC	TCT	ATG	TCT	GTG	AGG		GTG	TCT	CTC	
aa	Y	N		N	D	E	V	Y		G	S	S	H	G	S	M	S	V	R		V	S	L	
SP2	C	G	U	T	C	T	C	C	G	A	G	T	A	T	C	G	G	C	C	A	G	C	T	
	C	G	U	T	C	T	C	C	G	A	G	T	A	T	C	G	G	C	C	A	G	C	T	
codon	CAT	AGC		ATT	GAC	GAT	GCG	TCG		GAA	TCG	TTA	CAC	GGT	TCC	GTG	TCG	GCG	CAG		GTG	TCC	CTT	
aa	H	S		I	D	D	A	S		E	S	L	H	G	S	V	S	A	Q		V	S	L	
codon	CAT	AGC		ATT	GAC	GAT	GCG	TCG		GAA	TCG	TTA	CAC	GGT	TCC	GTG	TCG	GCG	CAG		GTG	TCC	CTT	
aa	H	S		I	D	D	A	S		E	S	L	H	G	S	V	S	A	Q		V	S	L	
FP1	C	G	U	T	C	T	C	C	G	A	G	T	A	T	C	G	G	C	C	A	G	C	T	+
	T	A	L	A	C	T	T	A	T	G	G	C	A	C	T	A	T	T	A	G	G	T	C	
codon	CAT	AGC		ATT	GAC	GAT	GCG	TCG		GAA	TCG	TTA	CAC	GGT	TCC	GTG	TCG	GCG	CAG		GTG	TCC	CTT	
aa	H	S		I	D	D	A	S		E	S	L	H	G	S	V	S	A	Q		V	S	L	
codon	TAT	AAC		AAT	GAC	GAT	GTG	TAT		GGA	TCG	TCA	CAC	GGC	TCT	ATG	TCT	GTG	AGG		GTG	TCT	CTC	
aa	Y	N		N	D	D	V	Y		G	S	S	H	G	S	M	S	V	R		V	S	L	
FP2	T	G	L	A	T	T	T	C	G	A	A	C	C	C	C	A	T	C	C	G	A	T	C	
	T	G	L	A	T	T	T	C	G	A	A	C	C	C	C	A	T	C	C	G	A	T	C	
codon	TAT	AGC		AAT	GAT	GAT	GTG	TCG		GAA	TCA	TCA	CCC	GGC	TCC	ATG	TCT	GCG	CGG		GTA	TCT	CTC	
aa	Y	S		N	D	D	V	S		E	S	S	P	G	S	M	S	A	R		V	S	L	
codon	TAT	AGC		AAT	GAT	GAT	GTG	TCG		GAA	TCA	TCA	CCC	GGC	TCC	ATG	TCT	GCG	CGG		GTA	TCT	CTC	
aa	Y	S		N	D	D	V	S		E	S	S	P	G	S	M	S	A	R		V	S	L	

g

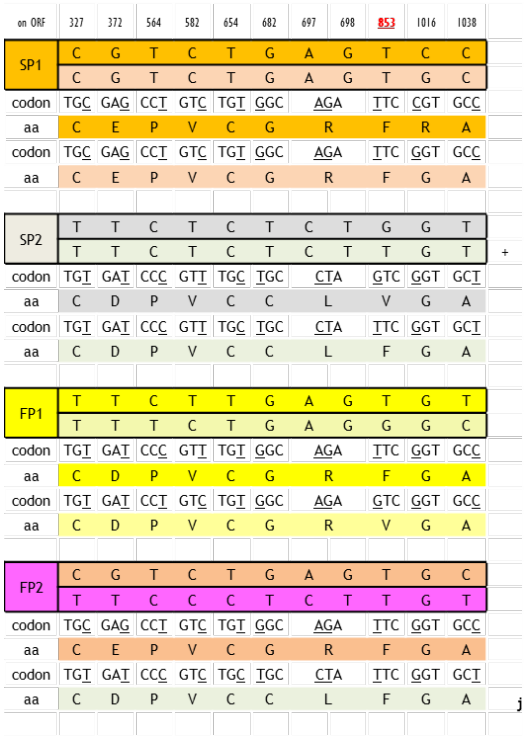


on ORF	58	105	159	324	639	759	760	828	906	1056	1149	1158	1180	1189	
SP1	C	T	C	G	G	C	A	A	G	T	T	T	C	C	
	G	C	T	A	G	G	G	G	A	C	C	T	G	G	
codon	GGC	TGT	CAC	CCG	TCG	CCC	AAC	GTA	TCG	GAT	ACT	ACT	CTT	CCG	
aa	G	C	H	P	S	P	N	V	S	D	T	T	L	P	
codon	GGT	TGC	CAT	CCA	TCG	CCG	GAC	GTG	TCA	GAC	ACC	ACT	GTT	GCG	
aa	G	C	H	P	S	P	D	V	S	D	T	T	V	A	
SP2	G	C	T	A	G	G	G	G	A	C	C	T	G	G	
	G	C	T	A	G	G	G	G	A	C	C	T	G	G	
codon	GGT	TGC	CAT	CCA	TCG	CCG	GAC	GTG	TCA	GAC	ACC	ACT	GTT	GCG	
aa	G	C	H	P	S	P	D	V	S	D	T	T	V	A	
codon	GGT	TGC	CAT	CCA	TCG	CCG	GAC	GTG	TCA	GAC	ACC	ACT	GTT	GCG	
aa	G	C	H	P	S	P	D	V	S	D	T	T	V	A	
FP1	C	T	C	G	G	C	A	A	G	T	T	T	C	C	+
	G	C	T	A	A	G	G	A	A	C	T	C	G	C	
codon	GGC	TGT	CAC	CCG	TCG	CCC	AAC	GTA	TCG	GAT	ACT	ACT	CTT	CCG	
aa	G	C	H	P	S	P	N	V	S	D	T	T	L	P	
codon	GGT	TGC	CAT	CCA	TCA	CCG	GAC	GTA	TCA	GAC	ACT	ACC	GTT	CCG	
aa	G	C	H	P	S	P	D	V	S	D	T	T	V	P	
FP2	G	C	T	A	A	G	G	A	A	C	T	C	G	C	
	C	C	T	G	A	G	G	A	G	C	T	T	C	C	
codon	GGT	TGC	CAT	CCA	TCA	CCG	GAC	GTA	TCA	GAC	ACT	ACC	GTT	CCG	
aa	G	C	H	P	S	P	D	V	S	D	T	T	V	P	
codon	CGT	TGC	CAT	CCG	TCA	CCG	GAC	GTA	TCG	GAC	ACT	ACT	CTT	CCG	
aa	R	C	H	P	S	P	D	V	S	D	T	T	L	P	

h



on ORF	63	265	372	417	453	894	940	1041	1173	1233	1251	1416	1446	1510	1512	1629	1672	1746	1914
SP1	C	A	C	A	A	A	A	G	C	A	C	T	T	C	T	G	G	A	G
	C	A	C	A	A	A	A	G	C	A	C	T	T	C	T	G	G	A	G
codon	GCC	ATT	AAC	GCA	TTA	GGA	ATG	GCG	GCC	TCA	ACC	AAT	GAT	CCT		TCG	GTT	TTA	CAG
aa	A	I	N	A	L	G	M	A	A	S	T	N	D	P		S	V	L	Q
codon	GCC	ATT	AAC	GCA	TTA	GGA	ATG	GCG	GCC	TCA	ACC	AAT	GAT	CCT		TCG	GTT	TTA	CAG
aa	A	I	N	A	L	G	M	A	A	S	T	N	D	P		S	V	L	Q
SP2	C	A	C	A	A	A	A	G	C	A	C	T	T	C	T	G	G	A	G
	A	A	C	A	A	A	A	G	C	G	C	C	T	C	G	G	G	A	G
codon	GCC	ATT	AAC	GCA	TTA	GGA	ATG	GCG	GCC	TCA	ACC	AAT	GAT	CCT		TCG	GTT	TTA	CAG
aa	A	I	N	A	L	G	M	A	A	S	T	N	D	P		S	V	L	Q
codon	GCA	ATT	AAC	GCA	TTA	GGA	ATG	GCG	GCC	TCG	ACC	AAC	GAT	CCG		TCG	GTT	TTA	CAG
aa	A	I	N	A	L	G	M	A	A	S	T	N	D	P		S	V	L	Q
FP1	C	A	C	A	A	A	A	G	C	A	C	T	T	C	T	G	G	A	G
	C	A	C	A	A	A	A	G	C	G	C	C	T	C	T	G	G	A	G
codon	GCC	ATT	AAC	GCA	TTA	GGA	ATG	GCG	GCC	TCA	ACC	AAT	GAT	CCT		TCG	GTT	TTA	CAG
aa	A	I	N	A	L	G	M	A	A	S	T	N	D	P		S	V	L	Q
codon	GCC	ATT	AAC	GCA	TTA	GGA	ATG	GCG	GCC	TCG	ACC	AAC	GAT	CCT		TCG	GTT	TTA	CAG
aa	A	I	N	A	L	G	M	A	A	S	T	N	D	P		S	V	L	Q
FP2	A	G	T	T	G	G	T	A	T	G	T	C	C	T	G	A	A	G	A
	A	G	T	T	G	G	T	A	T	G	T	C	C	T	G	A	A	G	A
codon	GCA	GTT	AAT	GCT	TTG	GGG	TTG	GCA	GCT	TCG	ACT	AAC	GAC	TCG		TCA	ATT	TTG	CAA
aa	A	V	N	A	L	G	L	A	A	S	T	N	D	S		S	I	L	Q
codon	GCA	GTT	AAT	GCT	TTG	GGG	TTG	GCA	GCT	TCG	ACT	AAC	GAC	TCG		TCA	ATT	TTG	CAA
aa	A	V	N	A	L	G	L	A	A	S	T	N	D	S		S	I	L	Q



on ORF	327	372	564	582	654	682	697	698	853	1016	1038
SP1	C	G	T	C	T	G	A	G	T	C	C
	C	G	T	C	T	G	A	G	T	G	C
codon	TGC	GAG	CCT	GTC	TGT	GGC	AGA		TTC	CGT	GCC
aa	C	E	P	V	C	G	R		F	R	A
codon	TGC	GAG	CCT	GTC	TGT	GGC	AGA		TTC	GGT	GCC
aa	C	E	P	V	C	G	R		F	G	A
SP2	T	T	C	T	C	T	C	T	G	G	T
	T	T	C	T	C	T	C	T	T	G	T
codon	TGT	GAT	CCC	GTT	TGC	TGC	CTA		GTC	GGT	GCT
aa	C	D	P	V	C	C	L		V	G	A
codon	TGT	GAT	CCC	GTT	TGC	TGC	CTA		TTC	GGT	GCT
aa	C	D	P	V	C	C	L		F	G	A
FP1	T	T	C	T	T	G	A	G	T	G	T
	T	T	T	C	T	G	A	G	G	G	C
codon	TGT	GAT	CCC	GTT	TGT	GGC	AGA		TTC	GGT	GCC
aa	C	D	P	V	C	G	R		F	G	A
codon	TGT	GAT	CCT	GTC	TGT	GGC	AGA		GTC	GGT	GCC
aa	C	D	P	V	C	G	R		V	G	A
FP2	C	G	T	C	T	G	A	G	T	G	C
	T	T	C	C	C	T	C	T	T	G	T
codon	TGC	GAG	CCT	GTC	TGT	GGC	AGA		TTC	GGT	GCC
aa	C	E	P	V	C	G	R		F	G	A
codon	TGT	GAT	CCC	GTC	TGC	TGC	CTA		TTC	GGT	GCT
aa	C	D	P	V	C	C	L		F	G	A

Fig. S4-4 The alignment of alleles of CGs in four parents. Allele of each gene from parents are represented only by indicating SNP positions. SNPs for HRM is highlighted with red and underlined. a. *ghERF*; b. *ghPG1*; c. *ghFDH*; d. *ghPER62*; e. *ghSS*; f. *ghCHI*; g. *ghsit*; h. *gh2-PS*; i. *ghcutin*; j. *ghPG9*.

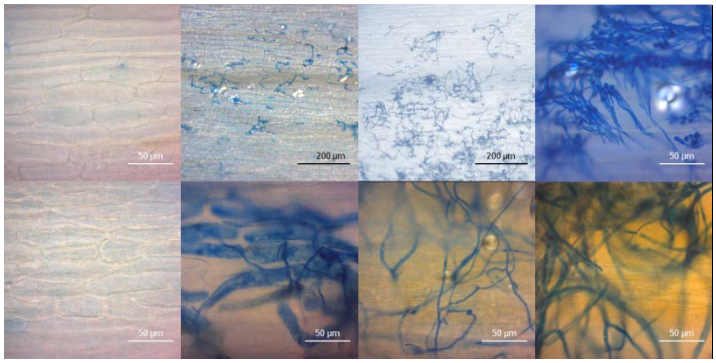


Fig. S4-5 Progress of disease in Botrytis-inoculated gerbera SP2 ray florets after trypan blue staining at 0, 6, 12, 24hpi; 24 hpi mock, 36, 48 and 72hpi hpi (from left to right, from top to bottom). At the 6hpi, the Spore of Botrytis is germinated and hyphal growth on ray florets. In 36, 48, 72 hpi, background can be seen in the microscope that ray florets are necrotic brown. Trypan blue staining was according to the protocol by Chung (2006).

Chapter 5 Two candidate genes, *ghPG1* and *ghsit*, contribute to Botrytis resistance in *Gerbera hybrida*

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Abstract

Botrytis cinerea can cause direct damage during the whole process of gerbera production. Two candidate genes *PG1* (polygalacturonase gene) and *sit* (*sitiens*, ABA-aldehyde oxidase gene) for resistance against Botrytis that were previously mapped in QTL regions for the *ray floret* disease resistance test in gerbera were studied. Tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) was used to characterize gene function in relation to Botrytis resistance in gerbera. Single candidate gene constructs and a two-gene-combined construct were developed and tested. Results showed that after 24h of Botrytis *in vitro* inoculation on the ray florets, a significantly delayed spread of lesions was observed on VIGS candidate gene silenced ray florets compared to controls. One gerbera genotype with two genes co-suppression had even smaller lesions, on ray florets than single gene silenced plants, although difference was not significant. Through this study, we conclude that gene silencing each of these two genes, results in decreased Botrytis invasion in gerbera and that these genes probably are among the determining genes leading to the variation of Botrytis resistance in gerbera QTLs.

Keywords

Gerbera gray mold; virus-induced gene silencing; TRV; VIGS

Introduction

Gerbera gray mold is a serious disease that occurs in the whole production process including the post-harvest life of gerbera and is caused by the necrotrophic pathogen *Botrytis cinerea*. Gerbera, especially cut flower cultivars, are vulnerable to Botrytis attack owing to the occurrence of high humidity during transport as well as that inflorescences are suffering from the active process of senescence once harvested. The Dutch Flower Auction Association (VBN) practice zero tolerance on gerbera affected by *Botrytis*, and all gerbera flowers with detectable Botrytis infection will be rejected and destroyed. Quality losses due to gerbera gray mold might affect the entire supply chain of gerbera from upstream (breeders) to downstream (consumers) (Bastiaan-Net *et al*. 2007; Dean *et al*. 2012).

Botrytis resistant or less susceptible gerbera cultivars are highly desirable for gerbera breeding companies due to the limitations for the usage of fungicides, particularly, in post-harvest. Several QTLs linked with Botrytis resistance were found from two mapping populations in a previous study (*Chapter 3*; Fu *et al*. 2017). The detected QTLs contributed minor effects on phenotypic variance similarly to other QTL mapping studies in *Arabidopsis* and tomato (Davis *et al*. 2009; Denby *et al*. 2004; Finkers *et al*. 2008; Finkers *et al*. 2007a; Finkers *et al*. 2007b; Rowe and Kliebenstein 2008). As in many QTL studies, the found QTL regions in Fu *et al*. (2017; *Chapter 3*) cover relative large genetic distances with high probability for recombination between QTL flanking markers. These circumstances might all reduce the reliability and usefulness of the markers. Moreover, gerbera, like many other ornamental plants, has a heterozygous background and a lack of adequate genetic information. Instead of going through the process of high-resolution fine mapping and identify tightly-linked markers for marker-assisted selection, we developed a candidate gene approach searching for possible causal genes. Four candidate genes were mapped co-localizing in previously identified QTL regions and three candidate genes were found near the QTLs. Upregulated expression of these candidate genes indicated that these genes were activated during Botrytis infection in gerbera (*Chapter 4*).

As co-localization is the first indication for putative candidate genes to be involved in resistance to Botrytis, gene function of these candidate genes needs to be further explored. There are several methods to characterize gene function. One of the methods is based on phenotypic screening of mutants (Koornneef *et al*. 1980) however such resources are not available for gerbera. Overexpression of genes can also be utilized to study gene function. Several genes, *GMYB10* (Elomaa *et al*. 2003), *GhCYC2* (Broholm *et al*. 2008), *GSQUA2* (Ruokolainen *et al*. 2010) and *Gh-SOC1* (Ruokolainen *et al*. 2011), were transformed to gerbera to identify gene functions in flower secondary metabolism (color) and floral development, which are important traits for this ornamental crop. Thus, overexpressed genes showed a phenotypic change which provided evidence for the genes' involvement in a trait. Similarly, downregulating a gene by genetic modification can also provide evidence for the functional effect of genes. For instance, downregulation of 2-pyrone synthase (2-PS) which is involved in the synthesis of the precursors of the polyketide-derived antifungal compounds present in gerbera plants resulted in increased susceptible to Botrytis (Eckermann *et al*. 1998; Koskela *et al*. 2011).

However, these transformation technologies for gene function analysis are often very genotype depending and considered time-consuming. Virus-induced gene silencing (VIGS) is a post-transcriptional gene silencing (PTGS) that is considered as one of the mechanisms for plant defense against virus invasion (Jiang *et al.* 2011). During virus replication in plants, double-stranded RNAs (dsRNAs) are produced. Plant cells recognize these dsRNAs as foreign invaders, and an enzyme named Dicer degrades the dsRNA into small interfering oligonucleotides (siRNA). The siRNA duplexes are composed of two 21~25 nucleotide (nt) RNA (MacDiarmid 2005) and bind to an RNA-induced silencing complex (RISC). The specific RISC cleaves viral transcripts with identical or highly similar sequences (Jiang *et al.* 2011). This plant defense strategy has been developed and adapted as a widely used tool to analyze the functional effect of gene silencing on plant phenotype (Jiang *et al.* 2011; Senthil-Kumar and Mysore 2014). A specific plant gene fragment inserted into a virus vector is multiplied in plants by viral replication. PTGS is induced by recognizing these plant transcripts as foreign invaders, so siRNA's are generated and these target the paticular plant gene resulting in gene silencing.

Advantages of VIGS over other gene knockdown methods are: i) VIGS vectors can be easily constructed and applied in a broad host range; ii) VIGS phenotypes can be quickly observed in a relatively short period; and iii) VIGS (by inserting a fragment from a conserved region) can be used to downregulate all members of a gene family (Jiang *et al.* 2011; Senthil-Kumar and Mysore 2014). Disadvantages may be: i) incomplete loss of gene expression, ii) occurrence of off-target gene silencing, iii) occurrence of a mild viral infection phenotype, iv) a variable silencing efficiency (Senthil-Kumar and Mysore 2014). Despite these limitations for VIGS applications, VIGS systems have been successfully applied in a large number of ornamental plants like, TRV-based VIGS on *Petunia hybrida* (Chen *et al.* 2004; Chen *et al.* 2005; Reid *et al.* 2009), *Rosa hybrida* (Ma *et al.* 2008), *Eschscholzia californica* (Wege *et al.* 2007), *Gerbera hybrida* (Deng *et al.* 2014; Deng *et al.* 2012), *Papaver somniferum* (Hileman *et al.* 2005; Wijekoon and Facchini 2012), *Aquilegla vulgaris* (Gould and Kramer 2007; Kramer *et al.* 2007), *Gladiolus grandifloras* (Singh *et al.* 2013); cucumber mosaic virus (CMV)-based VIGS on *Antirrhinum majus* (Kim *et al.* 2011); cymbidium mosaic virus (CymMV)-based VIGS on *Phalaenopsis* (Hsieh *et al.* 2013; Liao *et al.* 2004; Lu *et al.* 2007). And the system is considered an attractive approach for gene characterisation in ornamentals, particularly those not amenable to tissue culture or genetic transformation.

Two gerbera candidate genes named *ghPG1* and *ghsitiens* (shortened as *ghsit*), homologs of tomato polygalacturonase gene and ABA-aldehyde oxidase gene, were mapped in Botrytis resistance QTL regions detected from the *ray floret* test in gerbera (*Chapter 3*; Fu *et al.* 2017). Tomato polygalacturonase is responsible for pectin degradation during fruit ripening (Sitrit and Bennett 1998) and could be used by Botrytis for induction of plant susceptibility (Blanco-Ulate *et al.* 2014). The tomato *sit* mutant which is deficient in aldehyde oxidase for ABA biosynthesis (Taylor *et al.* 1988) was found to contribute to resistance against *B. cinerea* (Asselbergh *et al.* 2007). To investigate whether these two candidate genes *ghPG1* and *ghsit* contributed to Botrytis resistance in gerbera, we adopted a tobacco rattle virus (TRV)-based gene silencing system to study the function of the two genes.

Materials and Methods

Plant material

Gerbera plants (genotypes SP1, SP2, FP1) were obtained from the Gerbera breeding companies Florist B.V. and Schreurs B.V. and grown in greenhouse chambers (Unifarm, Wageningen UR, the Netherlands) under 16h light/8h night photoperiods with 21°C/19°C day/night temperature in relative humidity of ~75%, and 100 $W \cdot m^{-2}$ supplemental light when light intensity dropped below 150 $W \cdot m^{-2}$.

Amplification and isolation of target genes

Candidate gene sequences *ghPG1* and *ghsit* were identified from the gerbera EST database (*Chapter 2*; Fu *et al*. 2016). Similarly, sequences of reporter genes, two chalcone synthase genes (*ghCHS1* and *ghCHS4*) and one phytoene desaturase gene (*ghPDS*), which were used as the positive controls were retrieved from the database as well (Suppl. Fig. S5-1). *CHS* is responsible for flavonoids biosynthesis, and silencing one member (*ghCHS1*) of CHS family has resulted in partly color loss in gerbera inflorescence (Deng *et al*. 2012; Deng *et al*. 2014). Silencing *PDS* would produce a typical photobleaching in the green leaves, and in gerbera, photobleaching could be found in scapes (flower stems) or bracts (Deng *et al*. 2014). Gene-specific primers to generate a 300~700bp fragment were designed on Primer3 online. The *att*B1 and *att*B2 sequences were added, respectively, to the forward and reverse primers for amplification (listed in Suppl. Table S5-1 and Suppl. Fig. S5-1). To check whether they could hit any other continuous identical or reverse complementary sequence that may result in triggering off-target gene silencing (Xu *et al*. 2006), the expected fragments of target genes were divided into 20bp sequences and used as query to blast against our gerbera EST database (*Chapter 2*; Fu *et al*. 2016).

The fragments of the candidate genes (*ghPG1*, *ghsit*) and three reporter genes (*ghPDS*, *ghCHS1*, *ghCHS4*) for VIGS were amplified from gerbera DNA by using Phusion DNA polymerase (Thermo Scientific). PCR reaction mixture (25µl) included: 2µl DNA (20ng/µl), 5µl 5× Phusion HF Buffer, 1µl dNTP's (5mM), 1.25µl of forward and reverse primer each (10µM), 0.25µl Phusion DNA Polymerase and 14.25µl Milli-Q water. PCR was initiated at 98°C for 1min, then 35 cycles of 10s at 98°C, 30s at **62**°C and 30s at 72°C, and a final extension step of 7min at 72°C. To ensure that the PCR products were specifically amplified with expected size, PCR-specific products were checked on 1% agarose gel and were excised from gel and purified by using the QIAquick Gel Extraction Kit (Qiagen).

To co-suppress two candidate genes (*ghPG1*+*ghsit*), a construct was constructed consisting of the two gene fragments. Fragments of the first gene (A; *ghPG1*) were amplified using forward primers of A gene sequence added with *att*B1 sequence in 5' end (as forward primer, AF in Suppl. Table S5-1) and reverse primers A added with forward primer of the other gene (B, *ghsit*) sequence in 3' end (as reverse primer, BF+AR); fragments of B gene were amplified using forward primer of B gene with reverse primers A in 5' end (AR+BF) and reverse primers B with *att*B2 sequence in 3' end (BR). The two fragments were amplified separately and purified by cutting from the gel. The 25µl-reaction mixture for overlap extension PCR was 2µl fragment A, 2µl fragment B (equal masses), 5µl 5× Phusion HF Buffer, 1µl dNTP's mix (5mM), 0.25µl Phusion DNA Polymerase and 14.75µl Milli-Q water (without primers). The overlap

extension PCR was initiated at 98°C for 1min, then **12** cycles of 10s at 98°C, 30s at 62°C and 30s at 72°C, and one hold of 7min at 72°C. From this PCR, a 4µl reaction mixture was used as the template for a 50-µl PCR reaction (components were doubled and procedure for PCR were the same as the previous reaction). Two gene fragment constructs with expected size were excised from gel and purified by QIAquick Gel Extraction Kit and checked on 1% agarose gel.

VIGS vectors construction

The Gateway-compatible Tobacco rattle virus (TRV) two-component Agrobacterium mediated expression system was used for gene silencing as previously described (Liu *et al.* 2002). DNA fragments of reporter genes and candidate genes were individually cloned into pDONR207 which contains a gene for gentamycin resistance by using the Gateway® BP Clonase® II Enzyme Mix following the manufacturer's recommendation (Invitrogen) to generate entry vectors (Fig. 5-1). Entry vectors with the presence of CG fragments insertion can be grown on selection media with 25mg gentamicin and were verified by DNA sequencing using primers *att*L1 and *att*L2 listed in Suppl. Table S5-1. Entry vectors pDONR207 carrying the reporter genes and candidate genes fragments were cloned into the destination vector pTRV2 by using Gateway® LR Clonase® II Enzyme Mix (Invitrogen) to generate constructs *TRV2::ghPDS*, *TRV2::ghCHS1*, *TRV2::ghCHS4*, *TRV2::ghPG1*, *TRV2::ghsit* and *TRV2::ghPG1+ghsit*. A negative control TRV2 construct (*TRV2::ghGUS*) carrying a 648bp *GUS* fragment was also used and described by Song and Thomma (2016). The resulting *TRV2* constructs were confirmed by DNA sequencing (using TRV2 seq listed in Suppl. Table S5-1).

All *TRV1* and *TRV2* (*TRV2::GOI*) (*GOI*, Gene Of Interest, either single gene or two-gene combination) constructs were transformed to *Agrobacterium tumefaciens* strain GV3101 by electroporation. Transformed agrobacteria were inoculated on LB agar media supplemented with 25mg rifampicin (rif) + 25mg gentamicin (genta) + 50mg kanamycin (kana). Three single colonies for each construct were picked and cultured in the LB liquid media with rif25, genta25 and kana50, and inoculated in an incubator at 28°C, 200rpm overnight. Constructs were confirmed by normal PCR procedure by using 2µl agro-bacterial suspension as the template and the gene-specific primers (the primers for target gene fragments amplification), only with extending the time of initialization step to 10 minutes. One confirmed colony of each target gene construct was used for agro-infiltration.

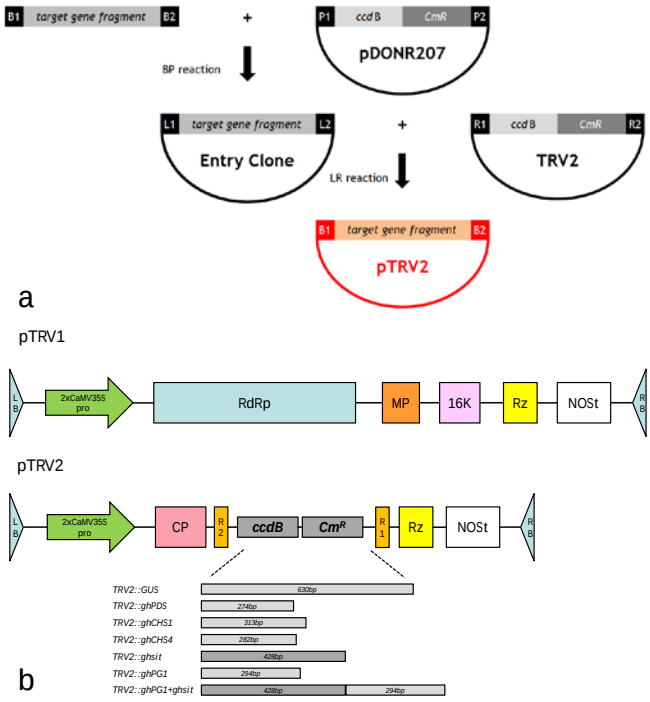

Fig. 5-1 The Gateway-compatible Tobacco rattle Virus (TRV)-based virus induced gene silencing (VIGS) system.
a. TRV-based VIGS vector construction. Target gene fragments (gene of interest, *GOI*) are amplified with gene-specific primers added with *att*B1 and *att*B2 sites. Gene fragments are cloned into pDONR207 to create entry clone by BP reaction. Entry clone pDONR207 with target gene fragment is cloned into TRV2 to generate *pTRV2::GO*. B1 and B2, *att*B1 and *att*B2 sites; P1 and P2, *att*P1 and *att*P2 sites; L1 and L2, *att*L1 and *att*L2 sites; R1 and R2, *att*R1 and *att*R2 sites; *ccdB,* cell killing gene; Cm^R, chloramphenicol-resistance gene.
b. TRV-based VIGS vector systems. LB and RB, left and right borders of T-DNA; 2 × 35S, duplicated CaMV 35S promoter; RdRp, RNA-dependent RNA polymerase; MP, movement protein; 16K, 16 kDa cysteine-rich protein; Rz, self-cleaving ribozyme; NOSt; nopaline synthase terminator; CP, coat protein; R1 and R2, *att*R1 and *att*R2 sites.

Agrobacterium infiltration on gerbera scape

A. tumefaciens carrying *TRV1* and confirmed *TRV2* constructs were incubated separately in LB liquid medium containing the same antibiotics grown overnight at 28°C shaking with 200rpm. Subsequently, a new culture was made by adding 100µl *TRV1* or *TRV2::GOI* to a 50ml blue cap centrifuge tube with 20ml YEB (every 1000ml containing 5g beef extract, 5g bacteriological peptone, 5g sucrose, 1g yeast extract and 2ml 1M $MgSO_4$) + 2µl 200mM acetosyringone + 20ul 50mg/ml kana + 200ul 1M MES. These cultures were grown at least 15 hours in 28°C shaker (200rpm) after which the O.D. value was measured. Cells were pelleted by 10min centrifugation at 3000 rpm. Pellets were resuspended with fresh MMA (every 1000ml MMA containing 20g sucrose, 5g MS salt without vitamins, 1.95g MES and 1ml 200mM acetosyringone; pH = 5.6) to adjust to an OD of 2. Equal volumes of agrobacteria carrying *pTRV1* and each modified *pTRV2* derivatives were mixed making the final OD of 1.0. After incubation at 28°C with 200rpm shaking for 1 hour, cultures in MMA were ready for agro-infiltration.

As in the procedure described by Deng *et al.* (2012) for gene silencing in gerbera inflorescences, the agro-infiltrates with 1:1 mixtures of *TRV1* and *TRV2::GOI* constructs were applied to scapes. When the scape was about 5cm in length (used in this study), the flower stem close to the flower bud was scratched by a metal nail file to remove the outside cell layer for around 1cm length and less than 1/3 of the outer perimeter of the stem. *Agrobacterium*-soaked cotton was covered on the scratched area bound loosely with a tape for agro-infiltration. Gerbera genotypes SP1, SP2, FP1 that previous were used as parents to construct two crossing populations (*Chapter* 3; Fu *et al*. 2017) were used for infiltration. Two independent experiments were carried out. Nine inflorescences for each single candidate gene silencing in genotypes SP2 and FP1 were infiltrated with *TRV1* + *TRV2::ghPG1* or *TRV1* + *TRV2::ghsit*. For *ghPG1* and *ghsit* co-suppression, 13~16 inflorescences were used for each parent (SP1, SP2 and FP1). Five inflorescences for each genotype were infiltrated with the negative control *TRV1* + *TRV2::GUS*; and three to six inflorescences for each reporter gene (*TRV1* + *TRV2::ghPDS; TRV1* + *TRV2::ghCHS1*; *TRV1* + *TRV2:: ghCHS4*) as positive control were treated simultaneously as indicated.

Expression analysis and disease test

To test the Botrytis infection on TRV-treated gerbera plants, flowers were harvested at around 2 to 3 weeks post Agrobacterium infiltration when the first and second whorls of disc florets were fully developed, which is generally accepted as the time for harvesting. Ray florets (in the sector right above the scar due to the scratching) from each inflorescence were collected for Botrytis inoculation and for total RNA isolation (i.e. flash frozen in liquid nitrogen (LN) then stored at -80 °C).

To analyze the efficiency of candidate gene silencing, LN-frozen single ray floret was ground and total RNA was isolated using Trizol reagent (Invitrogen) and cDNA was synthesized according to the iScript™ cDNA Synthesis Kit (Bio-Rad) for Real time quantitative PCR (described in *Chapter 4* in detail). Relative quantification (Livak and Schmittgen 2001) was used to analyze data from the real-time quantities PCR. Target gene and reference gene (*GAPDH*) were amplified using the cDNA of *TRV2::GUS* plants and *TRV2::GOI* plants. The change in expression of the target gene in silenced plants was normalized to the reference gene (*GAPDH*) relative to control plants (as 1, *TRV2::GUS* plant in this experiment) and represented as fold change. The method is called $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta CT = (C_{T,\,Target} - C_{T,\,GAPDH})_{\,TRV2::GOI} - (C_{T,\,Target} - C_{T,\,GAPDH})_{\,TRV2::GUS}$. Three ray florets from different silenced inflorescences and three technical replications for each treatment was performed.

For Botrytis resistance testing, single fresh ray florets were inoculated with a 2µl-droplet of *B. cinerea* (strain B05.10) spore suspension with a concentration of 5×10^5/ml. Ray florets were put on wet filter paper in plastic containers and containers were put in plastic bags to maintain a high humidity at room temperature (described in *Chapter 4* in detail). From individual inflorescence, for each treatment 5-6 ray florets were collected for the inoculation. The inoculation droplets resulted in a spreading lesion on the ray florets and lesion sizes of inoculated ray florets were obtained after 24 hours post infection from images using ImageJ. Data from different gene silenced treatments were analyzed by one-way ANOVA in SPSS (Version 21).

Results

Construction of the TRV vector for gene silencing

To characterize the function of candidate genes which co-localized with the Botrytis resistance QTLs in gerbera, we constructed a TRV-based VIGS system (Liu *et al*. 2002) which carried the target candidate gene fragments in *pTRV2*, to suppress the level of plant endo-gene expression. Fragments of *PDS* (phytoene desaturase gene) and *CHS* (Chalcone synthase gene) that both are widely used as reporter genes to recognize the silenced phenotypes in gerbera (Deng *et al*. 2014; Deng *et al*. 2012) and other crops, and a β-glucuronidase (*GUS*) gene fragment were also cloned into the TRV2 vectors.

Target gene fragments with lengths varying from 274bp to 428bp (Suppl. Fig. S5-1) were amplified from the four parental gerbera cDNA (*Chapter 2*; Fu *et al*. 2016). And a fragment with a length of 722bp combining two gene fragments (*ghPG1* 428bp + *ghsit* 294bp) was also developed by overlapping PCR. To confirm that fragments might not trigger any other unexpected non-target gene silencing; we divided the entire fragments of candidate gene and reporter gene into a series of continuous 20bp subsequences that were used as the query for BLASTn. In BLAST output, all 20bp-subsequences of target genes only aligned to the original contigs indicating no other off-target hits were found that could lead to possible non-target gene silencing based on the currently available DNA information. All the target genes were amplified in the four parental genotypes (*Chapter 4*). Since only a few SNPs were found between the target gene fragments in the four parents, target gene fragments from SP1 were used for subsequent TRV-VIGS vector construction. All entry vectors and TRV2 constructs that were generated in the two steps Gateway reaction have been sequenced and confirmed carrying the right target genes fragments (Fig. 5-1 and Suppl. Fig. S5-2).

Visible indications for successful silencing of reporter genes

Considering that successful target candidate gene silencing might not be visible, we constructed the reporter genes (*ghPDS*, *ghCHS1* and *ghCHS4*) and used them as the positive control to identify in which sectors of the inflorescences the silencing phenotype was present. Since *ghCHS1* and *ghCHS4* silenced plants did not give visible indications of gene silencing, they were therefore not used further in the tests. TRV2 constructs with the *GUS* gene fragment were used as negative control.

In *TRV2::ghPDS* silenced plants of SP2, a visible color change emerged above the scar by scratching for agro-infiltration and along on the green trunk of the elongated scapes until the bottom of the flower head that could be easily followed (Fig. 5-2a, b, c, as the arrows shown). Three of the six PDS-silenced SP2 plants exhibited a changed pink color in ray florets of the inflorescences with varying ray floret numbers (Fig. 5-2d). The ray florets from the original white inflorescences that were on sectors of the same side of the scar from the scratching and also the sectors of the opposite side of the scar turned to pink. Those pink ray florets/sectors defined in which parts of the flower head *PDS* had been silenced. The phenotype of PDS-silencing in SP1 plants varied, showing a diluted orange color in parts of the inflorescences (Suppl. Fig. S5-3a) or few petals (Suppl. Fig. S5-3b, c) on the scratching side, or bleaching on ray florets (Suppl. Fig. S5-3d, e, f). Quite a few flower scapes of FP1 were broken in the place of scape scratching as the stem

extended (Suppl. Fig. S5-4a, b) and few even fell off, before the flower blossoming. Out of the scape without stem breaking after flowering, no detectable phenotype was found in the reporter gene silenced inflorescences indicating that gene silencing in FP1 was likely ineffective.

Fig. 5-2 The phenotypes of SP2 plants after agro-infiltration with *TRV2::ghPDS* constructs. The crosses in (a) and (b) represent the position of scratching and the scar still remain with scape extension. The arrows on (a), (b), (c) track the potential transmission of the virus, from the scar left till the bottom of the flower head. Three gerbera flowers in (d) indicate the color changed sectors in the whole gerbera inflorescences and the number of pink ray florets is varied.

Gene expression in silenced ray florets

To quantify the suppression of the candidate and reporter gene expression after VIGS, we used a relative quantification method to determine the efficiency of silencing. The relative expression of target gene in negative control *TRV2::GUS* plants using the formula were set as 1. The change in expression of target genes was normalized to the reference gene (*ghGAPDH*) relative to *TRV2::GUS* silenced plant and represented as fold change.

The expression levels of *PDS* gene after normalization in *TRV2::ghPDS* plants showed a significant decrease ($P<0.05$) to *PDS* expression in control *TRV2::GUS* plants of SP1 and SP2 (Fig. 5-3a). There was a decrease in PDS-silenced FP2 of *PDS* without a statistically significant difference. The expression of *PG1* and *sit* were analyzed in the two-gene-silenced SP1 plant, and both genes were successfully silenced, albeit to a different extent (Fig. 5-3b). Significant silencing of *PG1* expression was only in SP2 *TRV2::ghPG1* plants, but not in TRV::*ghPG1+ghsit* plants. While the relative *sit* expression in SP2 TRV2::*ghsit* plants and *TRV2::ghPG1+ghsit* plants decreased significantly (Fig. 5-3c). The expression levels of neither *PG1* nor *sit* were found decreased statistical significantly on any gene silenced treatment in FP1 (Fig. 5-3d).

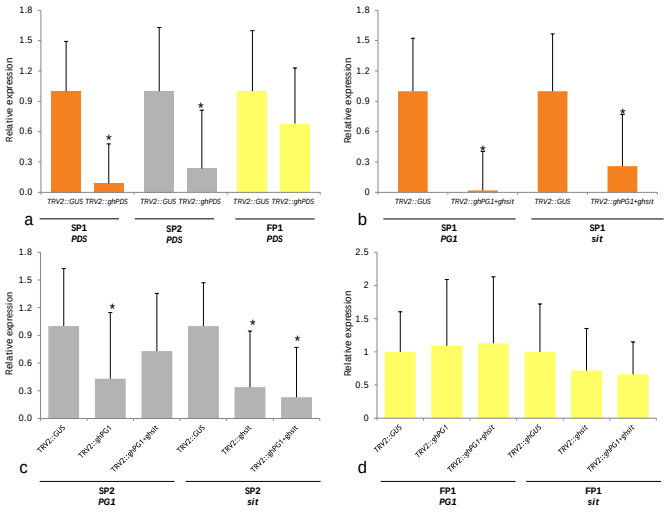

Fig. 5-3. Relative gene expression level of reporter gene PDS and candidate genes in silenced plants using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The change in expression of target gene in silenced plant which was normalized to reference gene (GAPDH) relative to control plant (as 1, *TRV2::GUS* plant in this experiment) and represent as fold change. The aster indicated the significant difference compared with the related gene expression on control TRV2::*GUS* plants (P<0.05).

a, relative *PDS* expression on three parental *TRV2::ghPDS* silenced plants. b, relative *PG1* and *sit* expression on SP1 *TRV2::GUS* silenced plants and *TRV2::ghPG1+ghsit* silenced plants;c, relative *PG1* expression on SP2 *TRV2::GUS* silenced plants, *TRV2::ghPG1* silenced plants and *TRV2::ghPG1+ghsit* silenced plants; relative *sit* expression on SP2 *TRV2::GUS* silenced plants, *TRV2::ghsit* silenced plants and *TRV2::ghPG1+ghsit* silenced plants; d, relative *PG1* expression on FP1 *TRV2::GUS* silenced plants, *TRV2::ghPG1* silenced plants and *TRV2::ghPG1+ghsit* silenced plants; relative *sit* expression on FP2 *TRV2::GUS* plants, *TRV2::ghsit* silenced plants and *TRV2::ghPG1+ghsit* silenced plants.

Disease test on candidate gene silenced ray florets

To test Botrytis resistance on ray florets after VIGS, single ray florets were collected for Botrytis inoculation. According to the results from the indicator gene, we collected 5-6 ray florets in the sectors just right above the scratching scar being around or less than 1/4 of the total ray florets (Suppl. Fig. S5-5a). After 24 h post inoculation, lesion sizes of each ray floret from target gene silenced plants and *GUS* gene silencing treatment can be observed using ImageJ. Lesion regions were identified and lesion size difference was analyzed with one-way ANOVA test (Suppl. Fig. S5-5b). The number of ray florets sampled from each parent and each silencing treatment for disease test was shown in Suppl. Table S5-2.

Compared with the mean lesion size of control treatment (*TRV2::GUS*), the mean lesion size on ray florets from TRV2::*GOI* silenced plants showed a decrease on the three genotypes used (Fig. 5-4a, b, c). Due to the material limitations, SP1 was only used for two genes (*TRV2::ghPG1+ghsit*) co-suppression. The lesion size of *TRV2::ghPG1+ghsit* co-silenced plants were reduced by 80% compared to *TRV2::GUS* silenced plants. The same amount (81%) reduction was also observed in SP2 with *TRV2::ghPG1+ghsit* co-silenced (Suppl. Fig. S5-6). There were significant differences (P<0.05) between *TRV2::ghPG1+ghsit* and *TRV2::ghsit* silenced plants compared to *TRV2::GUS* silenced SP2 plants, while no significant difference between *TRV2::PG1+sit* co-silenced plants compared to *TRV2::PG1* silenced plants was found. Although the lesion size of FP1 ray florets came with less reduction and more variation than other parents, it seems a bigger lesion reduction in FP1 came from the *TRV2::PG1* silenced plants. Significant differences could be

observed in the mean of lesion size between the four treatments of FP1, yet in each treatment, large variations existed.

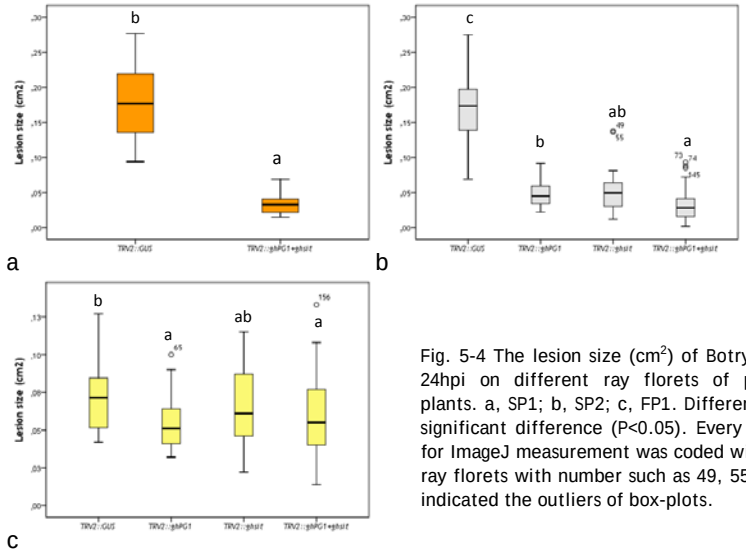

Fig. 5-4 The lesion size (cm^2) of Botrytis inoculation at 24hpi on different ray florets of parental silenced plants. a, SP1; b, SP2; c, FP1. Different letters indicate significant difference ($P<0.05$). Every single ray florets for ImageJ measurement was coded with a number, and ray florets with number such as 49, 55, 73, 74 *etc* were indicated the outliers of box-plots.

5

Discussion

The use of VIGS to study gene function in inflorescences

In this study, we adopted the TRV-based induced gene silencing system that was previously described for gerbera (Deng *et al.* 2014; Deng *et al.* 2012) to inspect the function of two candidate genes related to Botrytis resistance. Deng *et al.* (2012) developed different methods for agro-infiltration on gerbera, syringe infiltration and vacuum infiltration on newly developed leaves, and inoculating of the Agrobacteria on the surface of scapes after mechanical wounding. The latter was developed especially for studying the gene function in floral tissues, and two chalcone synthesis genes were successfully silenced using this method by the authors themselves (Deng *et al.* 2014). Similarly, direct injection of the Agrobacteria suspension was employed into the stalk of the raceme (floral bud) of *Phalaenopsis* for silencing genes related to floral morphological features (Lu *et al.* 2007), although they found leaf injection is more easily (Hsieh *et al.* 2013). Agro-injection was also applied through the peduncle that is attached to a tomato fruit to study the function of genes related fruit developing and ripening (Fu *et al.* 2005; Orzaez *et al.* 2009; Fantini and Giuliano (2016). Since these genes expressed in the later plant developmental stages, gene suppression can be utilized in the reproductive parts of a plant directly, e.g. the flowers and fruits, instead of young leaves. Deng *et al.* (2012) indicated that the best time for agro-infiltration was gerbera seedling at the stage when the leaf shape starts to turn from round to lobed. However, it will take at least three months from this stage to a mature gerbera that could be able to flower (information from Florist Holland B.V. and Schreurs Holland B.V.). Considering the time (from 5 cm scape to flowering only need 3-4 weeks) and ease of operation (high pressure exists in gerbera scapes that make injection by syringe difficult), we chose the agro-infiltration method using mechanical wounding on scapes.

Variations in responses exist for TRV-based VIGS infiltration among gerbera cultivars. Deng *et al.* (2012) described that responses could be with a typical virus induced silencing phenotype (photobleaching), without photo-bleached symptoms or with severe virus infection symptoms. The three genotypes we used for this study didn't show severe virus infection symptoms indicating that these genotypes might be tolerant for the side effect that TRV-VIGS could deliver. We observed a visible color changing phenotype in the reporter gene *ghPDS* silenced plants. The silenced phenotypes on the inflorescences were detected as described in Deng *et al.* (2014), on the side of the scape where it was wounded for agro-infiltration. The PDS-silenced plants in SP2 even showed a recognizable clue not only on the inflorescences but also on the scape by coloration which apparently tracks the virus movement. It also indicates the virus transmits mainly by means of the vascular system. Wege *et al.* (2007) also found patterns of photo-bleaching mainly occurred along the vascular system in California poppy, and there was a separated boundary of photobleached and unsilenced (green) tissues parallel to the vascular bundle. With these indications, we only sampled the ray florets above the scar in candidate gene(s) silenced plants to observe lesion size after Botrytis inoculation.

The TRV-based VIGS can be successfully performed at least in two gerbera parents (SP1 and SP2), since the phenotypes of the PDS reporter gene silenced in these plants can be readily recognized and a significant decrease in gene expression confirmed this. We sampled the ray florets from the inflorescence on the same side above the scar and inoculated the Botrytis on the silenced plant ray florets and detected reduced lesion sizes comparing with the control treatment. However, in FP1, we didn't notice a visible color change on the reporter gene silenced plants nor a significant decrease in gene expression, as was detected for SP1 and SP2. There was a large variation in FP1 between target gene silenced plants and control plants in lesion size reduction in ray florets after Botrytis infection. It indicated that the target genes might not be silenced in most FP1 inflorescences. We found quite a few inflorescence stems that were broken on the wounded place from the stem scratching. Apparently, FP1 is more sensitive to stem wounding. When stems are stretching, the difference in growth speed between the scratched side and the unscratched side of the stem as well as the gradually increased weight of flower head may lead to problems for stem integrity. Gerbera needs a straight-stand and firm stem to support the relative large flower head. Stem bending occurs in gerbera and was found correlated with low stem lignin levels (Perik *et al.* 2014). Actually, the stem in FP1 is harder than others (Y. Fu pers. observation) and high lignin content could be expected in this cultivar. The unbalanced growth of the two sides might be the reason for FP1 inflorescence stems to break after stem injury. The result might be that the virus cannot transmit upward, and that finally results in low efficiency and success of VIGS in FP1.

The possible involvement of PDS for gerbera flower coloration

We studied the VIGS system on flower inflorescences to characterize the previously identified candidate gene function. Since targeted candidate gene silencing would not be expected to give any direct visual phenotype, several reporter gene constructs were tested to track the timing and location of silencing. We expected to see recognizable phenotypes that occur in the absence of the reporter gene product in the stems and/or leaves or inflorescences to guide to the exact timing and extent of silencing. *PDS* encodes an enzyme in the carotenoid biosynthesis pathway and carotenoids protect chlorophyll from photo-bleaching.

PDS gene-silenced plants demonstrate a photo-bleached phenotype and as such *TRV2::PDS* VIGS constructs are widely used as the reporter gene for tobacco, petunia and other crops (Deng *et al.* 2012; Jiang *et al.* 2011; Senthil-Kumar and Mysore 2014). CHS is part of the flavonoid biosynthesis pathway contributing to the flower color. Silencing *phCHS* in the purple Petunia resulted in white corollas (Chen *et al.* 2004). In gerbera, there are three members (*ghCHS1, ghCHS3, ghCHS4*) in *CHS* gene family, and two of them (*ghCHS1, ghCHS4*) are highly expressed in gerbera ray florets (Helariutta *et al.* 1995; Deng *et al.* 2014). Only *ghCHS1* seems to be contributing to flavonoid biosynthesis in ray florets. Silencing *ghCHS1* in two gerbera cultivars containing different kinds of anthocyanin pigmentation (one pelargonidin and one cyanidin) resulted in a visible color loss, from orange or purple to milky white in partial sectors of gerbera inflorescences (Deng *et al.* 2014). The *ghCHS4* was successfully silenced in two gerbera cultivars with a deceased gene expression and flavonoids content as described in Deng *et al.* (2014), whereas no visible color change on ray florets of the two was observed.

In our experiments, no obvious phenotype on either *ghCHS1* or *ghCHS4* silenced plants of the three parental genotypes was observed. The possible reason might come from the color of the three genotypes themselves which are different from the two gerbera genotypes used by Deng *et al.* (2014). The white and yellow colored parents (SP2 and FP1) might not contain any anthocyanin and the orange of the parent (SP1) is probably due to carotenoids content. The SP1, *PDS* silenced plants produced the photo-bleached phenotype in the orange ray florets, yet not in green parts of the plants. Carotenoids contribute to the orange-red colors in flowers (Tanaka *et al.* 2008) and the gene *PDS* is responsible for synthesizing carotenoids. It seems highly likely that the reduction of flower pigment on SP1 was originating from the reduced expression of phytoene desaturase (PDS) and a likely subsequent reduction of carotenoids. To our surprise, silencing the of *PDS* in the white parent SP2 leads to the pink color of ray florets and also on the scape. In Chrysanthemum, a carotenoid cleavage dioxygenase gene (*cmCCD4a*) that could degrade carotenoid to a colorless compound was found to contribute to white color and suspension the expression of *cmCCD4a* produced a yellow-colored Chrysanthemum flower (Ohmiya *et al.* 2006). Deng *et al.* (2012) found the expression of *2-PS* gene was downregulated to a very low level in *PDS*-silenced gerbera. *2-PS* gene (previously named as *ghCHS2*) belongs to CHS superfamily of type III polyketide synthase and catalyzes gerbera specific secondary metabolites gerberin and parasorboside (Eckermann *et al.* 1998; Koskela *et al.* 2011). Therefore, we assume that the *PDS* gene for carotenoid biosynthesis somehow is involved in gerbera coloration, and that the balance between the carotenoids and flavonoids determine the final presented color of gerbera.

The function of candidate genes for Botrytis cinerea resistance

In our previous studies, we developed Botrytis disease tests on *whole inflorescence*, *bottom* and *ray floret* and found several QTLs for these tests and candidate genes related to these QTLs individually. Two of those candidate genes, *ghPG1* and *ghsit*, which were mapped in QTL regions from the *ray floret* test were further characterized in this study. The two candidate genes are the homologs of the genes responsible for Botrytis infection in tomato. Polygalacturonases (*PGs*) are cell-wall-degrading enzymes and participate in tomato ripening (Cantu *et al.* 2009). *PGs*, among one of the tomato ripening-associated genes, have been found to facilitate Botrytis susceptibility (Cantu *et al.* 2009). The ABA-deficient *sitiens* mutant in tomato is

impaired in the last step of ABA biosynthesis (Taylor *et al*. 1988; Rock *et al*. 1991). The *sitiens* mutant with reduced ABA level is more resistant to Botrytis than wild type tomato plants that have higher amounts of ABA (Audenaert *et al*. 2002). The *sit* mutant also accumulates H_2O_2 and changes cell walls timely and efficiently to resistance Botrytis infection (Asselbergh *et al*. 2007).

The normal function of these two genes might be beneficial to Botrytis infection, while when the gene expressions are suppressed, Botrytis might fail to take advantage of the genes for infection. Plant genes/alleles that facilitate pathogen infection are defined as susceptibility (S) genes/alleles and an exclusive S-gene list is given by van Schie and Takken (2014). These two genes are part of that list. In tomato, simultaneous suppression of two 'S-genes', *PG* (polygalacturonase) and *EXP1* (expansin), lead to a reduced susceptibility to *B. cinerea* (Candu *et al*.2008). Similarly, silencing two signaling components *EDS1* and *SGT1* by VIGS enhanced resistance to *B. cinerea* in *Nicotiana benthamiana* (Oirdi and Bouarab 2007). Silencing S-genes can limit the ability of the pathogen for infection and that was performed in this study. A remarkable reduction of lesion size was found in these S-gene silenced gerbera ray florets and resulted from slowing the spreading of Botrytis on ray florets 24hpi. Denby *et al* (2004) considered that the variations in lesion size of Arabidopsis ecotypes for Botrytis infection were caused by either time prior to lesion initiation or the lesion's growth rate. Suppression of these two genes might postpone the lesion initiation and presented a delayed invasion at 24hpi on the ray florets.

Genes contributing to fruit ripening are considered to induce *B. cinerea* susceptibility in tomato since the two processes: fruit ripening and successfully *B. cinerea* infection require the expression of cell-wall-degrading enzymes (Cantu *et al*. 2008). *B. cinerea* is probably hitchhiking with the process of fruit ripening to penetrate the plant cell. Gerbera inflorescences after cutting inevitably experience senescence. Loss of membrane integrity is an early stage of plant senescence (Fan *et al*.1997). ABA and ethylene participate in the regulation of plant senescence and promote this process. Thus, reducing the amount of ABA will prevent senescence. It is easy to understand that both of the two candidate genes are related with senescence and that senescent cell walls support Botrytis infection. The two genes interacted with Botrytis in a somehow similar way which might be the reason that no further decrease of the lesion size on SP2 for the two gene silenced constructs was found compared with *TRV2::ghsit* alone.

Based on the visible indications from *TRV2::PDS* gene silenced plants of SP2, there may only be a small part, of the ray florets on the flower inflorescence be silenced. We also constructed TRV2 vectors carrying the candidate gene *ghCHI* that mapped in a botrytis resistance QTLs from *whole flower* test. However, when we sprayed the Botrytis spore on the whole flower, no visible difference was found (data not shown). The phenotypes after Botrytis infection may be difficult to detect as whole inflorescences are sprayed for the botrytis test, but only a small part of the ray petals are silenced based on the reporter gene. To explore the function of candidate genes that were found on the *whole inflorescence* or *bottom* test, a stable transformation may be needed for confirming the role in botrytis resistance.

This study confirmed that the two candidate genes probably are among the determining genes leading to the variation of Botrytis resistance in gerbera. After 24h of Botrytis *in vitro* inoculation on the ray florets, a significantly delayed spread of lesions was observed on candidate gene silenced ray florets compared to

controls. As a crop without genome sequence, using a candidate gene approach could be an efficient method to pinpoint possible causal genes. VIGS here provided a rapid way to study the relationship between gene expression and susceptibility to Botrytis. Using markers developed from the causal genes themselves could make marker-assisted selection more accurate and can avoid the risk of loss of linkage due to recombination when using more distant markers. Further research will have to show the value of the combined effects of the best alleles for the two genes *ghsit* and *ghPG1* in gerbera with respect to botrytis incidence and resistance under normal greenhouse and postharvest conditions.

Supplementary Tables and Figures

Table S5-1 Primers used in this study

Primer name	Oligonucleotide sequence (5'-3')	note
ghPDS_B1_274_F	ggggacaagtttgtacaaaaaagcaggctAGTAAAGTTTGCGATTGGGCT	*att*B1 is underlined
ghPDS_B2_274_R	ggggaccactttgtacaagaaagctgggtAAGTCTCTCAGGTGGGTTGC	*att*B2 is underlined
ghCHS1_B1_313_F	ggggacaagtttgtacaaaaaagcaggctAATTGCGTCTATCAAGCGGA	
ghCHS1_B2_313_R	ggggaccactttgtacaagaaagctgggtAGGTGGTGCAGAAGATGAGG	
ghCHS4_B1_282_F	ggggacaagtttgtacaaaaaagcaggctTGCAGTTACTTTTCGTGGGC	
ghCHS4_B2_282_R	ggggaccactttgtacaagaaagctgggtTGAAAGGCCTCTACCAAGCT	
ghPG1_B1_294_F	ggggacaagtttgtacaaaaaagcaggctAAAGCCTCCCCACTTCTACC	AF
ghPG1_B2_294_R	ggggaccactttgtacaagaaagctgggtGCCGCTGTATCTAGCAAACC	
ghsit_B1_428_F	ggggacaagtttgtacaaaaaagcaggctCTGTCGCTGCACTGGTTATC	
ghsit_B2_428_R	ggggaccactttgtacaagaaagctgggtCAGCTGCTCCAACTTCGATC	BR
ghPG1+ghsit_BF+AR	GGTAGAAGTGGGGAGGCTTTCAGCTGCTCCAACTTCGATC	BF+AR
ghPG1+ghsit_AR+BF	GATCGAAGTTGGAGCAGCTGAAAGCCTCCCCACTTCTACC	AR+BF
*att*L1	TCGCGTTAACGCTAGCATGGATCTC	sequencing
*att*L2	ACATCAGAGATTTTGAGACACGGGC	sequencing
TRV2	GTTTTTATGTTCAGGCG	sequencing
ghPDS_qPCR_F	GTGGCCAAGTCAGGCTAAAT	qRT-PCR
ghPDS_qPCR_R	ACATAAGCATCGCCTTTGAT	qRT-PCR
ghPG1_qPCR_F	GTGGGGTTTTCTGGCTACG	qRT-PCR
ghPG1_qPCR_R	AGTATCATTCGCGCCGTTAC	qRT-PCR
ghsit_qPCR_F	TTCAACCACCTGTTTTGCAG	qRT-PCR
ghsit_qPCR_R	AATTCTGGGATTTGTTCAAGATT	qRT-PCR
ghGAPDH_qPCR_F	CCAGGAACCCAGAGGAGATACC	qRT-PCR
ghGAPDH_qPCR_R	GGAGCGGATATGATGACCTTCTTG	qRT-PCR

Table S5-2 The number of ray florets used for Botrytis disease test.

		Treatments			
		TRV2::GUS	*TRV2::ghPG1*	*TRV2::ghsit*	*TRV2::ghPG1+ghsit*
parents	SP1	27 (5)*	-	-	52 (10)
	SP2	22 (5)	21 (4)	20 (4)	84 (14)
	FP1	32 (5)	34 (6)	34 (6)	57 (10)

* the number of ray florets collected for disease test; the number in brackets are indicating from how many inflorescences the ray florets were collected.

>*ghPDS*; 274bp
AGTAAAGTTTGCGATTGGGCTCTTGCCAGCAATGTTAGGTGGACAGGCTTATGTTGAGGCTCAAGATGGTTTGAGTGTTCAAGACTGGATG
AGAAAGCAAGGCATACCAGATCGGGTTACTACTGAGGTGTTTATTGCCATGTCAAAGGCGTTAAACTTCATAAATCCAGATGAACTTTCTA
TGCAATGTATTCTCATTGCTTTGAACAGGTTTCTTCAGGAGAAGCACGGTTCAAAGATGGCATTTTTAGATGGCAACCCACCTGAGAGACT
T

>*ghCHS1*; 313bp
AATTGCGTCTATCAAGCGGATTATCCCGATTACTATTTTCGGATCACCAAGAGTGAACACATGGTGGATCTCAAAGAGAAATTCAAGCGCA
TGTGTGACAAGTCGATGATAAGGAAACGTTACATGCACATCACAGAGGAGTATCTTAAACAAAACCCTAACATGTGCGCATACATGGCGCC
GTCGCTCGACGTCCGGCAAGACCTGGTCGTCGTCGAAGTCCCAAAGCTCGGCAAAGAAGCCGCCATGAAAGCCATCAAAGAATGGGGACAC
CCCAAATCCAAGATCACCCACCTCCATCTTCTGCACCACCT

>*ghCHS4*; 282bp
TGCAGTTACTTTTCGTGGGCCTGATGAAACCCATCTTGATAGCCTTGTGGGCCAAGCATTGTTTGGTGACGGGGCTGCTGCTATCATAGTT
GGGTCCGACCCGTTGTTGGGCCAAGAAAAGCCTCTTTTTGAGATGGTTTATGCGGCCCAAACCATTCTCCCTGATAGCGAGGGGGCGATTG
ATGGGCATCTTCGTGAGGTCGGGCTTACCTTTCATCTTCTTAAGGATGTTCCTGGGCTTATATCAAAACACATCGATAAGAGCTTGGTAGA
GGCCTTTCA

>*ghsit*; 428bp
CTGTCGCTGCACTGGTTATCGACCCATTGCTGACGTCTGTAAAAGTTTCGCTGCTGACGTGGATATTGAGGATTTGGGGCTTAATTCTTTC
TGGAAAAAGGGCGAAAATGAAGACCCGGATGAAAAAATTCATAAACTACCCTTTTATGATTCGAAACAGATATGTACTTATCCAGAGTTCT
TGAAAAAAGAATATCAGAAATATGAGAAAACATCTTGGTATAGTCCGGTTTCTATAAAAGATCTTCAGAGCTTACTGGAATCAAGTTCAGC
TGAAAATGGGGTGACTGCAAAGTTAACTGTTGGTAACACAGGCATAGGTTATTATAAGGAAAATCAGCATTATGATAAGTACATTGATCTT
AGGTTTATCCCTGATCTCTCTATAATTAAGAAAGATGACTCTAAGATCGAAGTTGGAGCAGCTG

>*ghPG1*; 294bp
AAAGCCTCCCCACTTCTACCCCTGGAGTTCACTTTATTTACCAAACTAGCCTCCACCCACTCTCTTTCTTCTCACCTCTCCTCCTTCTGCG
CCACCGCCAACTTATTCTGCTTTTCCGACACCGGCACCACAGCCGGCGGCCAGTCAAATCCAAGCAAAGACTCAAACTTCGCCGTCTACGA
CGGCAAACGATTCACCAACTACGGCGCCGGCCGCATTTCCGGCGGAGACACGTTCAAGAACTACTCGGAAAACACCAACTTTGCCGTTAGT
GGGTTTGCTAGATACAGCGGC

>GUS; 648bp
CACTTACAGGCGATTAAAGAGCTGATAGCGCGTGACAAAAACCACCCAAGCGTGGTGATGTGGAGTATTGCCAACGAACCGGATACCCGTC
CGCAAGTGCACGGGAATATTTCGCCACTGGCGGAAGCAACGCGTAAACTCGACCCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTG
CGACGCTCACACCGATACCATCAGCGATCTCTTTGATGTGCTGTGCCTGAACCGTTATTACGGATGGTATGTCCAAAGCGGCGATTTGGAA
ACGGCAGAGAAGGTACTGGAAAAAGAACTTCTGGCCTGGCAGGAGAAACTGCATCAGCCGATTATCATCACCGAATACGGCGTGGATACGT
TAGCCGGGCTGCACTCAATGTACACCGACATGTGGAGTGAAGAGTATCAGTGTGCATGGCTGGATATGTATCACCGCGTCTTTGATCGCGT
CAGCGCCGTCGTCGGTGAACAGGTATGGAATTTCGCCGATTTTGCGACCTCGCAAGGCATATTGCGCGTTGGCGGTAACAAGAAAGGGATC
TTCACTCGCGACCGCAAACCGAAGTCGGCGGCTTTTCTGCTGCAAAAACGCTGGACTGGCATGAACTTCGGTGAAAAACCGCAGCAGGGAG
GCAAACAATGA

Fig. S5-1 The inserted target gene fragment sequences into the TRV2 vector. Underlined sequences are indicating primers sites.

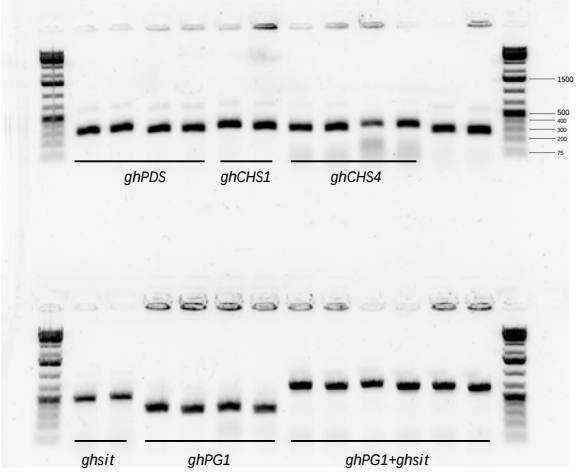

Fig. S5-2 The fragment size of target genes transformed to *Agrobacterium tumefaciens*. Agro-bacterial suspension with *TRV2::GOI* constructs were used as a template for PCR and the primers were the same as the primers for amplifying target genes.

Fig. S5-3. The variation for *PDS* silencing in SP1 inflorescences. a, the phenotype of PDS-silencing in SP1 plants showed a diluted orange color in parts of the inflorescences; b and c, one or two ray florets with a diluted orange color; d, e and f, bleaching on ray florets.

Fig. S5-4. The stem break happened on FP1. As stem extension and the increased flower head of gerbera, the flower stem of FP1 were broken at the site of scratching for agro-infiltration.

Fig. S5-5. The ray florets sampled for Botrytis inoculation (a) and the area circled for measuring the lesion size, the lesion size of the petals from top to bottom are 0.0024cm^2, 0.0068cm^2 and 0.0105cm^2.

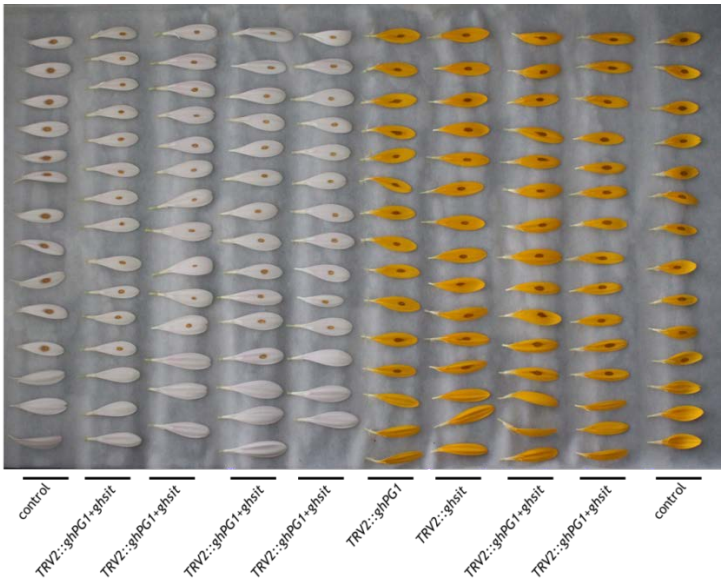

Fig. S5-6. Ray florets from SP2 (white cultivar) and FP1 (yellow cultivar) inoculated with Botrytis at 24hpi (part). Controls were *TRV2::GUS* silenced plants and the other treatments were indicated. For each row, the first five ray florets were sampled from one plant and the following 5 (or 6) were the sample from another plant. The bottom three ray florets were inoculated with MQ.

Chapter 6 General discussion

Gerbera gray mold, caused by *Botrytis cinerea,* is a serious and unpredictable problem that can happen in all steps of gerbera cut flower production and post-harvest life. Breeding gerbera with improved Botrytis resistance is one of the most important objectives gerbera breeders would like to achieve. Plant resistance to *B. cinerea* is considered a quantitative disease resistance (QDR) which is under polygenic control, and is highly affected by the environment. Classical breeding is based on a selection of desired phenotypes and has been used for improving many traits in ornamentals. However, for QDR improvement by classical breeding may turn out to be much more difficult and time-consuming. The lack of 'real' Botrytis resistant gerbera and the difficulty to test resistance form obstacles for obtaining better performing varieties. Hence, classical breeding that employs only breeding based on phenotypes for Botrytis resistance in gerbera might be inefficient.

The main aim of this thesis was to make the first step to unravel the genetics of *Botrytis cinerea* resistance in *Gerbera hybrida*. The entire research took a top-down strategy. The study went from the generation of four parental transcriptome data sets, development of SNP markers (*Chapter 2*), construction of genetic maps and mapping of QTLs to identify the potential locations for Botrytis resistance (*Chapter 3*). This was combined with candidate gene searching in other crops, querying and mapping homologous genes (*Chapter 4*) and characterizing the candidate genes, which co-localized with QTLs (*Chapter 5*). The whole process not only helped us to unravel the genetics of Botrytis resistance in gerbera and develop genetic tools for gerbera improvement, but also could serve as guidance for developing genetic resources for other ornamental plants that start from scratch. In this chapter, the general discussion, I would like to discuss the findings of this thesis in view of results yielded from recently published studies.

Development of genomic and genetic resources for ornamental crops

The transition from phenotype-based breeding to genotype-based breeding requires a large amount of sequencing information and genetic resources. With these sequencing data, molecular markers can be developed for establishing the relationship between phenotypic variation and sequence polymorphisms. Once the connection of genotype and phenotype is established, molecular markers will assist phenotype-based classical selection. In general, the application of marker-assisted selection (MAS) for ornamental crops has been lagging behind other agricultural and horticultural crops (Arens *et al*. 2012; Smulders *et al*. 2012). It results partly from the fact that some very important breeding goals for ornamentals, like flower color or pattern, are easy to select for as the morphological trait. Moreover, the floriculture has traditionally been an industry with many small companies and hundreds of different species (and cultivars). The costs and time needed for the development of molecular markers for every specific ornamental crop limits the use of MAS. In addition, most ornamental crops are highly heterozygous with a complex genetic background which makes that, although markers might be available, it generally is much more difficult to apply them broader (Debener 2009). Most ornamental crops have a relatively large genome size. According to the *RBG Kew Plant DNA C-value database* (Bennett and Leitch 2002), the amount of DNA in the unreplicated haploid nuclear genome (known as the 1C-value) for widely used cut flowers, like *Rosa* (average) is 0.74 picograms (pg); *Gerbera hybrida* is 2.56 pg; *Phalaenopsis* (average) is 3.67 pg and *Chrysanthemum* (average) is 8.71 pg. Many ornamentals are polyploids as well, such as most

roses, lily, carnation, freesia, gladiolus, alstroemeria and chrysanthemum. It results from evolution, selection and hybridization (either intraspecific or interspecific) during the hundreds and sometimes thousand years of domestication. It is not difficult to imagine that a great number of duplicated sequences probably exist in the genomes of ornamentals.

It could be the best time to start MAS in ornamental crops with the introduction of next generation sequencing and high-throughput genotyping. Development of transcriptome data based molecular marker discovery might be an economical option and can achieve many valuable genetic resources, which could be employed for gene identification. In *Chapter 2*, we generated a consensus contig with a total length of 51,360,054 bp. Based on the 1C-value, the whole genome size of *G. hybrida* is estimated to be about 5007 Mb (1 pg ≈ 9.78×10^8 bp), and our transcriptome data thus would represent 1% of the whole genome. Although only a small percentage of sequence data, they are regarded as the best representative of the genome to understand gene activity. Hence, targeting on genic regions can decrease the complexity of large genomes and reduce the redundancy of sequencing repeat regions. Also, the genic regions are expected to have lower polymorphism rates and this makes the development of markers more accessible and results in more widely applicable markers.

Start from generation of transcriptome data

With the rapid progress of high-throughput next-generation sequencing technologies (NGS, e.g. Illumina Sequencing), quite a lot of ornamental genomic resources were generated from transcriptomes in recent years such as from *Rosa* (Dubois *et al.* 2012; Gao *et al.* 2016; Kim *et al.* 2012; Koning-Boucoiran *et al.* 2015; Yan *et al.* 2015; Zhang *et al.* 2016b); *Chrysanthemum* (Hong *et al.* 2015; Song *et al.* 2016a; Song *et al.* 2016b; Xu *et al.* 2013); *Lilium* (Shahin *et al.* 2012; Zhu *et al.* 2016); *Tulipa* (Shahin *et al.* 2012; Moreno/Leeggangers 2016); *Paeonia* (Li *et al.* 2015b; Wu *et al.* 2014; Zhang *et al.* 2015; Zhou *et al.* 2013) and *Gerbera* (Fu *et al.* 2016; Kuang *et al.* 2013; Laitinen *et al.* 2008; Laitinen *et al.* 2005; Laitinen *et al.* 2007; Li *et al.* 2015). Each transcript from expressed sequence tags (ESTs) sequencing represents a specific gene and can be annotated to provide a general awareness of the potential gene function into different categories, molecular function, biological process and cellular component. Producing these transcriptome data always comes with several particular targets in these studies, such as predicting certain candidate genes involved in the hormonal regulation pathway; related to flower color variation; activated under different biotic/abiotic stress; expressed during developmental stages; or for molecular marker development (*Chapter 2, 3 & 4*).

In *Chapter 2*, we generated a genetic resource by preparing cDNA libraries of all four gerbera parental genotypes and performed sequencing on an Illumina HiSeq platform. A total number of 36,770 consensus contigs with an average length of 1397 bp were produced and used for gene annotation. Around 29,198 (80%) of the consensus contigs showed BLAST hits based on sequence similarities and approximately 55% of the contigs have strong similarities (smaller than 1e-100) that could provide a direct association with potential genes/proteins/functions. These data are comparable with those reported by Laitinen *et al.* (2005) where 58.5% of the 8098 Unigenes remained 'unknown' and Kuang *et al.* (2013) where 44% of the Unigenes is smaller than 1e-100. The better outcome of our results is most probably due to the fact that

more and more plant sequence data have become available very recently which would help to get an updated BLAST hit. Another reason in our favor could be that we sequenced cDNA from both leaves and flower buds, and assembled four parental genotypes to produce the final consensus contigs (UniGene).

Although our transcriptome/RNA-sequences were obtained from unchallenged/untreated materials by gerbera gray mold, we still could recognize almost all the genes of the phenylpropanoid and flavonoid biosynthesis pathway that could produce plant secondary metabolites responsible for plant defense responses (Dixon 2001; Dixon *et al.* 2002). Also, we found the genes related to the biosynthesis and regulation of the phytohormone jasmonate (JA) and ethylene (ET) which also play a role in plant defense against *B. cinerea* (Thomma *et al.* 2001) (*Chapter 2*). Additionally, these transcriptome data provided us all gene sequence information we needed for SNP marker development (*Chapter 2 & 3*) and, candidate genes identification (*Chapter 4*). Furthermore, the EST data provided housekeeping gene sequences for RT-qPCR normalization and indicator genes for gene silencing experiments (*Chapter 4 & 5*). By annotating the transcriptome to KEGG (Kyoto Encyclopedia of Genes and Genomes) data, we generated a rough impression of what genes/transcripts are active in which pathway. We believe once a gene function is identified in other pioneer/model crops, transcriptome data could immediately provide a valuable resource for retrieving homologous genes in gerbera and provide an insight to explore gene function.

Towards the genetic dissection of complex traits

SNP marker development

Transcriptome data can also generate numerous transcripts with sufficient read-depth to guarantee high quality single nucleotide polymorphisms (SNP) identification (Shahin *et al.* 2012; Kim *et al.* 2014). Since each transcript represents an expressed gene, SNPs are derived from the coding region of a gene and directly exhibit DNA polymorphism in the genes. Development of SNP markers for the highly heterozygous ornamentals is very feasible and 200–1000 SNP markers will be sufficient to construct a genetic map for QTL mapping (Smulders *et al.* 2012). SNPs are valuable resources for genetic research and widely accepted as markers for genetic linkage map construction or as newly added markers for existing linkage maps as shown in recent years for ornamentals like rose (Bourke *et al.* 2017; Vukosavljev *et al.* 2016), lily (Shahin *et al.* 2011), tulip (Tang *et al.* 2015) and gerbera (Fu *et al.* 2017). The applications of SNP markers in these studies were done with the aim to produce a high density linkage map, increase the marker density on existing linkage maps or integrate different linkage maps.

Markers developed from SNPs have a benefit from their abundant numbers, especially in the case of the highly heterozygous ornamental crops. In *Chapter 2*, we achieved in total 398,917 polymorphic loci (SNPs existing either in two alleles of a parent or between parents) from all consensus contigs in the process of SNP detection. SNPs derived from transcriptome sequencing data for marker development were assessed according to several criteria such as high-quality sequences, at least two reads, no other SNPs present on flanking sequence of 50 bp on both sides, *etc*. Selected reliable SNPs were genotyped by Kompetitive Allele Specific PCR (KASP) on the two entire populations. However, because of the high heterozygosity of gerbera, some SNPs showed unexpected segregation patterns that were identified as SNP markers with null-alleles. Quite a few SNP markers (about 5-6% in both gerbera populations) were identified as

containing null-alleles. However, most of these markers could be rescored and mapped (*Chapter 3*). Null-alleles were also noticed in other ornamental plants, such as lily (Shahin *et al.* 2011), rose (Vukosavljev *et al.* 2012) and tulip (Tang *et al.* 2015). In *Chapter 4*, some primers designed for candidate gene amplification and high resolution melting (HRM) analyzes failed to distinguish the allelic variation in populations due to a similar reason. From the melting curves it became evident that they apparently were generated from amplicons containing multiple SNPs. Actually, we adopted the criteria for SNP selection for linkage group construction (*Chapter 3*), and tried to target single SNPs for HRM without flanking SNPs, yet unexpected SNPs could still pop up. One possible reason is that the default setting for computational SNPs detection always required a minimal number of high quality reads. Low quality reads are removed, but this might also filter out some rare SNPs or SNPs in less well covered flanking regions. Filtering with a low-threshold setting for SNPs detection might be beneficial.

SNP markers we used for linkage group construction and QTL mapping (*Chapter 3*) and candidate gene mapping (*Chapter 4*) are bi-allelic and not multi-allelic, and they can only represent two possible alleles. When looking at the parents from the populations, we noticed in many cases more than two alleles are present (*Chapter 4*). It might minimize the transition of the QTL analyzes results from our working populations to other genotypes. Thus, in *Chapter 4*, we sequenced the full length of several candidate genes that co-localized with the detected QTL regions, and identified multiple alleles. We expected that the candidate gene allelic information combined with the QTL analysis could be applied to a greater extent to different gerbera genotypes as the initial screening for a genome-wide association study (GWAS).

Genetic linkage groups of Gerbera hybrida

In *Chapter 3*, successfully genotyped SNPs markers were used for genetic linkage map construction. As a cross pollinated plant, four gerbera maternal and paternal genetic maps containing 30, 29, 27 and 28 linkage groups were constructed simultaneously for subsequent studies. *G. hybrida* is characterized as a diploid with a chromosome number of 2n = 50 (Cappadocia and Vieth 1990; Ahmim and Vieth 1986; Reynoird *et al.* 1993; Bennett and Leitch 1997; Marie and Brown 1993; Sitbon 1981). All parental linkage groups constructed could be integrated into a consensus genetic map covering 24 of the 25 expected chromosomes, only linkage group (LG) no. 25 could not be assigned. This could be due to the chromosome size of this particular LG and also to the total number of markers used in our study. Most of the chromosomes of *G. hybrida* contained markers, but we still probably need to add more markers to also identify the missing linkage group.

Teeri *et al.* (2006) indicated that *G. hybrida* carries 50 chromosomes or chromosomal fragments from different *Gerbera* species as a functional diploid. It results from the origin of the commercial gerbera (*Chapter 1*). However, plants in the family of the *Compositae* are believed to have an ancestral chromosome base number of x = 9. On the meta-tree of *Compositae* which is used for understanding the systematics, evolution, and biogeography of this family (Funk *et al.* 2009a; Funk *et al.* 2005; Funk *et al.* 2009b), the tribe Mutisieae (*Gerbera* belongs to) and its close tribes/clades (Barnadesieae, Stifftia, *etc.*) are listed as the *Basal Grade* (Ortiz *et al.* 2009). Plants in these branches are hypothesized as paleopolyploid with the derived base number x_2 = 27 from basal number x = 9 (Semple and Watanabe 2009).

Chromosome counts reported for genera in Mutisieae varied from 8, 9, 10, 11, to 25, 26, 27 and 36, and it indicates that polyploidy and dysploidy have occurred multiple times in this tribe (Semple and Watanabe 2009). We assume *Gerbera* might be an ancient hexaploid but lost 4 chromosomes that finally make the *G. hybrida* nowadays as the functional diploid we know. In *Chapter 5*, several genes from the same gene family were mapped in different parental linkage groups. These duplicated genes existed in the gerbera genome and are probably derived from polyploidization events.

Phenotyping for Botrytis resistance

Botrytis disease severity in gerbera was evaluated using three different tests, *whole inflorescence*, *bottom* (of disc florets) and *ray floret*. According to these phenotypic data from the two populations, we estimated the broad-sense heritability (H^2) of Botrytis resistance for these three tests in S population as 28.24%, 32.6% and 38.76%, respectively, while H^2 in F population for the three tests is 38.83%, 46.41% and 48.70%. After calculating the broad-sense heritability, we could notice that a great part of phenotypic variation after *B. cinerea* infection is caused by environmental factors. Selection of individuals only based on phenotype after Botrytis infection would be insufficient and biased.

Although these are two independent populations constructed by two breeding companies and grown in different environments, H^2 of all tests in F population are higher than in S population. There might be more genetic variation in F population since it is a cross between a cut flower genotype and a garden used gerbera with wild characteristics, while S population is produced by two gerbera genotypes with similar qualities as cut flower. The H^2 of *ray floret* tests on the two populations are all higher than the other two tests. It might be because we added the potato dextrose broth (PDB) for *ray floret* test which ensured a higher germination rate of Botrytis spores. The germination of Botrytis spores is indicating the start of a successful infection that could also be included in the environmental variation/non-genetic factors in the formula of H^2. Since *ray floret* test always had a higher H^2, we think the *ray floret* test is a more repeatable test and we also used *ray floret* test in *Chapter 4 & 5* (also considering the amount of material available and required and relative ease of VIGS and disease testing). The candidate genes (shown with statistical significance) related to *ray floret* (test) is different from the other two tests (*Chapter 4*). It might also be because of the added PDB for the Botrytis spore germination, or because different parts of the gerbera inflorescence might have different mechanisms to response to Botrytis infection. Van Kan (2006) indicated that Botrytis needs to decompose host biomass for its own use as the energy, and plants with high pectin content would be beneficial for Botrytis infection. Since in the *ray floret* test energy could be obtained from the PDB, other genetic variation in gerbera would be more important in the response to Botrytis.

QTL mapping for Botrytis resistance

The big difference between our study and most of the researches at analyzing ornamental transcriptome data is that we were not concentrating on evaluating differential gene expression patterns under different conditions, but aimed to develop SNP markers based on these EST data to construct linkage groups and for QTL mapping. When plants are under different conditions, stresses or developmental stages, or with a different origin, a large number of genes/transcripts might be activated and revealed with a differential

expression level. So which genes would be the switch for these downstream genes and finally lead to the phenotypic variation in each individual? For a disease resistance study with a necrotrophic pathogen and resistance controlled by multiple QTLs such a question is not likely to be addressed by differential expression studies.

With the advent of various and abundant molecular markers (e.g., SNP markers used in *Chapter 3*), construction of genetic maps and QTLs mapping has become easy to perform. Via development of two F1 segregating populations and SNPs markers, four parental genetic maps were constructed separately for QTL mapping (*Chapter 3*). QTL mapping was performed with these phenotypic data and a total of 20 QTLs (including one identical QTL for *whole inflorescence* and *bottom* tests) were identified in the four parental linkage maps. Markers that are identified as tightly linked with genes or QTLs of interest could transfer, track and select the favorable allele(s) in breeding for genetic improvement (St. Clair 2010). Individuals not containing the desired marker alleles could be dismissed or disposed of, as a preliminary screening to save space and time for breeding companies. Collard *et al.* (2005) listed the advantages of MAS, like saving time, ignoring the environmental variation, choosing wanted genotypes at an earlier stage, pyramiding multiple genes, preventing linkage drag, selecting traits with low heritability *etc.* There is no scientific report on MAS application in crop improvement for Botrytis resistance yet, and QTL analysis on Botrytis resistance or susceptibility has been performed only in a limited number of crops like tomato (Davis *et al.* 2009; Finkers *et al.* 2008; Finkers *et al.* 2007a; Finkers *et al.* 2007b), chickpea (Anuradha *et al.* 2011), *Brassica rapa* (Zhang *et al.* 2016a) and gerbera (*Chapter3*; Fu *et al.* 2017). MAS has been used to increase the favorable QTL alleles for other traits, such as stripe rust in barley, common bacterial blight in common bean, Fusarium head blight in wheat, which were reviewed by St.Clair (2010) and build up our confidence on the application of MAS for improving Botrytis resistance in gerbera.

Finding markers for candidate genes related to a complex trait

Studies from pioneer plants on Botrytis resistance

Identification of the causal gene(s) related to Botrytis resistance can benefit greatly from the basic researches on interactions between Arabidopsis and Botrytis. A successful infection of *B. cinerea* comprises several steps, penetrating the host cell wall, killing the host cell and decomposing the host biomass as well as the involvement of sensing and signaling (van Kan 2006). A series of genes in the host Arabidopsis were defined by using existing Arabidopsis mutants and series of transgenic techniques (Denby *et al.* 2004; Ferrari *et al.* 2007; Ferrari *et al.* 2003; Govrin and Levine 2000; Kliebenstein *et al.* 2005; Lionetti *et al.* 2007; Mengiste *et al.* 2003; Thomma *et al.* 1999; Veronese *et al.* 2006). The variable resistance/susceptibility to *B. cinerea* in Arabidopsis is influenced by multiple loci which were detected at the gene level (Denby *et al.* 2004; Rowe and Kliebenstein 2008). These massive studies provide a primary understanding of Botrytis resistance and source for candidate genes for gerbera or other crops.

The plant defense mechanism against necrotrophic pathogens is considered to be different from defense against biotrophic pathogens which would promote a hypersensitive response (HR) to prevent an infection (Glazebrook 2005). HR is a form of cell death and might not be effective to defend against a necrotrophic pathogen, because the death of the host cells facilitates the infection of *B. cinerea*. Moreover,

B. cinerea itself carries weapons to kill the host cell (van Kan 2006). This was exemplified on an Arabidopsis HR-deficient mutant *dnd1* in which it was found that the growth of *B. cinerea* on leaves was suppressed (Govrin and Levine 2000). The production of camalexin, a secondary metabolite phytoalexin, is also involved in Arabidopsis/Botrytis interactions, despite showing variation in different Botrytis isolates and Arabidopsis genotypes (Kliebenstein *et al.* 2005). There are also two QTL analysis studies on Botrytis resistance/susceptibility for Arabidopsis to discover the complex genetic basis of this quantitative disease resistance (Denby *et al.* 2004; Rowe and Kliebenstein 2008). Denby *et al.* (2004) identified 12 QTLs in an RIL (recombinant inbred line) population using two Botrytis isolates. While comparing the region of Botrytis susceptibility QTL on the physical map with the genomic location of certain genes related to Botrytis infection, four previously identified genes (*BOS1*, *EIN2*, *JAR1* and *PGIP1/2*) were found to be co-localized with the detected three QTLs. Rowe and Kliebenstein (2008) also located candidate genes in the QTL regions and found overlaps with the QTLs from Denby *et al.* (2004).

Pinpointing candidate genes in ornamentals

As a model plant, Arabidopsis has a completed genome sequence with detailed gene information. The corresponding position between a QTL region and possible candidate genes are readily available. In some plants with a genome sequence, narrowing down the QTL region by fine mapping could also contribute to the efficiency of MAS. This is because the linkage between the flanking markers of a QTL and the causal gene allele can be broken by recombination (Andersen and Lubberstedt 2003; Poczai *et al.* 2013). Narrowing down the QTL region and even better to be able to develop polymorphic markers based on the causal gene(s) underlying a QTL can increase the effective use of MAS in breeding. Thus, identifying the causal gene in QTL regions and development of markers targeted from candidate gene is an alternative promising approach.

To identify a potential functional gene and develop functional gene targeted markers on plants without prior knowledge of the whole genome, for example, ornamentals can be based on a candidate gene (CG) approach. The CG approach is based on the hypothesis that analogs of known functional genes from other species could control traits of interest in the focal species (Collard *et al.* 2005; Pflieger *et al.* 2001). For ornamentals, in which ESTs have been produced and are available these could be used for candidate gene homolog identification. CG homologs can either be used for studying the genetic basis of particular traits or for marker development. Markers that are developed from polymorphic sites in a functional gene are defined as functional markers (FMs) (Andersen and Lübberstedt 2003). Probably due to the limited functional genetic resources, Poczai *et al.* (2013) found about 90% of the previous studies which were involved with molecular markers used arbitrarily amplified DNA markers, and only a small part of the studies was adopted with gene-targeted markers or functional markers. FMs are in complete linkage with the favorable allele (Andersen and Lübberstedt 2003), so FMs are of much higher predictive value on phenotypic trait variation than random DNA markers. With increased research on model plants and genetic resources available in ornamental plants, the use of functional gene targeted markers will be increasing in all plant species irrespective of the status of their genome sequence availability. For instance in rose (*Rosa hybrida*) several genes, controlling ornamental characteristics and disease resistance *etc.*, have been mapped (Byrne 2009; Foucher *et al.* 2008). Twenty-five homologs of Arabidopsis genes in the

gibberellin (GA) pathway that potentially controls recurrent blooming trait in rose were mapped (Remay *et al*. 2009). Spiller *et al*. (2011) constructed an integrated rose genetic map from four individual maps via several bridge SSR and gene-targeted markers, and the position of CGs and previous detected QTLs were recognized. The co-localization of several candidate genes with QTLs was also shown in recent studies (Kawamura *et al*. 2015; Moghaddam *et al*. 2012; Otagaki *et al*. 2015; Roman *et al*. 2015). Recently Bourke *et al*. (2017) showed a high-density SNP genetic map of rose that synteny with *Fragaria vesca* is very high and the genome sequence of this species could be used to find functional gene positions in rose as well. This information indicates that the CG approach is valuable for molecular breeding approaches in ornamental crops.

The effectiveness of the candidate gene strategy

With the idea of the candidate gene strategy, we also developed markers from candidate genes that are related to Botrytis resistance (*Chapter 4*). The homologs of candidate Botrytis resistance genes from other crops, like a lot of genes from Arabidopsis, but also from tomato and grape, two major hosts of *B. cinerea*, were confirmed in gerbera based on protein similarities. Unlike the CG markers in rose that are SSR markers, the SNPs we used directly originated from the gerbera ESTs contigs (*Chapter 2*). Twenty-nine candidate genes, out of 71 CGs could be successfully genotyped by HRM and were mapped on the parental linkage maps (*Chapter 3)* of which seven candidate genes could be mapped on both populations. Using the same phenotyping data for QTLs detection (*Chapter 3*), we found different genetic groups which were separated by allelic variation of 17 candidate genes (markers) and showed a significant difference ($P<0.05$) on the disease score from either the *whole inflorescence*, *bottom* (of disc florets) or *ray floret* tests. Apparently, candidate genes are worth to give more attention, especially with the indication of statistically significant difference or when they are located in the vicinity of the QTLs detected. These promising candidate genes could be categorized into various types and some of them were included in *Chapter 2*. Looking at the *bottom* test, genes from secondary metabolite biosynthesis pathways (stilbene synthase, polyketide synthase), phytohormone biosynthesis (ABA aldehyde oxidase) and signal transduction (elongator subunits 2), cuticle biogenesis (long-chain acyl-CoA synthetase, 3-ketoacyl-CoA synthase) and plant cell wall-degrading enzyme (polygalacturonase) are associated. For *whole inflorescence* and *ray floret* tests, fewer gene categories were involved. Secondary metabolites are expected to play an important role in the defense against necrotrophs, but no gene was found in the secondary metabolite biosynthesis pathways with a significant difference on *ray floret* test in any population. I assume it probably results from the different inoculation method we used for the *ray floret* test or also could be due to the different defense mechanisms for different parts of gerbera inflorescences.

Statistical analysis of the candidate gene allelic variation on these disease scores in our study (*Chapter 4*) is like the single-marker analysis (Collard *et al*. 2005). A single marker separates a (mapping) population and the disease score mean of two or more groups can be conducted by *t*-test or ANOVA. Markers are assumed to be linked with traits of interest or QTLs when one group is significantly better/different than/from the other group. The single-marker analysis could be performed without the genetic maps, although linkage maps were already constructed by then, and also QTLs (without CGs marker) have been analyzed (*Chapter 3*). After redoing QTLs analysis with new CGs mapped on the parental maps, we found

that some candidate genes which showed a statistical difference by *t*-test were not detected as the QTLs or co-localized with QTLs region. MQM mapping (also known as the composite interval mapping) we used for QTLs detection is considered to have more statistical power, because it could estimate the possible recombination between markers and QTLs, and calculate multiple QTLs *etc*. (Collard *et al*. 2005; Zeng 1994). Therefore, interval mapping (composite or simple) is widely accepted by researchers as the methodology for QTL mapping. Rebai *et al*. (1995) proposed that interval mapping gives more powerful results when markers are sparse or with increasing intervals, otherwise, the single marker analysis would also be powerful enough. The candidate gene analysis is indeed the single-marker analysis, whereas the marker is developed from the motif of a candidate gene sequence itself, recombination to be happening in that gene itself would be rare. Thus, I very much believe that these candidate genes are promising to use.

Like the QTL analysis in *Chapter 3* where QTLs were detected from both parents, favorable alleles of candidate genes were also mapped on both parents (*Chapter 4*). Apparently, Botrytis resistance could be contributed by these candidate genes with additive genetic effects, and pyramiding more possible genes could be positive for gerbera improvement. The significant difference of CGs and the co-localization of QTLs with CGs indicates that these candidate genes could be involved in resistance to Botrytis and provide a more precise possibility to use MAS in gerbera breeding in the future.

The characterization of candidate genes mapped in the QTLs region

With the ongoing researches in model plants and pioneer plants and the increasing availability of EST data from ornamentals, finding a homolog for a specific gene that was previously identified in a model plant to start with might not be difficult anymore. The challenge is finding whether the gene functionally contributes to the phenotypic variation. A co-localization of putative candidate genes with QTLs provides favorable evidence that these candidate genes might indeed be responsible for the difference in resistance between genotypes. Gene function characterization is complementary to our initial QTL and CG studies (*Chapter 2 & 3 & 4*). There are several methods to study gene function. We followed a tobacco rattle virus (TRV) based gene silencing system, that was already previously described for gerbera (Deng *et al*. 2014; Deng *et al*. 2012), to inspect the function of two candidate genes thought to be involved in Botrytis resistance (*Chapter 5*). These two CGs are the homologs of the genes responsible for Botrytis infection in tomato and were mapped in the QTL region related to Botrytis resistance in gerbera *ray floret* test. Since the expression of these two genes was beneficial to Botrytis infection in tomato, Van Schie and Takken (2014) included them in the list of susceptibility (S) genes/alleles.

B. cinerea is notorious as an opportunistic fungus (Corbaz 1978; Prins *et al*. 2000). The best time for Botrytis to attack host plants is when plants are at their most vulnerable, like tomato in the process of fruit ripening or gerbera inflorescences experiencing senescence after cutting. Moreover, the expression of cell-wall-degrading enzymes is needed for tomato fruit ripening (Cantu *et al*. 2008) and the expression of these genes probably is used by *B. cinerea* for a successful infection. In the ABA-deficient tomato mutant *sitiens*, accumulation of H_2O_2 was observed earlier and defense-related transcript expression was higher than in wild type plants (Asselbergh *et al*. 2007). The two candidate genes (*ghPG1* and *ghsit*) mapped in the QTLs region are related to Botrytis infection and seem to function as susceptibility genes. Silencing the two

genes in gerbera by VIGS, we found a smaller lesion size indicating a possible higher resistance to Botrytis for gerbera ray florets. Denby *et al* (2004) considered that the variation on lesion size of Arabidopsis ecotypes was due to either time prior to lesion initiation or the lesion's growth rate. The function of PGs is originally for the plants' own biological processes, but the genes might also promote the penetration of Botrytis, thus knocking down these genes may have postponed the lesion formation in gerbera ray florets. The accumulation of ABA transfers gerbera plants in a more senescing state, leaving them vulnerable to Botrytis. Therefore, silencing ABA will increase resistance.

Obviously, other candidate genes not yet analyzed or not co-localized with QTLs we found in our two populations might also contribute to the resistance of Botrytis, and probably function in other genotypes or take effect in different situations. Antimicrobial secondary metabolites like, camalexin found in Arabidopsis, stilbenes from grape and gerberin from gerbera, can detoxify a variety of phytotoxic metabolites produced by Botrytis (van Baarlen *et al*. 2007; Koskela *et al*. 2011). Gene(s) that influence the accumulation of these phytoalexins or the amount of phytoalexins could slow down the speed of Botrytis growth or be able to inhibit the Botrytis growth. In gerbera, I would expect the only CG *ghCHI* (chalcone isomerase) which was mapped in both populations with a statistical difference and also detected as the QTL would be especially interesting and could be used as a marker directly. Since we already know that the resistance to Botrytis is a quantitative disease resistance, selecting more candidate gene (markers) could be additive to the effect of resistance to Botrytis in Gerbera.

Concluding remarks and future perspectives

In this study, we sequenced the cDNAs of four gerbera genotypes which were used as the parents for two segregating populations in assessing Botrytis resistance. Transcriptome data were annotated and we especially focused on the potential function and biological process of transcripts related with Botrytis resistance. SNPs were detected in each transcript and selected for as SNPs markers that are employed for genotyping in two segregating populations and construction of the first gerbera genetic maps. We also performed QTL analysis on the two populations and subsequently added candidate genes markers. Two candidate genes which were co-localized with QTLs were confirmed explicitly and suggest that the candidate gene approach is effective to pinpoint potential gene(s) for developing functional gene markers or transgene applications for ornamental improvement. The whole study provides a complete procedure to develop the genetic resources for carrying out MAS in a non-model crop and unravel a complex quantitative trait step by step from the beginning. To deploy an effective MAS program for gerbera might need more validation in a wide-scale gerbera genetic breeding pool to determine a best-practice strategy to select genotypes with a variety of quality characteristics. A follow-up of this research can be initiated immediately by the SNPs detected in every candidate gene co-located with Botrytis resistance QTLs, and the two candidate genes that have been functionally identified in this study.

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Summary

Gerbera hybrida is one of the top five cut flowers. It is well-known to people for its variation in flower color and patterning. Gerbera breeding at the moment is done using conventional methods which are based on a phenotypic selection. This has drawbacks in breeding speed and efficiency, especially for complex traits like disease resistance. Gerbera gray mold, promoted by high humidity during the production in greenhouses or by an accumulation of condensate during transportation, is a considerable threat to the gerbera production. Gerbera gray mold is caused by *Botrytis cinerea* and plant resistance to *B. cinerea* is considered to be a polygenic trait that needs the contribution of multiple loci, and on top of that is highly affected by the environment. Conventional breeding might be inefficient for improving Botrytis resistance in gerbera.

Given the importance of gerbera in floriculture and breeding as well as its potential to be a model species to study flower development in composed (*Compositae*) flowers, there is a demand for genomic resources. To develop genomic resources, the transcriptomes of four parents of two gerbera populations were sequenced using Illumina paired-end sequencing. In total, 36,770 consensus contigs with an average length of 1397 bp were generated. SNPs within and between the four genotypes were detected on the consensus contigs. These contigs were also the starting point for transcriptome annotation. A series of genes from the phenylpropanoid and flavonoid biosynthesis pathway were identified since they are involved in the production of plant secondary metabolites responsible for plant defense responses. In addition, key genes in the biosynthesis and regulation of phytohormone which likewise play a role in plant defense against *B. cinerea* were identified. Transcriptome data provides a resource for genetic dissection and an insight to explore gene functions for this ornamental crop.

To identify the QTL regions leading to the phenotypic variation in Botrytis resistance, and establishing a relationship between marker genotype and phenotypic variation for marker assisted selection (MAS), genetic linkage maps were constructed with SNP markers in the two F1 segregating populations. SNPs markers were developed from the transcriptome data of the four parents and were genotyped in the populations. Botrytis disease severity in gerbera was evaluated using three different tests, *whole inflorescence*, *bottom* (of disc florets) and *ray floret*. QTL mapping was performed using the four individual parental maps. A total of 20 QTLs (including one identical QTL locus for *whole inflorescence* and *bottom* test) were identified in the parental maps of the two populations. The number of QTLs found and the explained variance of most QTLs detected reflects the complex mechanism of Botrytis disease response.

Narrowing down the QTL region and identifying the causal gene(s) underlying a QTL could maximize the effective use of MAS in breeding. To develop functional gene-targeted markers on plants without genome sequence like for gerbera, a candidate gene (CG) approach was developed. Homologs of known functional genes involved in Botrytis resistance from other species were obtained in gerbera and SNP markers identified and mapped. Twenty-nine candidate genes were mapped and seven candidate genes could be

mapped on both populations. QTL detection for Botrytis resistance and candidate gene mapping was accomplished in the same two populations and using the same phenotyping data as before. Several candidate genes showed a significant difference between allelic groups in the disease score from the *whole inflorescence*, *bottom* (of disc florets) and *ray floret* tests independently and seven candidate genes were located in the vicinity of the QTLs detected. The co-localization of QTLs with CGs gives an indication that these candidate genes could probably be involved in resistance to Botrytis and provide a more precise possibility to use MAS in gerbera breeding in the future.

A tobacco rattle virus (TRV) based gene silencing system which was previously described in gerbera was used to inspect the function of two candidate genes. The two CGs are the homologs of the genes responsible for Botrytis resistance in tomato and both mapped in QTL regions related to Botrytis resistance in gerbera *ray floret* test. Silencing the two genes by VIGS, showed smaller lesion sizes upon Botrytis infection on gerbera ray florets compared with the controls. The variation in lesion size is considered to be caused by either different interval of time before lesion initiation or to the lesion's growth rate. The expression of candidate gene PGs, for plants' own biological process, would promote the penetration of Botrytis and knock down the gene might postpone the lesion formation; and lesser accumulation of ABA will prevent gerbera to enter into senescence and makes them more resilient to Botrytis.

The entire research went from the generation of four parental transcriptome data sets to development of SNP markers (*Chapter 2*), construction of genetic maps and to mapping QTLs for Botrytis resistance (*Chapter 3*). This was further on combined with candidate gene searching in other crops, querying and mapping homologous genes (*Chapter* 4) and characterizing the candidate genes which co-localized with QTLs (*Chapter* 5). The whole process not only helped us to unravel the genetics of Botrytis resistance in gerbera and develop genetic tools for gerbera improvement, but also could serve as guidance for developing marker-assisted selection for other ornamental plants from the beginning.

Acknowledgments

I came to the Netherlands without a clear idea of the genetic map and QTLs mapping. But at some point of my PhD, I realized when I was like 11 years old, I stood on a desk in front of a map of the world on the wall. My father helped me to memorize the knowledge I learned from the course of geography, and even ask me to point out where is the country Mauritius. My father specialized in cartography, and he probably knows more about the importance of a map and how to find a location on a map. I think that is why the second day when he visited me in the Netherlands, after a glance at the Google map, he cycled to Ede from Wageningen alone in this totally unfamiliar place. I really appreciate my father who let me form a right worldview starting from the map of the world and keep curious about the world.

First I would like to thank **Dr. Jaap van Tuyl** gave me the opportunity to come to the flowering Netherlands and continue the studies on ornamentals. Dear Jaap, I really admired your profound knowledge and enthusiasm on ornamentals and bulbs. Thanks for all your guidance and contributions on my experiments and thesis. **Dr. Paul Arens**, thank you told me the importance of genetic maps and encourage me to be an independent researcher! Dear Paul, thank you for allowing me to come to your office without an appointment in advance. I am reallllly appreciate your constructive suggestions, precise comments, considerable effort and time for my every progress, every rehearsal of presentation and after the presentation, every experimental design and every word of my papers and thesis chapters, in the past four and half years. **Prof. Dr. Richard Visser**, thanks for the extensive discussion especially during my second and third year of PhD, and your time on my thesis in the last journey of my PhD. Dear Richard, thanks for your encouragement when I felt insecurity and reminder when I was over-optimistic.

Thanks **Marleen** Cobben as my external supervisor, but more like my big sister. Dear Marleen, I really love to talk with you and share my life with you and thank you for guiding me for the life planning. Thanks **Awra** Shahin for your always valuable suggestions in the experiments and discussion, and your smile from the daily life. Thanks funny **Alex** van Silfhout making the life much easier. I am happy to belong to two research groups under **Frans** Krens and **Rene** Smulders. Thanks to all the colleagues from these two groups for the work discussion. Thanks **Rene** for the specific suggestion for my presentation and **Herman** van Eck for the worthwhile discussion. Thank you **Wendy** van't Westende, for guiding me at the very beginning of my lab-life and helping me to do experiments effectively. Thank you **Danny** Esslink for your assistance on dealing with the bioinformatic data. Thanks **Linda** Kodde, **Jan** Schaart, **Gert** van Arkel, **Christel** Denneboom, **Danny** schipper, **Marjon** Arens, **Gereard** Bijsterbosch, **Johan** Bucher, **Patrick** Hendrickx, **Dianka** Dees, and **Bernadette** van Kronenburg *etc.* for your kindness helps when I was working in the lab. Thanks **Annelies** Loonen and **Michela** Appiano for the guidance on VIGS. Thanks **Dirk Jan** Huigen and **Doret** Wouters for the arrangement of greenhouse in Unifarm. Thanks **Harm** Wiegersma from Unifarm for taking care of the gerbera plants. Thanks **Herma** Koehorst-van Putten for the yoga course and concerns.

Thanks **Martin** Beer, **Ronny** Egberts and other people from Florist BV and Schreurs BV for the cooperation on the project and helps on sampling and all Gerbera things. Thanks **Meng** Chen, **Wen** Fang, **Kevin** Windels, **Hani** Wijaya, **Joyce** van Assen, and **Eline** Jansen, who worked with me on the experiments.

Thanks secretaries of plant breeding, **Nicole** Trefflich, **Letty** Dijker, **Janneke** van Deursen and **Danielle** van der Wee for handling some tedious documents of mine. ***I would like to thank all the colleagues in Plant Breeding!!*** *I did not talk to most of you yet, but when I met you every time in the department, your smile, hello, hoi, hi, good morning, bye, nice weekend, doi, goedemorgen make me very delighted.*

I stay in the office E2.176 for almost five years, and I met my nice officemates **Clarie** Kamei, **Peter** Vos, **Ahmed** Abd-El-Haliem, **Jarst** van Belle, and **Zhe** Yan. Thank you for sharing the funny jokes and stories with me. I do not know why I sneezed a lot in the office, but I received a lot of *bless you* from you guys. Thanks to the helps from the PhD students in Plant Breeding, **Nur** Fatihah, **Ashikin** Kemat, **Atiyeh** Kashaninia, **Geert** van Geest, **Coralina** Aguilera Galvez, **Peter** Ding, **Charlie** (Changlin) Chen, **Peter** Bourke, **Ernest** Aliche (xueba), **Marcela** Viquez-Zamora, **Dalia** Carvalho, **Mas** Muniroh, **Sri** Sunarti, **Naser** Askari, **Mehdi** massoumi, **Cynara** Romero, *etc*.

I am happy to meet many Chinese friends who make my life so much fun here in Wageningen. I will always remember the stories between **you** and me. Thank you all: Dong Xiao/栋哥, Yin Song/学霸, Xi Chen/嘻嘻 & Weicong Qi/维聪, Kaile Sun/大表姐, Zhao Zhang/钊哥, Yue Lu/玥姐, Xiaomei Yang/杨二爷, Feng Zhu/峰峰朱 & Guojing Tian/大靓靓, Chunzhi Zhang/春春, Mengli Xi/席老师, Xiaoxue Sun/小雪, Xueping Chen/陈老师, Xiao Lin/四少, Lisha Zhang/丽莎师姐, Xu Cheng/师父, Junyi Xu/小君君, Lujie Chen/璐洁, Lijing Liu/小婧婧, Jiao Mei/美娇, Yuehan Dou/豆豆, Yifan Zhang/帆爷, Dongli Gao/冬冬, Fengjiao Bu/阿娇, Xiaohua Du/杜老师, Lianwei Qu/屈老师, Lujun Zhang/章老师, Yajun Wang/亚军师兄 & Wen Cui/崔文, Peirong Li/佩荣, Huayi Li/华一, Xuexue Shen/雪雪, Haiyan Xiao/海燕, Jinbin Wu/斌斌, Jinling Li/金岭, Zhichun Yan/小鲜肉, Tao Zhao/涛哥, Yan Wang/王燕, Yu Du/杜宇, Juan Du/杜鹃, Mengjing Sun/梦婧 & Wei Du/杜伟, Chunxun Song/春旭 & Wei Qin/伟哥, Ningwen Zhang/凝文师姐 & Ke Lin/林柯师兄, Mengyang Liu/梦洋, Nan Tang/唐楠, Xuan Xu/许萱, Jia Ning/Jessie, Hao Hu/胡昊别, Yongran Ji/老季, Juncai Chen/才哥, Guozhi Bi/毕姥爷, Yuanyuan Zhang/媛园, Fang Gou/苟芳, Yongfu Tang/永富 & Jiacui Chen/家翠, Hucheng Li/虎臣, Haiyan Ma/海燕, Guiling Ren/贵玲, *etc. etc.*

Thanks all my dearest friends in China, especially Nan Wang/多多, Jie Yu/小于子, Jin Liu/金姐, Minna Zeng/敏子, Le Li/乐姐, Feng Xiao/牛奶, Dan Tan/旦姐, *etc*. we used to spend a lot of time together, although I am now far away from you, you still encourage me and care about me a lot.

I am happy to born in a supportive family and thanks to my parents never hesitated to say how proud you are of me. Moreover, thanks to my other half Tonggao, thanks for your sacrifices and tolerating my sometimes childish behavior!!

Yiqian Fu 傅伊倩 18th, May, 2017

Curriculum Vitae

Yiqian Fu was born on July 8, 1987 in Huarong, Hunan province, China. In 2009, she accomplished her bachelor degree at the Horticulture in ornamentals module at Shandong Agricultural University, and her BSc thesis was *Characteristic physiological features of hyperhydric Chrysanthemum 'Jinba'*. At the same year, she started her master study at the Landscape Plant and Ornamental Horticulture at Beijing Forestry University and after a three-year master education program, she completed the MSc thesis entitled *In vitro conservation of wild lily*. In September 2012, she applied the fellowship from China Scholarship Council to continue the study on the ornamental plant. This thesis presents the outcomes of her four and half year PhD research on *Unraveling the genetics of Botrytis cinerea resistance in Gerbera hybrida*.

The following is a list of her publications in refereed journals:

Fu, Y., van Tuyl, J., Visser, R., Arens, P., 2015. The Use of a Candidate Gene Approach to Arrive at Botrytis Resistance in Gerbera, XXV International EUCARPIA Symposium Section Ornamentals: Crossing Borders 1087, pp. 461-466.

Fu, Y., Esselink, G.D., Visser, R.G.F., Van Tuyl, J.M., Arens, P., 2016. Transcriptome analysis of *Gerbera hybrid*: including *in silico* confirmation of defense genes found. Frontiers in Plant Science 7:247.

Fu, Y., van Silfhout, A., Shahin, A., Egberts, R., Beers, M., van der Velde, A., van Houten, A., van Tuyl, J.M., Visser, R.G.F., Arens, P., 2017. Genetic mapping and QTL analysis of Botrytis resistance in *Gerbera hybrida*. Mol Breeding 37, 13.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Yiqian Fu
Date: 19 June 2017
Group: Laboratory of Plant Breeding
University: Wageningen University & Research

1) Start-up phase	*date*
▶ **First presentation of your project**	
Title: QTLs mapping for Botrytis resistance in Gerbera hybrida	15 Apr 2013
▶ **Writing or rewriting a project proposal**	
▶ **Writing a review or book chapter**	
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1.5 credits**

2) Scientific Exposure	*date*
▶ **EPS PhD student days**	
EPS PhD student day, Amsterdam, NL	30 Nov 2012
EPS PhD student day, Leiden, NL	29 Nov 2013
EPS PhD student day 'Get2Gether', Soest, NL	29-30 Jan 2015
EPS PhD student day 'Get2Gether', Soest, NL	28-29 Jan 2016
▶ **EPS theme symposia**	
EPS Theme 2 symposia: Interactions bewteen Plants and Biotic Agents, Utrecht, NL	24 Jan 2013
EPS Theme 2 symposia: Interactions bewteen Plants and Biotic Agents and the Willie Commelin Scholten Day, Amsterdam, NL	25 Feb 2014
EPS Theme 4 symposia: Genome Biology, Wageningen, NL	03 Dec 2014
EPS Theme 2 symposia: Interactions bewteen Plants and Biotic Agents and the Willie Commelin Scholten Day, Utrecht, NL	20 Feb 2015
EPS Theme 2 symposia: Interactions bewteen Plants and Biotic Agents and the Willie Commelin Scholten Day, Leiden, NL	22 Jan 2016
EPS Theme 4 symposia: Genome Biology, Wageningen, NL	16 Dec 2016
▶ **Lunteren days and other National Platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	22-23 Apr 2013
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	13-14 Apr 2015
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2016
▶ **Seminars (series), workshops and symposia**	
Plant Sciences Seminar by Guusje Bonnema and Arnaud Bovy 'The role of Plant Breeding in improving quality of crop plants'	11 Jun 2013
Seminar by Kuang Hanhui 'Using the Nicotiana-TMV system to study resistance gene evolution and plant genome stability'	11 Sep 2013
Seminar by Teemu Teeri 'Deep RNA sequencing and flavonoid metabolons in Gerbera hybrida' in UVA	12 Sep 2013
Seminar by Salvadore Ceccarelli 'The efficiency of plant breeding'	16 Sep 2013
Plant Sciences Seminar by Robert Hall 'Metabolomics in the lab: a myriad of applications' and Nicole van Dam 'Metabolomics in the wild: Assessing the functional diversity of plant chemical defences in non-model systems'	08 Oct 2013
Mini-symposium: How to Write a World-class Paper	17 Oct 2013
CALN 2013 Annual Meeting 'Innovative Horticultural Industry and Food Safety in the Netherlands: past-present-future'	09 Nov 2013
Seminar by Li Jiayang 'Understanding the molecular mechanisms underlying rice tillering'	15 Nov 2013
Seminar by Dani Zamir 'Geno-Pheno in plant breeding'	10 Feb 2014
Seminar by Jeff Endelman 'Genome-wide Marker-assisted Selection'	25 Jun 2014
Seminar by Emma Huang 'Genetic analysis in MAGIC: advantages and challenges'	25 Jun 2014
WEES Seminar by Joy Bergelson 'Maintaining an ancient balanced polymorphism for resistance amidst diffuse interactions'	26 Sep 2014
Plant Breeding Research Day 2014	30 Sep 2014
Plant Sciences Seminar on Bioinformatics by Dick de Ridder and Paul Kersey	07 Nov 2014
Extra Plant Sciences Seminar by Claude Fauquet 'The application of plant biotechnology for preventing diseases'	04 Dec 2014
Seminar by Chih-Hang Wu 'Helper NLR proteins of the NRC family in solanaceous plants'	05 Mar 2015
Seminar by Sonia Osorio 'The uncovering of new transcription factors involved in ripening and post-harvest life of tomato and strawberry fruits'	15 Apr 2015
Plant Sciences Seminar on "Into the Battle between Plants and Viruses, but what about EVEs?" by Richard Kormelink	12 May 2015
Seminar by Christine Hackett 'Linkage analysis and QTL mapping in autotetraploid potato using SNP dosage data'	07 Sep 2015
QTL mapping within the Genstat environment: an overview of the QTL mapping library	08 Sep 2015
Plant Breeding Research Day 2015	29 Sep 2015
Seminar by Xiaowu Wang 'Subgenome Parallel Selection Drives Diversification and Convergent Morphotype Evolution in Brassica crops'	21 Oct 2015
Seminar on 'Genomics in plant and animal breeding and conservation: Strategies for success in plants, birds, and livestock'	04 Nov 2015
Seminar by Rossana Henriques 'And yet they oscillate: functional analysis of circadian long non-coding RNAs'	16 Nov 2016
Seminar by Jane Parker 'Plant intracellular immunity: evolutionary and molecular underpinnings'	21 Jan 2016
Workshops JoinMap® 4.1 : Introduction to mapping & Trouble shooting	19 May 2016
Workshops Sino Dutch Vegetable Breeding Sector Innovation	22 Sep 2016
Lectures by Edze Westra and Jennifer Doudna: 'Rewriting our genes?'	30 Sep 2016
1st Symposium 'WURomics: Technology-Driven Innovation for Plant Breeding'	15 Dec 2016

CONTINUED ON NEXT PAGE

►	**Seminar plus**	
►	**International symposia and congresses**	
	Symposium 'Roses, what in it for us' on Floriade, Venlo, NL	26 Sep 2012
	Conference Next Generation Plant Breeding, Ede, NL	12-14 Nov 2012
	3rd International Symposium on the genus Lilium, Zhangzhou, China	31 Apr- 03 May 2014
	6th European Plant Science Retreat for PhD Students, Amsterdam, NL	01-04 Jul 2014
	Symposium 'All-inclusive Breeding: Integrating high-throughtput science', Wageningen, NL	16 Oct 2014
	XXV Eucarpia Symposium on Ornamentals, Melle, Belgium	28 Jun-02 Jul 2015
►	**Presentations**	
	Talk: XXV Eucarpia Symposium on Ornamentals, Melle, Belgium	28 Jun-02 Jul 2015
	Poster: 6th European Plant Science Retreat for PhD Students, Amsterdam, NL	01-04 Jul 2014
►	**IAB interview**	
►	**Excursions**	
	PhD Trip: Rijk Zwaan excursion	27 Sep 2013
	PhD Company visit: Genetwister and In2 Care	19 Sep 2014
	PhD Company visit: Enza Zaden	12 Jun 2015
	PhD Company visit: TomatoWorld	14 Oct 2016
	Subtotal Scientific Exposure	*18.3 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Mixed model based genetic analysis in GenStat: from QTLs mapping and assosiation mapping to genetic prediction	02-04 Sep 2013
	An Introduction to Mass Spectrometry-based Plant Metabolomics	09-13 Dec 2013
	Genome Assembly	28-29 Apr 2015
	Introduction to R for statistical analysis	18-19 May 2015
►	**Journal club**	
	Participant of literature discussion group at Plant Breeding	2012-2016
►	**Individual research training**	
	Subtotal In-Depth Studies	*6.6 credits**

4) Personal development		*date*
►	**Skill training courses**	-
	Practical English	Oct 2012-Feb 2013
	Scientific Writing	Nov 2014-Jan 2015
	Presentation Skills	10, 17, 24 Oct 2014
	Efficient Writing Strategies	Sep-Oct 2015
	Interpersonal Communication for PhD candidates	19-20 Nov 2015
	Career assessment	2016
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*6.9 credits**

TOTAL NUMBER OF CREDIT POINTS*	**33.3**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

The research was conducted at Plant Breeding, Wageningen University & Research, and was made possible by a financal grant from the Foundation Technological Top Institute Green Genetics (3CFL030RP). Financial support from Wageningen University for printing this thesis is gratefully acknowledged. Yiqian Fu is sponsored by CSC Fellowship.

Cover Design: Yiqian Fu

Layout: Yiqian Fu

Printed by: GVO drukkers & vormgevers, Ede (NL) | gvo.nl

Gerbera Alma from Florist B.V. was used for the cover design and word cloud on the back cover was made by wordart.com.