The sexual advantage of smelling and tasting good:
the chemical cues that plants use to seduce insect pollinators

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Abstract

Most plants rely on insects for sexual reproduction. Millions of years of coevolution between plants and insects have created a diverse range of flowers. These flowers use several signals such as scent, colour and shape, to attract their respective pollinators. However, evidence of which signals are actually exploited by pollinators remain scarce. The plant species *Arabidopsis thaliana* provides an excellent opportunity to investigate signals exploited by insects, since there is extensive knowledge about the genome and there are many mutants available. This thesis aimed to use mutants to study the effect of different signals on the attraction of hoverflies (*Episyrphus balteatus*). A dual-choice assay was created where the preference of the hoverfly for different *Arabidopsis* mutants was tested. Colour and nectar were the two characteristics with the highest influence on pollinator behaviour. Red flower colour influenced the first choice (*P = 0.006)*, whereas nectar slightly influenced the time spent per individual flower (*P = 0.068)*. Since hoverflies are important for pollination in the field, breeding programs should take into account that these traits influence pollination. Successful pollination events are important for high yields in plant species that require cross-pollination for fruit set. Future research should be conducted to investigate the role of other scents and pollen on pollinator attraction.

Contents

Introduction

Pollen and seeds can be dispersed by wind, water and animals. Most plant species, around 75% of angiosperms, rely on insects to sexually reproduce (Faegri and van der Pijl, 2013). Coevolution between plants and insects has promoted the development of intricate pollination systems and a large diversity between flowers (Fig. 1, Bronstein *et al.*, 2006). One extreme example, made famous by Charles Darwin, is the orchid A*ngraecum sesquipedale* from Madagascar, which can only be pollinated by a specific moth with an extremely long tongue (Arditti *et al.*, 2012). However, most pollinator systems are generalised. To attract a large range of pollinating insect species, plants make use of a range of different signals such as scents, nectar, different shapes, colours and flower temperature and reward pollinators with nectar and pollen (Dyer *et al.*, 2006; Faegri and van der Pijl, 2013).

Most flowers emit a complex mixture of volatile compounds to attract pollinators. Volatiles are small molecules that do not exceed a molecular weight of 300 Da. For flowers, this mixture mainly consists of terpenoids, benzenoids, phenylpropanoids and fatty acid derivatives (Dudareva and Pichersky, 2006). Floral scent does not only differ per species but can also differ within species. Volatile emission can be affected by environmental factors such as light and temperature, as well as by the age of specific flower and plant, the occurrence of fertilization and the presence of herbivores (Dudareva and Pichersky, 2006). The petals, stamen, pistil and sepals all emit volatiles that contribute to the flower bouquet (Effmert *et al.*, 2006). One single flower can emit up to 100 different compounds although most flowers emit between 20 to 60 volatile compounds (Dudareva and Pichersky, 2006). Each pollinator is attracted to a different volatile blend, but some generalisations can be made for particular animal groups. For example, Diptera seem to be attracted to fatty acid derivatives and to compounds containing alcohol groups and nitrogen. Lepidoptera prefer fatty acids esters and hydrocarbons, whereas food-seeking bees like variable bouquets, which are abundant in terpenoids (Dobson, 2006). Flowers pollinated by hummingbirds, on the other hand, only have a weak or no scent at all and therefore attract the animals with other signals such as the colour of the corolla (Dobson, 2006).

Figure 1. *Illustration of the diversity in flower shape and colour.*

Colour is also an important trait involved the attraction of pollinators to flowers. It must be taken into account that the vision of insects is very different from that of humans. Flowers that appear white to the human eye may look very different to insects because of the reflected UV light (Kevan *et al.*, 1996). Insect vision ranges roughly from ultraviolet to the red part of the electromagnetic spectrum (300-700 nm). Some insects are innately attracted to one type of colour. Bees, for instance, are known to prefer purple flowers (Raine and Chittka, 2007). Flowers which are pollinated during the night are usually pale so their visibility is higher (Baker, 1961) and they generally also produce heavier fragrances (Stuurman *et al.*, 2004). A change in flower colour can cause a shift in the type of pollinator (Bradshaw and Schemske, 2003). Flavonoids are the main compounds colouring flowers (Brouillard, 1988). Different kinds of anthocyanins can colour a flower pink, orange, red, blue or violet, whereas flavonols can colour flower in yellow. Flowers can also be coloured yellow by chalcones, aurones and carotenoids.

Nectar and pollen serve as reward for pollinators and are an important food source for many insects, such as bees and butterflies. The main constituents of nectar vary between species. Nectar is generally rich in sugars, amino acids and they contain proteins, lipids and vitamins in smaller volumes (González-Teuber

and Heil, 2009). The pollen is rich in protein and attaches to insects while they feed on flowers. Short-tongued bees and flies prefer nectar that is rich in hexoses and long tongued insects prefer nectar rich in sucroses (Heil, 2011). Rewards shape the preference of pollinators. If a flower is rich in nectar and pollen, pollinators will associate high rewards with the scent, shape and colour of those flowers. Because of the high nutritional value of nectar, it is very appealing to bacteria, fungi and other insects that consume the reward without providing the pollinator service. These organisms are considered nectar robbers. Therefore, nectar also contains secondary metabolites with antimicrobial properties, such as terpenoids, which repel nectar robbers (González-Teuber and Heil, 2009).

Arabidopsis thaliana (*Arabidopsis*) is a member of the Brassicaceae family frequently used as a model plant for scientific research, because of the diploid genome and short growth cycle (Rhee *et al.*, 2003). *Arabidopsis* produces small (± 0.5 cm) white flowers that only open for a few hours per day. Although *Arabidopsis* is mainly considered a self-pollinating species, there is evidence showing that the plant is visited by insect pollinators. A survey conducted by Hoffmann *et al.* (2003) showed that 0.3%-2.4% of flowers were visited by pollinators, although it is not clear whether these visits resulted in pollination. Additionally, recombination observed in the *Arabidopsis* genome is an indirect evidence that *Arabidopsis* is cross-pollinated, since it cannot be explained by mutations alone. Most populations are polymorphic, which is not expected from a population that only reproduces by selfing (Nordborg *et al.,* 2005). This probably means that at least a part of *Arabidopsis* plants reproduce by cross-pollination. Most of the insects recorded on *Arabidopsis* flowers were solitary bees, members of Diptera (such as hoverflies) and thrips. There is a brief moment in the development of flowers in which the stigma protrudes before the stamen matures (Chen *et al.*, 2003), during which cross-pollination is possible. *Arabidopsis* flowers have two lateral and two median nectaries at the base of the stamen, which produce a small amount of nectar which might serve as a reward for pollinators (Davis *et al.*, 1998).

Arabidopsis flowers mainly emit terpenoids (>60%), but also aldehydes and alcohols (Chen *et al.*, 2003). Terpenoids can play a role in pollinator attraction, but they are also known for their antimicrobial activity (Dorman and Deans, 2000). In some plants they are released upon herbivory to attract their predators or parasitoids (Turlings *et al.*, 1995; Schnee *et al.*, 2006). The major monoterpenoids emitted by *Arabidopsis* flowers are β-myrcene, limonene, linalool and the major sesquiterpenes are β-caryophyllene, thujopsene, α-humulene, β-farnesene, β-chamigrene and cuparene. Terpenoid emission in *Arabidopsis* follows a diurnal pattern as it increases during the day and decreases during the night. Overexpression of terpenoids in *Arabidopsis* leads to a decreased attractiveness to herbivores (Aharoni *et al.*, 2003), but it is unknown if it affects the attraction of pollinators.

Aim of the thesis

Flowers come in all kinds of different sizes, smells and colours and because of this, attract different types of insects. However, within this complex sort of cues, it remains unknown what are the specific cues exploited by pollinators when searching for flowers that provide the best reward. This thesis investigated whether a few specific volatile compounds are exploited by the generalist pollinator *Episyrphus balteatus* when choosing flowers from *A. thaliana* plants. It was also investigated whether nectar availability and flower colour influence the attractiveness these pollinators. This was performed by using several different knock-out and overexpression lines of *Arabidopsis*, which were subjected to insect preference assays to test preferences between mutant and wild type plants. Hoverflies of *E. balteatus* have been observed to visit *Arabidopsis* flowers (Fig. 2, Hoffmann *et al.*, 2003) and were attracted to the pure compound of linalool (Boachon *et al.,* 2015).

Figure 2. *Episyrphus balteatus visiting Arabidopsis thaliana.*

Research questions

- Are the ordered mutants homozygous and do the volatiles of mutants correspond with the expected phenotype?
- Is *A. thaliana* a suitable study system to test pollinators attractiveness?
- Does either scent, colour or nectar availability influence the preference *E. balteatus* when looking for a food source?
- Is *E. balteatus* an efficient pollinator of *A. thaliana?*

Study system

Hoverflies

Hoverflies belong to the insect family *Syrphidae* and are common worldwide. Their bee or wasp-like appearance is assumed to have evolved to deter predators. Hoverflies can be discerned from bees because they have two wings instead of four. This makes it possible for them to hover in the air, which is how they received their name (Reemer *et al.,* 2009). Whereas adults of hoverfly species feed mainly on nectar and pollen, there is a large variety in the food source of larvae of different hoverfly species. Some species of hoverfly larvae feed on (decaying) plant or fungal material and others eat small insects such as aphids, thrips or caterpillars. After larvae hatch from the egg they go through three larval stadia (Fig. 3). After the third stadium the larvae pupate and in approximately eight days they eclose as adult flies. Hoverflies are important pollinators in nature and visit a range of different flowers in their lifetime (Cowgill *et al.,* 1993).

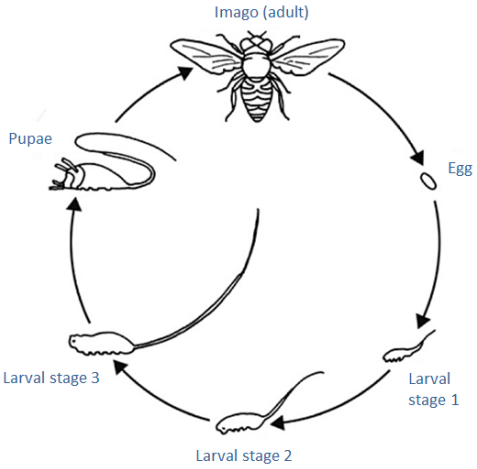

Figure 3. *Life cycle of hoverflies (Adapted from Reemer et al., 2009).*

The hoverflies species used in this thesis is *E. balteatus.* This species is very common in Europe, North Africa and North Asia. *Episyrphus balteatus* larvae feed on aphids and are therefore used as a biocontrol agent (Miñarro *et al.*, 2005; MacLeod, 1999). *Episyrphus balteatus* flies have a wasp-like appearance due to the presence of yellow and black stripes on their abdomen. The adults are known to visit a range of different plant species although they do prefer some flowers over others(Cowgill *et al.*, 1993; Goulson and Wright, 1998). Hoverflies can exploit both visual and odour cues. Hoverflies were able, for instance, to distinguish aphid-infested plants from non-infested plants exploiting olfactory cues emitted by the plants (Bargen *et al.*, 1998; Verheggen *et al.*, 2008). When *E. balteatus* has the choice between pure compounds of linalool, lilac aldehydes and lilac alcohols, they prefer linalool over lilac compounds (Boachon *et al.*, 2015). Hoverflies innately prefer yellow flowers (Wacht *et al.*, 1996; Primante and Dötterl, 2010). *Episyrphus balteatus* females show preference for artificial flowers coloured yellow over flowers that were coloured white, cream, green-yellow and blue. The artificial yellow flowers emit light in the region between 360 and 440 nm (Sutherland *et al.*, 1999). The same publication shows that *E. balteatus* exhibits no preference between different amounts of pollen, but does prefer nectar with a higher sugar concentration.

Lines used for the behavioural experiments with hoverflies

Scent: *35S:CYP76C1* (At2g45560)

35S:CYP76C1 is a complement/overexpression line, described by Boachon *et al.* (2015). It was created by inserting a vector containing the gene *CYP76C1* driven by the 35S promoter in a *cyp76c1* knockout line by floral dip. The gene *CYP76C1* is part of the cytochrome P450 family and is a monoterpenol-metabolizing

oxygenase. The *cyp76c1* knockout line emits high levels of linalool and less lilac aldehydes and lilac alcohols. The volatile mixture of the complemented/overexpression mutant *35S:CYP76C1* contains almost no linalool and half the amount of lilac aldehydes and lilac alcohols.

Fluorescence/colour: *tt4* (At5g13930)

Arabidopsis has small white flowers and enzymes of the flavonoid biosynthetic pathway are active in petals. The chalcone synthase (CHS), the first enzyme in the biosynthesis of flavonoids, is codified by the gene At5g13930 (Dong *et al.,* 2001). The gene was initially isolated in a screening of *Arabidopsis* mutants for producing seeds with a transparent seed coat and therefore named *transparent testa4*. In the same screening it was observed that the petals from mutant plants glow when exposed to UV light (Fig. 4)

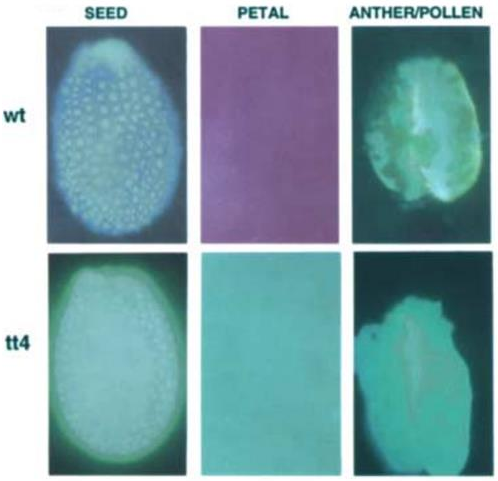

Figure 4. *Fluorescence of the seed, petal and anther/pollen under 365 nm in Arabidopsis thaliana Col-0 (WT) and tt4 (Shirley et al., 1995).*

Nectar: *Atsweet9* (At2g39060)

The *sweet9* lacks a functional sucrose efflux transporter, which makes it unable to exudate nectar (Lin *et al.*, 2014). This transporter is located in the nectary parenchyma. Hoverflies feed on pollen and nectar and are hypothesized to dislike flowers lacking nectar, however they are not expected to detect nectar cues from a distance. Hoverflies are thus predicted to visit flowers lacking nectar, but to spend less time on them. Females feed on pollen at the time of yolk deposition in the eggs and feed on nectar during mating time and oviposition, whereas males mainly feed on nectar (Reemer *et al.,* 2009).

Colour: Red flowers

To investigate whether there is an effect of colour the attractiveness of hoverflies to flowers, Col-0 plants were dyed red. Flowers were coloured with food colouring as previously described by Cook *et al.* (2013) in *Brassica napus.* This study revealed that *B. napus* flowers that were dyed red and blue were less attractive to the pollen beetle *Meligethes aeneus (*Cook *et al.*, 2013).

Materials and methods

Plant material

T-DNA knock-out lines of *Arabidopsis* genotyped and/or used in the experiments were obtained from the European *Arabidopsis* Stock Centre (NASC, http://*Arabidopsis*.info/). These lines were transformed using *Agrobacterium tumefaciens* (Alonso *et al.*, 2003). The selected mutant lines have insertions in genes that control the production of either volatiles, nectar or colour (see Table 1). A few lines that overexpress volatiles were previously produced in the laboratory of Plant Physiology, Wageningen University (the Netherlands). Seeds of *tt4* were donated by Takayuki Tohge (Max Planck Institute, Germany) and the mutation was calcium-ion induced (Shikazono *et al.*, 2003).

Table 1. *Overview of the transgenic lines genotyped and/or used in the experiments. The locus at which the T-DNA is inserted and the gene function are also shown. Mutant lines used in the behavioural assays are highlighted in blue.*

Cue	Gene	Name and Function	T-DNA Line	Type of mutant	Location	Phenotype (in relation to pollination)	Notes
Odour	At1g61120	TPS04, a geranyllinalool synthase	Salk_013858	T-DNA	300-UTR5	Lower emission of E,E)-geranyllinalool and the homoterpene TMTT (4,8,12-trimethyltrideca-1,3,7,11-tetraene)	Herde et al., 2008
			Salk_013880	T-DNA	300-UTR5		
	At1g61680	TPS14, catalyzes geranyl diphosphate to linalool	Salk_039462	T-DNA	Exon	Lower emission of S-linalool	TPS14 line 2 (Ginglinger et al., 2013)
			Salk_114189	T-DNA	Exon		
			Salk_059820	T-DNA	Exon		TPS14 line 3 (Ginglinger et al., 2013)
	At2g24210	TPS10, a monoterpene synthase	Salk_108420	T-DNA	Exon	Lower emission of beta-myrcene and (E)-beta-ocimene	Ginglinger et al., 2013
			Salk_041114	T-DNA	Exon		
			tps10xtps14	Double Mutant			tps10Xtps14 double knock-out (Ginglinger et al., 2013)
	At2g45560	CYP76C1, catalyzes the oxidation of linalool, and metabolizes α-terpineol	Salk_001949	T-DNA	Exon	Higher linalool and lower levels of lilac aldehydes/alcohola emission	Boachon et al., 2015
			35s:CYP76C1	Complement		Phenotype described for the mutant (above) are partly restored to wild type levels	
	At2g45580	CYP76C3, converts linalool into hydroxylated or epoxidated products	Salk_077330	T-DNA	Exon	30% increase in linalool emission	metabolizes two linalool enantiomers to form different but overlapping sets of hydroxylated or epoxidized products (Ginglinger et al., 2013)
			Salk_056876C	T-DNA	Intron		
			Salk_027343C	T-DNA	1000-Promotor		
			cyp76c3	T-DNA			
			35S:CYP76C3			Decrease in linalool emision	
	At3g14540	TPS19, unknown function	Salk_151809	T-DNA	Exon	Unknown	Tholl and Lee, 2011
	At3g25810	TPS24, monoterpene synthase	Salk_142794	T-DNA	Intron	No emission of α-pinene, sabinene, β-pinene, β-myrcene, limonene, and (E)-β-ocimene	Chen et al., 2003
	At3g53300	CYP71B31, converts linalool into oxygenated derivatives	Salk_009366	T-DNA	Exon	No change in phenotype	Ginglinger et al., 2013
			Salk_034144	T-DNA	Exon		
	At4g13280	TPS12, a sesquiterpene snthase	Salk_020880	T-DNA	Exon	Lower emission of (Z)-gamma-bisabolene and the E-nerolidol and alpha-bisabolol.	Catalyzes the conversion of farnesyl diphosphate to (Z)-gamma-bisabolene and the additional minor products E-nerolidol and alpha-bisabolol. Expressed in roots, damaged leaves and flower stigmata (Ro et al., 2006)
			Salk_052466	T-DNA	Exon		
	At4g13300	TPS13, a sesquiterpene synthase	Salk_011441	T-DNA	Exon	Lower emission of (Z)-gamma-bisabolene, E-nerolidol and alpha-bisabolol	Ro et al., 2006
	At4g16730	TPS2, synthesizes (E)-beta-ocimene and (E,E)-alpha farnesene	Salk_062519	T-DNA	Intron	No (E)-beta-ocimene synthase activity	
	At4g16740	TPS3, synthesizes (E)-beta-ocimene and (E,E)-alpha farnesene	Salk_152097	T-DNA	Intron	low farnese, low ocimene	A monoterpene synthase catalyzing jasmonate- and wound-induced volatile formation in Arabidopsis thaliana. Constitutively expressed in floral tissues (Fäldt et al., 2003, Huang et al.,
	At4g20230	TPS9, unknown function	Salk_141559	T-DNA	Exon	Unknown	Tholl and Lee, 2011
			Salk_035057	T-DNA	300-UTR5		
	At5g23960	TPS21, synthase of sesquiterpenes	Salk_138212	T-DNA	Exon	No emission of group A sesquiterpenes	Tholl et al., 2005
			35S:AtTPS21 (gDNA)	Overexpressor		Increased emission of group A sesquiterpenes	
	At5g44630	TPS11, synthase of sesquiterpenes	Salk_151777	T-DNA	Exon	No emission of group B sesquiterpenes	Tholl et al., 2005
			SAIL_728_G04	T-DNA	Intron	No emission of group B sesquiterpenes	
	From chrysanthemum	Chrysanthemyl diphosphate synthase	*CDS57*				
	From strawberry	linalool/nerolidol	*35S:ipFaNES 4-1*	Overexpressor		Higher expression of linalool and nerolidol	Aharoni et al., 2003
	From strawberry	linalool/nerolidol	*FPS1L+pFaNES*	Overexpressor		Higher expression of linalool and nerolidol	Aharoni et al., 2003/Kappers et al., 2005
Nectar	At2g39060	Encodes for a sucrose efflux transporter that is expressed in the nectaries	SK225	T-DNA		No nectar production	Lin et al., 2014
Colour	At5g13930	Transparent Testa Glabra 4	Salk_020583	T-DNA	Exon	Glowing petals under UV light	Shirley et al., 1995
	-	Coloured red with food colouring	Colombia-0	-	-	Red flowers	Cook et al., (2013)

Plant growth

Arabidopsis seeds were stratified in water in the dark at 4 °C for five days. Plants grown for genotyping were planted on rock wool and grown in a climate chamber (22 °C, 60-70% relative humidity, L12:D12). Plants grown for the behavioural assays were planted in soil in 5.9 cm square pots. *35S:CYP76C1*, *tt4* and Col-0 plants were grown in a climate chamber (22 °C during day, 17 °C during night, 65% r.h., L16:D8). *Sweet9* and Col-0 were grown in a greenhouse compartment (23 °C ± 2 °C, 50-70% r.h., L16:D8).

Figure 5. *Hoverflies of E. balteatus in a rearing cage.*

Insect rearing

Episyrphus balteatus pupae were obtained from Katz Biotech (Germany) or Biopol Natural (the Netherlands) and reared in a cage in a greenhouse compartment (Fig. 5; 22 °C ± 1 °C, 50-70% r.h., L16:D8). Adult hoverflies were provided with a source of water, sugar and pollen. A Brussels sprout plant (*Brassica oleracea* var. *gemmifera*), infested with aphids (*Brevicoryne brassicae)* was kept in the cage because the aphids stimulate females to complete maturity (Lucas-Barbosa *et al.*, 2015). Insects used for the dual-choice assays were starved for 3-5 hours prior to the experiments.

Genotyping

Transgenic plants were assessed for homozygosity before they were used for further experiments. Genomic DNA was extracted from plants and genotyped by PCR. For this, leaf tissue was frozen in liquid nitrogen and ground with a MM400 mixer mill (Retsch, Germany). Subsequently, 250 µL of Shorty buffer (0.2 M Tris/HCl pH 9.0; 0.4 M LiCl; 25 mM EDTA; 1% SDS) was added. The tubes were inverted and centrifuged at 11.000 rpm for 5 minutes. The supernatant was transferred to a new tube and an equal amount of volume of isopropanol was added to precipitate the genomic DNA. Afterwards the tubes were inverted several times and centrifuged at 13.000 rpm for 10 min. The supernatant was removed and the DNA was washed with 500 µL 70% ethanol at 13.000 rpm for 3 min. Once the pellet dried at room temperature, it was resuspended in 50 µL sterile H_2O. The samples were stored at -20 °C.

PCR was conducted with both genomic primers that anneal upstream and downstream the site of T-DNA insertion, as well as primers that anneal at the left border of T-DNA. 1 µL of DNA was added to a mixture of 3 µL 5x FIREPol Master Mix (Solis Biodyne, Estonia), 0.5 µL forward primer, 0.5 µL reverse primer and 10 µL H_2O. The primers used to genotype each specific line are shown in the supplements (Tab. S1). The PCR programme used was: 5 min at 95 °C, 40 cycles of 30 s at 95 °C, 40 s at 54 °C and 90 s at 72 °C followed by a final extension of 5 min at 72 °C. PCR reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems, USA). The PCR products were subsequently visualized in 1% agarose gel with ethidium bromide. A picture was taken using the Biorad Universal Hood II (Bio-rad Laboratories, USA) and Quantity One software (Bio-Rad Laboratories, USA). The genotyping of *sweet9* was performed by Touchdown PCR (TD-PCR; Korbie and Mattick, 2008), which increases specificity of the amplifications through tight control of the temperature setting. The TD-PCR programme used was: 5 min at 95 °C, 15 cycles of 30 s at 95 °C, 45 s at 67 °C, 90 s at 72 °C (with the 67 °C dropping with one degree per cycle), 20 cycles of 30 s at 95 °C, 45 s at 53 °C, 90 s at 72 °C and then a final extension of 10 min at 72 °C and 15 min at 4 °C. Homozygous lines show amplification products only when the combination of T-DNA specific primer and genomic primer were used. Heterozygous lines showed a band for both primers and lines that do not contain T-DNA only show a band when the genomic primers were used (see Fig. 3).

Volatile analysis

To test if the expected effect of the knocked-out or overexpressed gene could be quantified in terms of flower volatile composition, volatiles were collected from the headspace of flowers of mutants and

compared with volatiles emitted by Col-0. Volatiles were collected from Col-0 and transgenic lines and subsequently analysed by GC-MS. To do this, 15 mature flowers were collected in a 50 mL clear glass vial, sealed with aluminium/PTFE septum (Grace, The Netherlands). The closed vials were left in the growth chamber for 30 min and then stored at -80 °C. Volatiles were trapped on Tenax liners (Camsco) connected for 30 min to vacuum pumps (Pas-500 Personal Air Sampler) at an air flow rate of 100 $mL \cdot min^{-1}$. Vials without flowers were used as an additional control. The samples were desorbed in a TD-100 thermal desorption unit (Markes) for 5 min at 240 °C and focused in a general purpose hydrophobic trap, kept at 0 °C. The cold trap was subsequently heated at 40 °C per second to 260 °C and subsequently held at 260 °C for 4 min. Samples were analysed in a 7890B gas chromatography system equipped with 7200 Accurate-Mass Q-TOF detector (Agilent Technologies, USA). Separation of volatiles was performed on a DB5 capillary column (length: 30 m, diameter: 0.250 mm, film: 1 µm, Agilent Technologies, USA). The temperature programme was set for 2 min at 40 °C, followed by a ramp of 10 °C/min to 280 °C and held for 4 min. Column flow was 1.2 mL/min helium. The MS measured volatiles between the mass range of 50-350 Da with an acquisition rate of 5 spectra per second.

Insect behaviour assay

The hoverfly preference was studied using two-choice bioassays, similar to the one described by Lucas-Barbosa *et al*. (2015). One hoverfly was released at a time in a flight chamber containing one wild type and one mutant plant, or the wild type and a red coloured wild type. Ten flies were tested with each pair of plants. Parameters monitored were the first choice of the female hoverfly, the number of flowers visited, the duration of flower visits, time spent on the leaves and time spent flying or sitting still. This data was collected with the use of a hand-held computer (Psion Workabout Pro) programmed with 'The Observer' (version 10; Noldus Information Technology, Wageningen, the Netherlands). Flower visitation by the hoverflies was monitored for 12 minutes. In total one plant-pair was used per day with 4-7 replications per combination. Only female hoverflies were used because they are assumed to be better in distinguishing different odours (Sutherland *et al*., 1999; Primante and Dötterl, 2010). Insect response was tested only in the morning when *Arabidopsis* flowers were fully opened. Used plants were 5-7 weeks old. Each hoverfly was only used once and subsequently discarded.

Red flowers were obtained as described by Cook *et al*. (2013). Col-0 plants were taken out of the pot and soil was washed off. Each plant was subsequently placed in 50 mL of 50% solution of food colouring and water. Plants that served as control were placed in 50 mL of water. After 20 h the plants were taken out of the solution and used for the behavioural assay. Hoverfly preference was tested against non-coloured plants of Col-0.

Pollination efficiency

To test whether hoverflies can successfully pollinate *Arabidopsis*, Col-0 flowers visited by hoverflies that previously fed on *tt4* flowers were marked and the siliques that developed from these flowers separately harvested. The seeds collected from individual siliques were sterilised with 25% bleach and 70% ethanol and thoroughly rinsed with water before sawing on 0.5x MS medium, pH 5.5, containing 3% agar. Plates were kept in a climate chamber (24 °C, L16:D8) for 7 days after which the genomic DNA was extracted and the plants were genotyped to verify if they are heterozygous.

Statistics

The collected data of the behavioural assays were not normally distributed and a non-parametric test was performed to analyse the results. In the dual-choice assays samples were considered related and the Wilcoxon signed-rank test was used to evaluate the differences in means. A binomial test was used to test whether distribution of data collected for the first choice of the hoverflies differed from a 50:50 ratio.

Results

Genotyping

Genomic DNA was extracted from all plants and tested for homozygosity by PCR. Homozygous lines that contain the T-DNA inserted in both homologous chromosomes show a product of amplification when the BP primer that anneals to the T-DNA is used in combination with the genomic RP primer but not when both LP and BP genomic primers are utilized (Fig. 6). Heterozygous plants that carry the T-DNA inserted in only one chromosome, show both products of amplification (two bands).

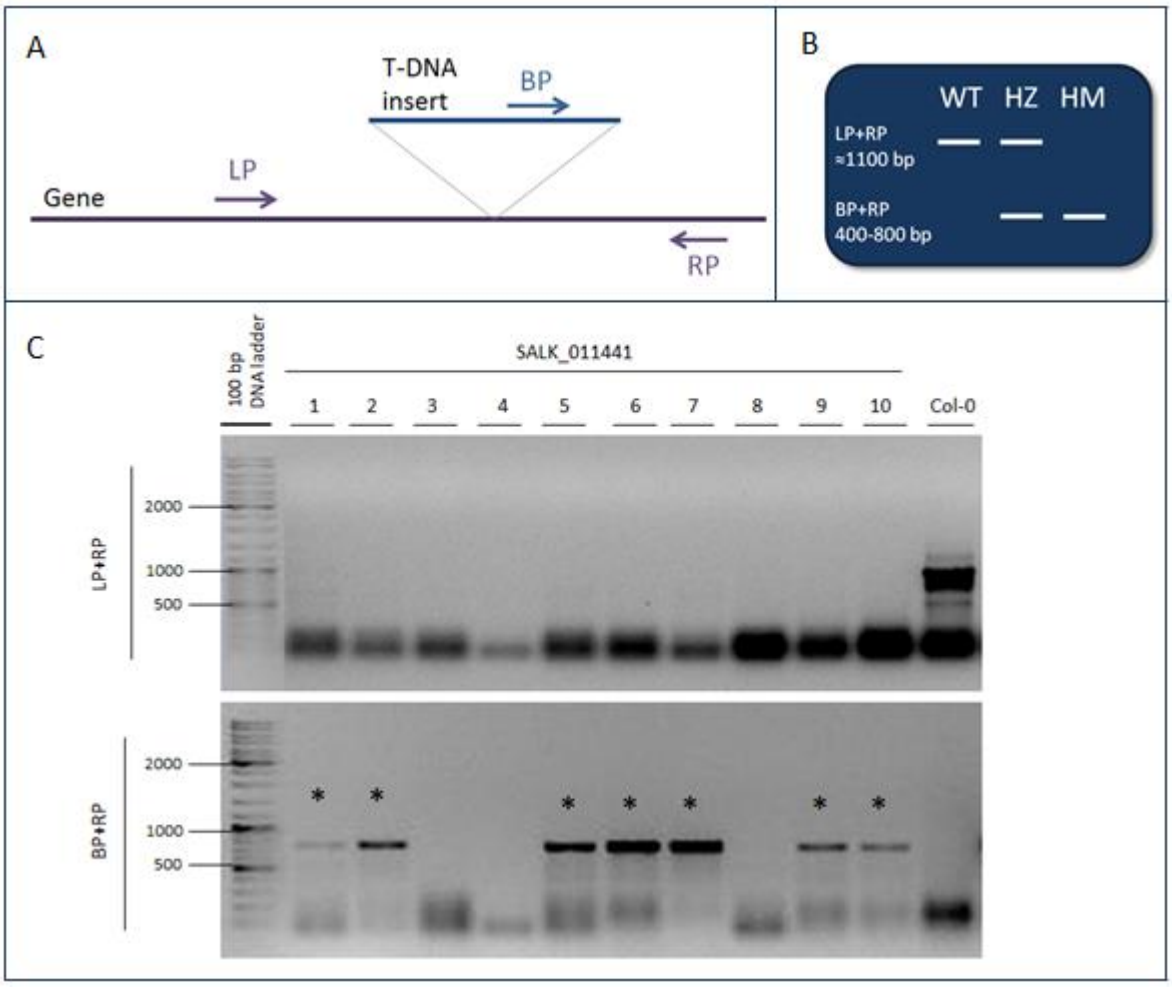

Figure 6. *Typical example the results of a genotyping experiment.* ***(A)*** *Representation of the T-DNA insert in the gene and the position of the primers. LP = Left genomic primer, RP = Right genomic primer, BP = T-DNA border primer.* ***(B)*** *Expected bands with the LP+RP primer and BP+RP primer. In the wild type (WT) there was only a band when the LP+RP primers were used. Heterozygous plants (HZ) showed a band for both the LP+RP and BP+RP primers and homozygous plants (HM) only showed a band when BP+RP were used.* ***(C)*** *Electrophoresis gel result for SALK line 011441. In the upper gel the PCR was run with LP+RP and only Col-0 showed a band at the correct size. In the lower gel the PCR was run with BP+RP and bands were shown in lanes 1, 2, 5, 6, 7, 9 and 10 (marked with an asterisk). This means that these lines are homozygous, as they only showed a band when BP+RP were used.*

Genotyping information for each T-DNA lines tested in this thesis can be found in Tab. 2.

Since the batch of *sweet9* seeds purchased at the ABRC Stock Center did not contain any homozygous plants, 100 identified heterozygous seeds were planted and screened for the presence of homozygous in

the F2 segregating population. Three F2 plants out of the hundred were identified as homozygous (Fig. S1).

Table 2. *Number of homozygous and heterozygous plants identified in the screening. Lines indicated with an asterisk were obtained from the Laboratory of Plant Physiology, Wageningen (the Netherlands) and these were previously checked for homozygosity.*

T-DNA Line	Total plants	Homozygous	Heterozygous	T-DNA Line	Total plants	Homozygous	Heterozygous
Salk_013858	26	4	22	**Salk_034144**	9	0	7
Salk_013880	20	-	-	**Salk_020880**	21	10	11
Salk_039462	21	-	-	**Salk_052466**	10	1	9
Salk_114189	21	-	-	**Salk_011441**	10	7	0
Salk_059820	8	0	0	**Salk_062519**	10	6	3
Salk_108420*	5	5	0	**Salk_152097**	21	-	-
Salk_041114*	5	5	0	**Salk_141559**	9	-	-
tps10xtps14*	5	5	0	**Salk_035057**	10	7	2
Salk_001949*	5	5	0	**Salk_138212***	5	5	0
35s:CYP76C1*	5	5	0	***35S:AtTPS21****	5	5	0
Salk_077330	21	-	-	**Salk_151777**	9	1	1
Salk_056876	19	2	17	**SAIL_728_G04**	9	-	-
Salk_027343	10	1	9	***CDS57****	5	5	0
cyp76c3*	5	-	-	***35S: FaNES****	5	5	0
35S:CYP76C3*	5	5	0	***FPS1L+pFaNES****	2	2	0
Salk_151809	9	0	9	***AtSWEET9***	20	0	12
Salk_142794	9	5	4	***TT4***	23	7	16
Salk_009366	20	0	20				

cyp76c3 was initially planned to be used in the hoverfly assays as well, but this mutant showed a delayed flowering phenotype compared with control plants of Col-0 grown in the same conditions. The first flowers of this mutant appeared at least a month after Col-0 flowered, even when a cold treatment of 2 to 3 weeks was applied (4 °C, in the dark). The *tt4* and *sweet9* approximately flowered a week later than Col-0.

Volatiles

Flower volatiles were measured for several lines to determine whether the expected phenotype of the mutant lines could be confirmed. This was not feasible for every line because there were large variations in emission of volatiles between the biological replicates. Only the lines that showed the same volatile emission pattern for multiple replicates will be shown here.

The expected low levels of linalool for *35S:CYP76C1* were confirmed (Fig. 7). The anticipated lower levels of lilac aldehydes were less pronounced and not visible for every replicate (Fig. S2). Lilac aldehydes and alcohol levels in were not detected in every replicate of Col-0.

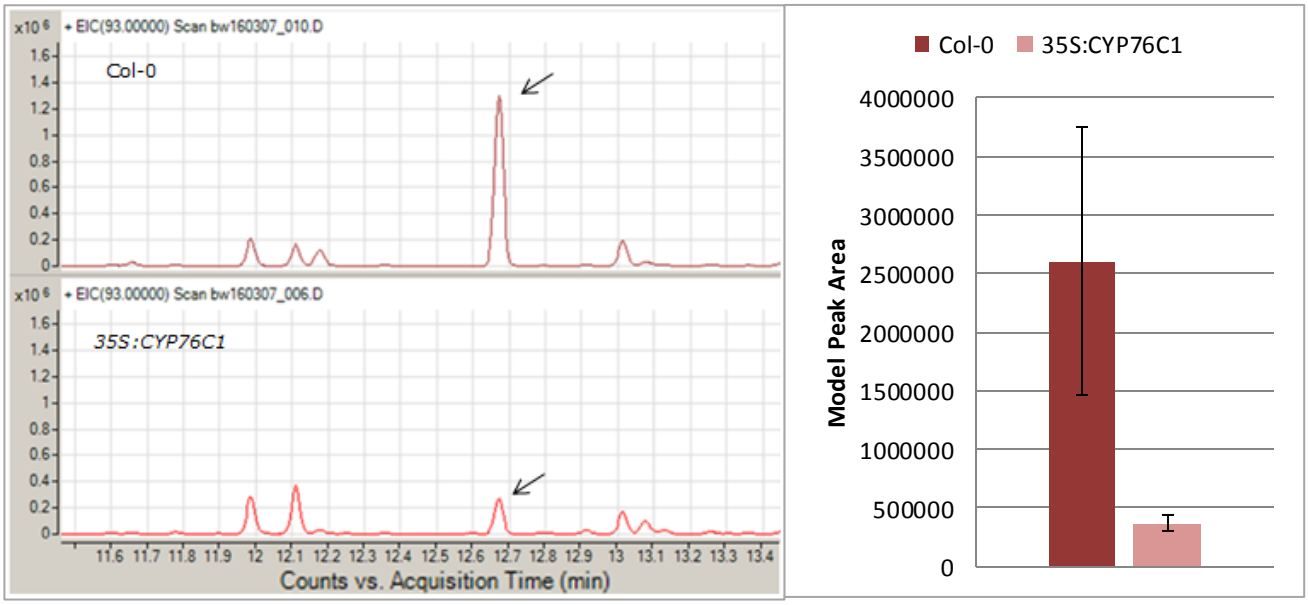

Figure 7. *Typical chromatogram of Col-0 (upper) and 35S:CYP76C1 (lower). The peak for linalool is indicated with an arrow. Area size of the linalool peak is shown in the graph on the right.*

The mutant *35S:AtTPS21* overexpresses a sesquiterpene synthase and therefore higher emission of (*E*)-*β*-caryophyllene, humulene and *α*-copaene is expected. This was confirmed by GCMS (Fig. 8) and the results were consisten for the different replicates..

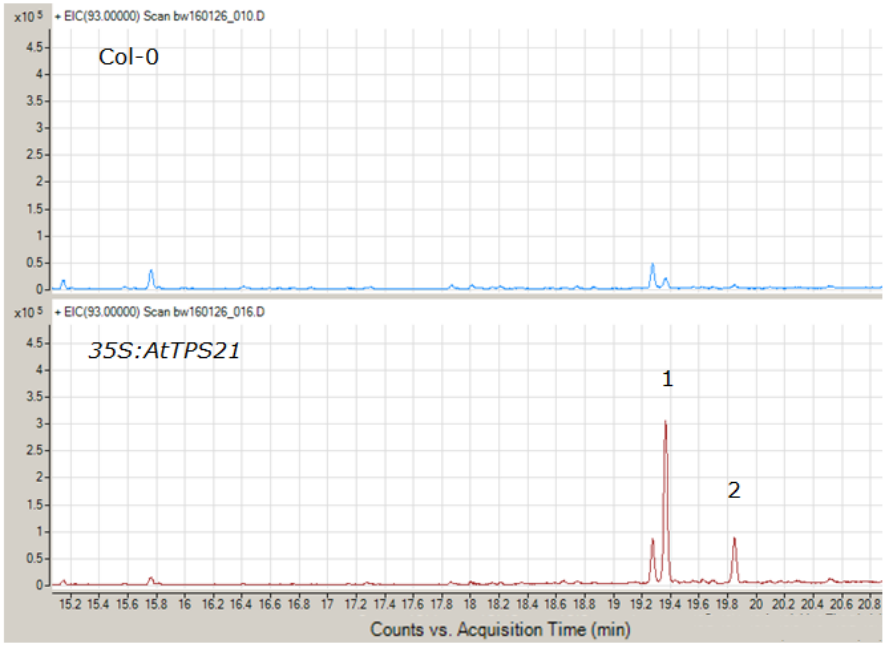

Figure 8. *Typical chromatogram of Col-0 (upper) and 35S:AtTPS21 (lower). The high peaks are (E)-β-caryophyllene (1) and humulene (2).*

Volatile composition of *tt4* did not differ from Col-0 (Fig. S3). This was as expected, since chalcone synthases are not known to be involved in volatile emission

Hoverfly assays

Hoverfly preference assays were tested in dual choice assays with 4 assays in total using Col-0 as a reference. In the analysis of the results, the emphasis will be on the first choice of the hoverfly, the number

of flowers visited by the hoverfly and the amount of time spend on the flowers, in total and per individual flower.

Scent

To investigate the role of a specific scent on the attraction of hoverflies, *35S:CYP76C3* was tested against the wild type. In total 51 hoverflies were released, of which 59% responded by visiting either Col-0 or *35S:CYP76C1.* Hoverflies landed as frequently on flowers of Col-0 and they did on flowers of the mutant line (Fig. 9, Binomial distribution, $P = 0.5$). Furthermore, the hoverflies spent as much time on the flowers of Col-0 as they did on the flowers of mutant line *35S:CYP76C1* (Fig. 9, Wilcoxon signed-rank test, $P = 0.345$). The time spent on a single flower by the hoverflies was similar for the two lines (Fig. 9, Wilcoxon signed-rank test, $P = 0.138$). Hoverflies visited a similar number flowers of *35S:CYP76C1* than of Col-0 (Fig. 9, Wilcoxon signed-rank test, $P = 0.081$). The lower linalool emission of *35S:CYP76C1* did not make the flowers less attractive to the hoverflies.

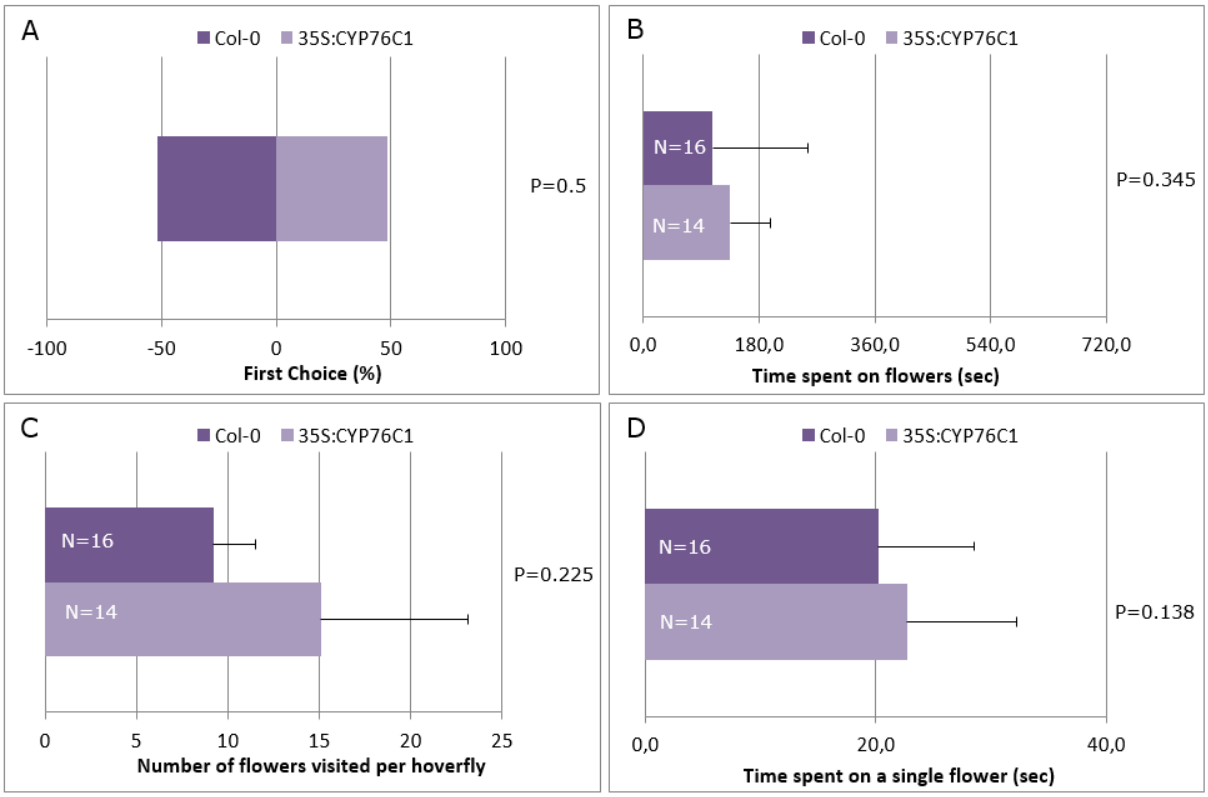

Figure 9. *First choice (A), time spent on flower (B), number of flowers visited per hoverfly (C) and the time spent on a single flower (D) (mean + SD), per 12 minutes of observation for Col-0 and 35S:CYP766C1. N stands for the number of hoverflies that visited each plant. Significance was tested with a Wilcoxon signed rank test for A until C and with a binomial distribution test for D. In total 59% of the hoverflies made a choice within 5 min (n=51).*

Fluorescence

To test the influence of fluorescence, in total 65 flies were released to choose between Col-0 and *tt4*. Of these hoverflies, 65% made a choice. Hoverflies spent an equal amount of time on all flowers and per single flower and visited an equal number of flowers (Fig. 10, Wilcoxon signed-rank test, P = 0.176, P = 0.091 and P = 0.091 respectively). There was a small tendency towards *tt4* as first choice of the hoverflies, but not big enough to be significant (Fig. 10, Binomial distribution, P = 0.180). The higher fluorescence of *tt4* petals did not lead to an increased or decreased attractiveness to the hoverflies.

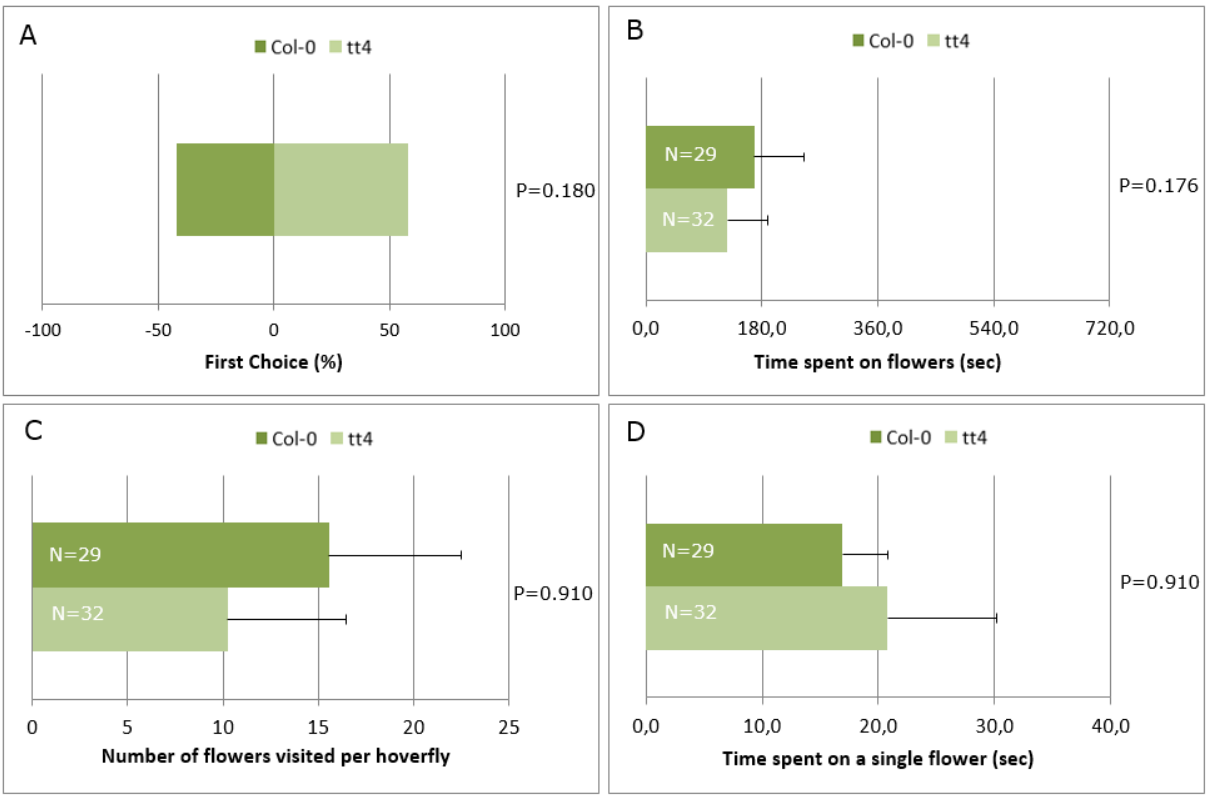

Figure 10. *First choice (A), time spent on flower (B), number of flowers visited per hoverfly (C) and the time spent on a single flower (D) (mean + SD), per 12 minutes of observation for Col-0 and tt4. N stands for the number of hoverflies that visited each plant. P values are shown next to each graph. Significance was tested with a Wilcoxon signed-rank test for A until C and with a binomial distribution test for D. In total 65% of the hoverflies made a choice within 5 min (n=65).*

Nectar

Since there were three homozygous *sweet9* plants and one plant was kept for collecting seeds, only two plants were available for this assay. Therefore, plants were used a second time, four days after the first assay. Due to the high turnover rate of *Arabidopsis* flowers, all the old flowers were already developed into siliques and the hoverflies were able to visit new flowers. In total 37 flies were released, of which 48% made a choice. Hoverflies landed first on Col-0 as many times as they landed first on *sweet9* (Binomial distribution test, P = 0.180). The lack of nectar had the highest influence on the time spent per individual flower. The time spent per individual flowers of *sweet9* was almost significantly lower than the time spent per individual flower of Col-0 (Fig. 11, Wilcoxon signed-rank test, P = 0.068). Hoverflies visits to Col-0 flowers lasted as long as visits to the mutant line lacking nectar. The number of flowers visited by the hoverflies was also similar when comparing the mutant line with Col-0 (Fig. 11, Wilcoxon signed-rank test, P = 0.715 and P = 0.273 respectively).

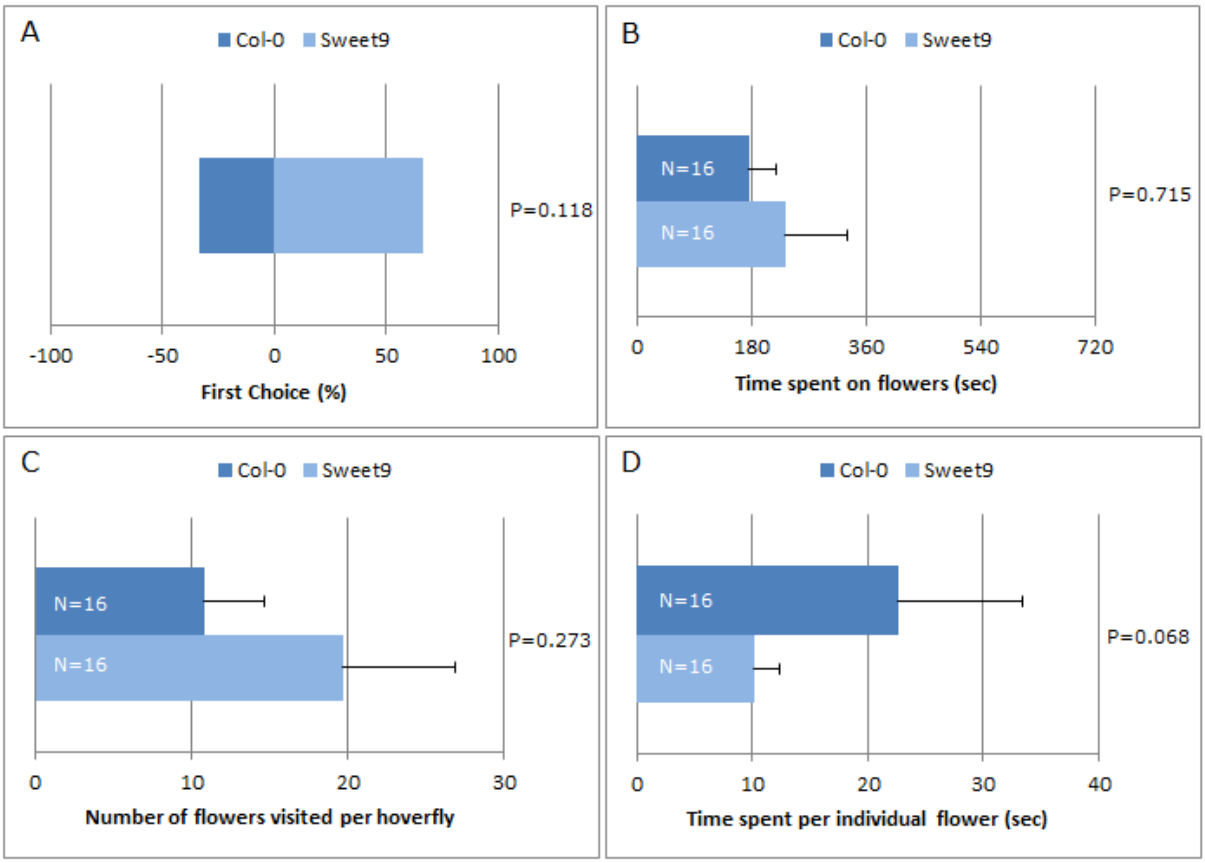

Figure 11. *First choice (A), time spent on flower (B), number of flowers visited per hoverfly (C) and the time spent on a single flower (D) (mean + SD), per 12 minutes of observation for Col-0 and sweet9. N stands for the number of hoverflies that visited each plant. P values are shown next to each graph. Significance was tested with a Wilcoxon signed rank test for A until C and with a binomial distribution test for D. In total 48% of the hoverflies made a choice within 5 min (n=37).*

Colour

To determine the effect of colour, Col-0 was coloured red with food colouring. Flowers did not turn completely red, but the veins and the leaves and siliques also showed a red hue. This had a significant impact on the first choice of the hoverflies (Fig. 12, Binomial distribution test, $P = 0.006$). Most hoverflies landed on Col-0 flowers first. However, once the hoverfly visited the red Col-0, these insects spent as much time on red flowers as on the non-coloured flowers (Fig. 12, Wilcoxon signed-rank test, $P = 0.465$). Time spent per individual flower (Fig. 12, Wilcoxon signed-rank test, $P = 0.715$) and number of flowers visited was also not influenced by the red colour(Fig. 12, Wilcoxon signed-rank test, $P = 0.713$).

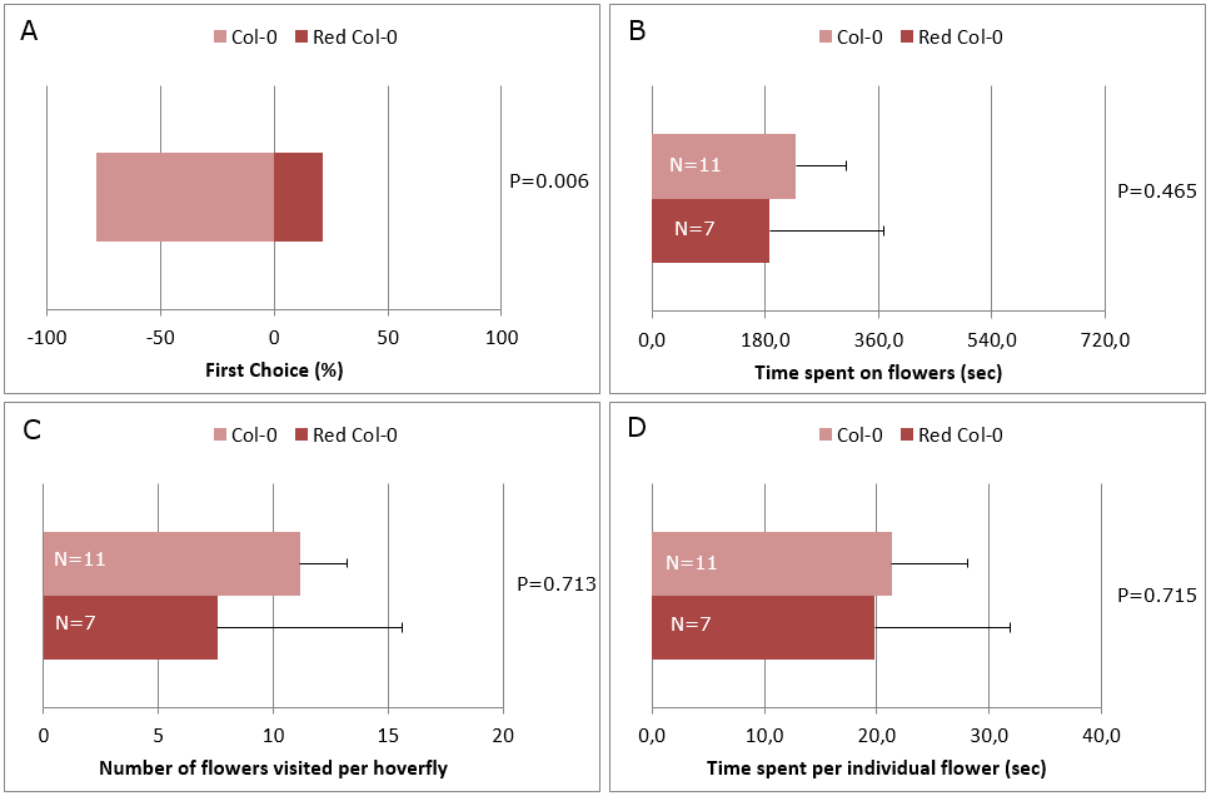

Figure 12. *First choice (A), time spent on flower (B), number of flowers visited per hoverfly (C) and the time spent on a single flower (D) (mean + SD), per 12 minutes of observation for Col-0 and Red Col-0. N stands for the number of hoverflies that visited each plant. P values are shown next to each graph. Significance was tested with a Wilcoxon signed rank test for A until C and with a binomial distribution test for D. In total 41% of the hoverflies made a choice within 5 min (n=33).*

Pollination efficiency

Although it has been shown that hoverflies visit *Arabidopsis* flowers (Hoffmann *et al.*, 2003), it is still unclear whether these visits contribute to pollination. To test for pollination efficiency, seedlings obtained from Col-0 flowers visited by hoverflies previously feeding on *tt4* were genotyped. The *tt4* utilized in the experiments is a carbon-ion induced mutant in Col-0 background putatively carrying a deletion in the At5g13930 gene (Shikazono *et al.*, 2003). No suitable genotyping primers are known for this line. Therefore, three primer pairs that span over the whole sequence of the TT4 gene have been designed (Fig. 13) with the purpose of identifying suitable markers for *tt4*. As seen in Fig. 14 when primer 1173F was used in a reaction with either primer 1174R or 1176R no products of amplification were generated if *tt4*

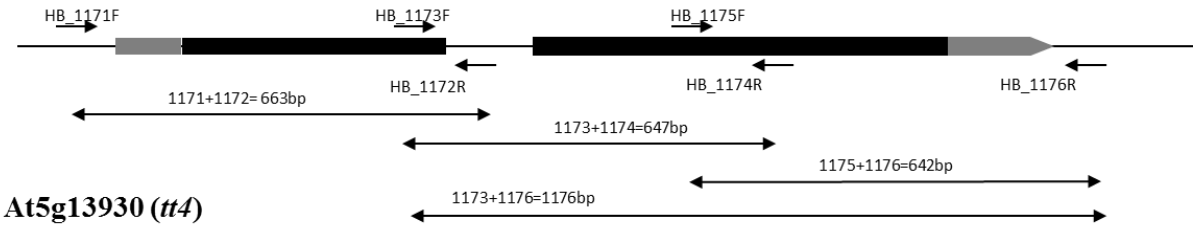

Figure 13 *The location of the primers on the genomic DNA to genotype the progeny of Col-0 that was possibly crossed with tt4 after pollination by Episyrphus balteatus.*

genomic DNA was the template. This means that a large mutation between nucleotide 281 and 802 of At5g13930 gene is present in *tt4*. Given that the lack of a product of amplification is not sufficient to determine if the *tt4* mutated allele has been inherited, the genotyping of the Col-0 x *tt4* progeny was not feasible.

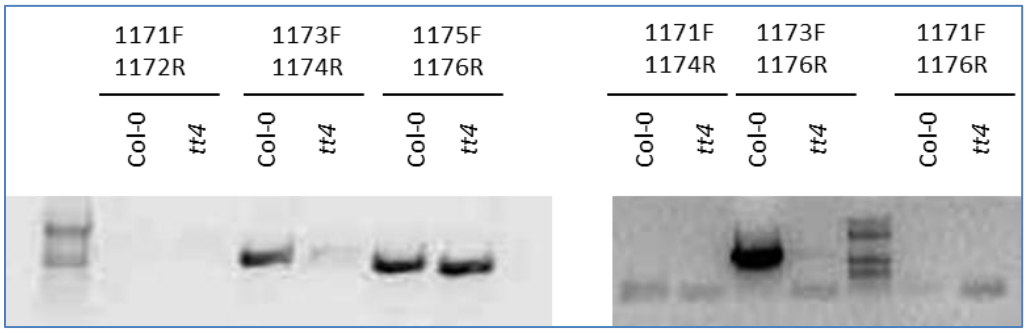

Figure 14 *PCR products of the different primers used to genotype the At5g13930 locus (tt4).*

Discussion

The aim of this thesis was to evaluate the relative importance of signals that plants use to attract pollinators. The effect of scent, fluorescence, colour and nectar availability were investigated using several *Arabidopsis* mutants, coloured plants and *E. balteatus* hoverflies. My results show that red colour influences the first choice of the hoverflies. Lack of nectar has shown to decrease the time spent per individual flower. Low linalool and fluorescence had no effect on the preference of *E. balteatus.* Seeds of flowers visited by hoverflies were collected to see whether these *Arabidopsis* plants were efficiently pollinated, but it was not possible to determine the genotype of the progeny. Volatiles of mutants were collected, but the expected phenotype was not found in all mutants. Chromatograms of biological replicates showed large variations in the emission of volatile compounds. The volatile collection should be fine-tuned to prevent this in the future.

Effect of scent

The lack of linalool in the flower volatile blend of *Arabidopsis* does not influence the preference of *E. balteatus*, although it was previously shown that hoverflies are attracted to the pure compound of linalool (Boachon *et al.,* 2015). Literature data show that volatile compounds can have different effects on insect preference when they are present alone or in a blend (Bruce and Pickett, 2011). For example, black bean aphids were repelled by several compounds when they were provided separately, but were attracted to the blend of these compounds (Webster *et al.,* 2010). Linalool could also be attractive to hoverflies as a pure compound, but have different effects when it is present in a volatile blend. My results show that lack of linalool in bouquet of Arabidopsis flowers did not render plants less attractive to the hoverflies.

It could also be that the difference in linalool between the wild type and the mutant was not large enough for the hoverfly to be noticed. Indeed, the amount of volatiles released from *Arabidopsis* is much lower than the volatile emission from flowers which primarily rely on insects for their reproduction, such as *Clarkia breweri* (Chen *et al*., 2003). It could also be the case that the difference in linalool between Col-0 and the mutant was not large enough for the hoverfly to be noticed. Chen *et al*. (2003) argued that terpenes in *Arabidopsis* might be more important for other functions than the attraction of pollinators. Linalool and other terpenes react with reactive oxygen species (Calogirou *et al*., 1999) and might therefore be involved in the protection of flowers against bacteria and fungi. Boachon *et al*., (2015) also suggest that linalool functions as deterrent for florivores and pollen thieves instead of attracting pollinators.

Not many studies investigated the role of scent in food foraging of hoverflies. Most studies focus on the oviposition site foraging behaviour of *E. balteatus*. This hoverfly species only lays eggs near aphids, since aphids are the primary food source of the hoverfly larvae. Aphids elicit the production of volatiles in potato (*Solanum tuberosum*) and this increases the visits and oviposition by *E. balteatus* (Harmel *et al*., 2007). However,in broad beans, the hoverflies only responded to aphid volatiles but not to plant volatiles (Francis *et al*., 2005). There is an orchid which takes advantage of the preference of *E. balteatus* for aphid volatiles (Stökl *et al*., 2011). This orchid species mimics aphid alarm pheromones and tricks *E. balteatus* into pollinating its flower. Considering this information, hoverflies might be more focused on aphid odours than plant odours.

A recent study showed that *E. balteatus* is not able to distinguish pollinated from unpollinated flowers in *Brassica nigra,* although the volatile profile differs between pollinated and unpollinated plants (Lucas-Barbosa *et al.,* 2015). This suggests that these insects rely more on visual than olfactory cues when looking for a food source. *Pieris brassicae* butterflies on the other hand did show a preference for unpollinated flowers and these insects are indeed known to exploit odour cues (Lucas-Barbosa *et al.,* 2015)

Effect of fluorescence

It was hypothesised that fluorescence of the *tt4* petals would increase or decrease the visibility of the flowers to hoverflies and that the hoverflies would show a difference in first choice. Nonetheless, the experiments showed no initial preference of the hoverflies. The time spent on flowers and number of flowers visited were not affected. This was as expected, since the *tt4* mutation does not alter scent, nectar and pollen availability. Although many flowers exhibit fluorescence, evidence regarding the influence of fluorescence of flowers on pollinator attraction remains elusive. It is unsure whether fluorescence is still visible against different backgrounds, such as a blue sky or against vegetation (Iriel and Lagorio, 2010). Fluorescence could also be favoured by natural selection because it can protect tissues from being damaged by harmful light intensities (Holovachov, 2015). This trait might not be exploited by pollinators, although this deserves to the further investigated. It is known, for instance, that the floral parts of grasses emit fluorescence patterns (Baby *et al.*, 2013). It was assumed by Baby *et al.* (2013) that this fluorescence plays a role in the attraction of pollinators or pests. The fluorescence of males stages of flowers of several species of bamboo corresponded with the visitations by pollinators (Baby *et al.*, 2013). The species *Mirabilis japa* uses contrasting fluorescence patterns, which might increase its appeal towards pollinators (Gandia-Herrero *et al.*, 2005). UV fluorescence is used to catch prey by the carnivorous plants from the genera *Nepenthes, Dionaea* and *Sarracenia* (Kurup *et al.*, 2013). When the fluorescent areas were covered, the quantity of prey caught by the plants were drastically decreased.

Effect of nectar availability

It was hypothesised that hoverflies spend less time on flowers without nectar, as there is less food for them to feed on. Hoverflies were not expected to perceive the lack of nectar of a flower from a distance unless this could be associated with changes in odours or visual cues from the flowers . Indeed, the first choice was not affected and hoverflies landed as often on the flowers of the mutant line as they did on flowers of Col-0. Hoverflies tended to spend less time on a flower of the mutant line *sweet9* mutant that on a flower of Col-) (P = 0.068). Hoverflies were also observed to feed on pollen, and pollen availability in *sweet9* could explain why flowers of *sweet9* remain attractive to the hoverflies. A way to test the influence of pollen on insect behaviour would be to excise the pollen of flowers of Sweet9 (Barragan, 2014) and in this case the hoverflies presumably will spend less time on the flowers without pollen and prefer flowers with pollen.

Effect of colour

To test whether flower colour influence the preference of hoverflies, Col-0 plants were coloured red with food colouring. Most hoverflies preferred landing first on flowers of non-coloured Col-0 than on the Col-0 plants with red flowers (P = 0.006). This corresponds with earlier observations that *E. balteatus* discriminates between colours (Sutherland *et al.*, 1999). Most flies do not perceive the colour red (Woodcock *et al.*, 2014). A large part of red flowers in nature are pollinated by birds that are able to observe red colours (Rodríguez-Gironés and Santamaría, 2004). Only 21% of all hoverflies tested landed on a plant with red flowers. Once they found the red flowers the time they spent visiting flowers was not affected, and this is what I would expect because the colouring of the flowers does not affect scent, nectar or pollen. Since colour affects the behaviour of *E. balteatus,* which is an effective pollinator of *B. napus* (Jauker and Volkmar, 2008), the suggestion of Cook *et al.* (2013) to colour *B. napus* red to reduce herbivory could have serious implications for pollination. It should be tested whether the yield of red coloured *B. napus* decreases in the field.

Pollination efficiency

An effective way to test for pollination efficiency, is to measure the outcrossing rate in the offspring of a cross between genetically different parent lines. In the case of this thesis Col-0 and the *tt4*, which are genetically different at the At5g13930 locus, were utilized as pollen receptor and as pollen donor, and hoverflies used as carriers for pollen. If Col-0 received pollen from a hoverfly that previously fed on *tt4*, the progeny would be heterozygous at the At5g13930 locus. Because the *tt4* utilized in this experiment

has been previously identified by complementation (Shikazono *et al.*, 2003) and not characterized with genotyped with allele specific markers or sequencing, it was not possible to complete the genotyping of the progeny in time. However, in this thesis it has been shown that the mutation underlying the *tt4* phenotype is a large deletion positioned between nucleotides 281 and 708. Successively, pollination efficiency could be tested in the F2 population by scoring the seeds which have the *tt4* genotype. *tt4* seeds have a transparent seed coat due to the lack of CHS.

Concluding remarks and future implications

Of all the examined signals that *Arabidopsis* could potentially use to attract pollinators, colour and nectar were the most influential ones. The role of scent should be investigated further, since only one mutant in scent was used in this study. The fact that nectar availability did not affect the time spend on flowers significantly, points to an important role of pollen in the attractiveness of flowers to hoverflies.

In contrast to the artificial flowers used by Sutherland *et al.* (1999) and the testing of pure compounds by Boachon *et al.,* (2015) this study used mutant plants that lack or overexpress a given trait to test the effect of this specific trait on the attraction and food preference by hoverflies while conserving all other traits characteristic of *Arabidopsis* flowers. This was possible thanks to the large selection of mutants available for *Arabidopsis*. In this thesis only the effect of linalool on *E. balteatus* behaviour was examined. Further research should focus on the other components of the volatile blend of *Arabidopsis.* For instance, hoverflies could distinguish between plants with and without herbivores and preferred plants without herbivores. Volatile analysis showed that the infested plants had a different blend than the control plants (Lucas-Barbosa *et al.* 2015). The volatile blend of these plants give an indication which volatile compounds might influence the preference of *E. balteatus.* Control plants emitted a higher level of benzyl alcohol, which might be more attractive to *E. balteatus.* Another plant to study plant-pollinator interactions could be *Arabidopsis lyrata*, which is a close relative to *Arabidopsis* Col-0, but relies solely on cross-pollination for its reproduction (Abel *et al.*, 2009). The genome of *A. lyrata* has been sequenced a few years ago (Hu *et al.*, 2011).

Hoverflies are important pollinators in nature. During the past few decades several pollinators, especially bumblebees and honeybees suffered from population declines (Goulson *et al.*, 2008). Nevertheless, in the Netherlands the population of hoverflies has remained relatively stable (Biesmeijer *et al.*, 2006). With the decline of bees, hoverflies might become more important pollinators. Already a third of pollination services are done by other species than bees (Rader *et al.*, 2016) and this may increase in the future. Hoverflies perform better in agricultural landscapes than bees (Jauker *et al.*, 2009). Breeders need to take into account that future pollination services could shift from bees to other insects. This thesis shows that a red flower colour decreases the attraction of the flower to *E. balteatus*. Colouring flowers red to decrease herbivore damage as suggested by Cook et al., (2013) would not be prudent. Breeding for better disease resistance should not compromise pollinator attraction as this could lead to lower yields. Therefore, it is important to investigate the cues that plants use to seduce pollinators.

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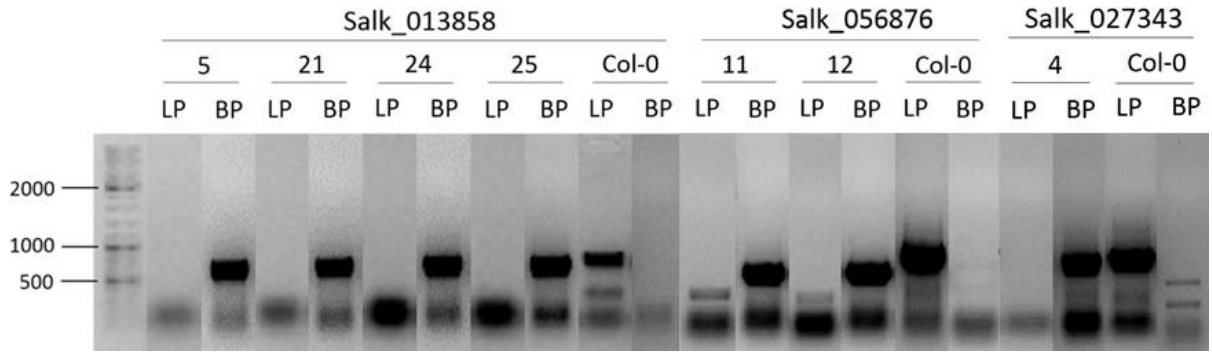
Supplemental data

Table S1. *Primers used for genotyping. The three primers at the bottom of the list (LBb1.3, LBa1 and LBb1) are BP primers. In this case the forward primer will be substituted by one of the three T-DNA primers and the regular reverse primer is used.*

Line	Forward primer	Reverse primer
SALK_013858	CAAGTGGTCGAAGAAAGAACG	TAGACAACCTTGGAACATCGG
SALK_013880	CAAGTGGTCGAAGAAAGAACG	TAGACAACCTTGGAACATCGG
SALK_114189	TCATCATTATTGGTTTGTTCCG	ATCTCATGGAGATCACCGTTG
SALK_039462.41.95	CTAATCGAACTCTGGCGAATG	CTTTGTTTCTCAGTGGGCAAG
SALK_059820.44.15	TGCGCCAAAGACTTCATTATC	TGGTTGAGGCTTGAAGTTTAATG
SALK_027343.14.80	AGCCTTTTGCAGGTTTAAAGC	TAGTGAGAGGTCCATTGGACG
SALK_056876	CACAACACAGTGGTTCACCTG	GTACGGCACAAAGAGATTTGG
SALK_077330	TCGGAAACATATTCCAACTCG	ACCGTTTTGACCAATCACTTG
SALK_142794	GCAGCAACTATAGCCACGATC	CATGTGTTGAAGAAAAAGGTGAAG
SAIL_361_G11	GTGGAACAGAGCAAGAAATCG	TATTTTGTTTGGGCTGGACAC
SALK_151809C	ATCAATTGGGAGATCGAGACC	ACATGGAAGCAACAAGAATGG
SALK_034144	GCTGGTGCAAGAGACAGAAAC	CTTTCTTGGCGATGTTCAAAG
SALK_009366C	TCCAACGTTTAGGATCACGTC	ATGCTCAAACACAAACCTTGG
SALK_020880	TTGGCCTACAATTTTGGTTTG	TGCTAATTGTGATGGTATTGCAG
SALK_052466	TCGACCCTAGCCATAACTCAG	TCTTGATCTTGTCAAATGGGC
SALK_011441	TTTTTGTTCTCTTGGCTGGG	CCACTCATAAGCTTCCTTCCC
SALK_062519	ATCGTCCACCTCTATGGGATC	AATGGTACGGCGTCTCTAGTG
SALK_152097	AACACGTCTCTTGAGATGATGG	CAGCACGAATATCTCCTCTCG
SALK_141559	ATCTTTTGTTGTCGCCAAATG	ATTTGCATTATCGCCGTAATC
SALK_035057	TGAGAAAACGTTGGTTTACGG	AATCTAGGCCAAAACTCGTCC
SALK_151777	TATTTTGGTAGGTGGTGGACG	GGTGGTTGTAAACATCATCCG
SAIL_728_G04	GGTGGTTGTAAACATCATCCG	TATTTTGGTAGGTGGTGGACG
QRB1 (for Col-3 background)	CAAACTAGGATAAATTATCGCGCGCGGTGTCA	ATTAGGCACCCCAGGCTTTACACTTTATG
SALK_020583	TCGAATAGACCTGTCCAGCAC	CTTCTCTGGACACCAGACAGG
SK225	CTTTGTCGGATTTAGAAGGCC	ATTTGCAATGTCGTCTCCAAG
LBb1.3	ATTTTGCCGATTTCGGAAC	
LBa1	TGGTTCACGTAGTGGGCCATCG	
LBb1	GCGTGGACCGCTTGCTGCAACT	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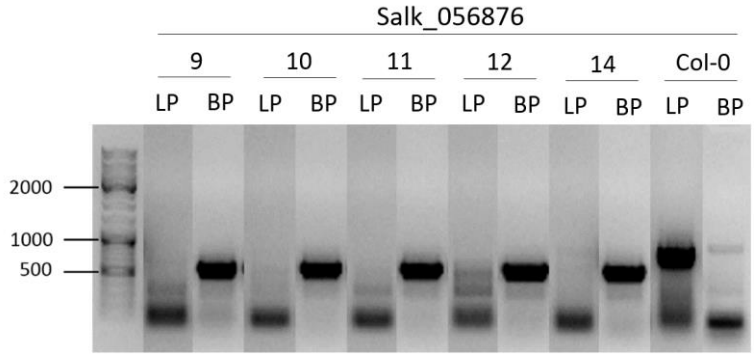
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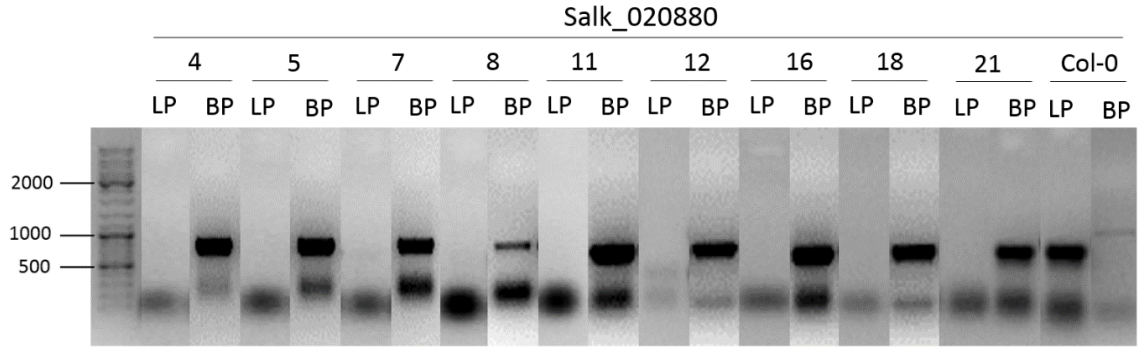
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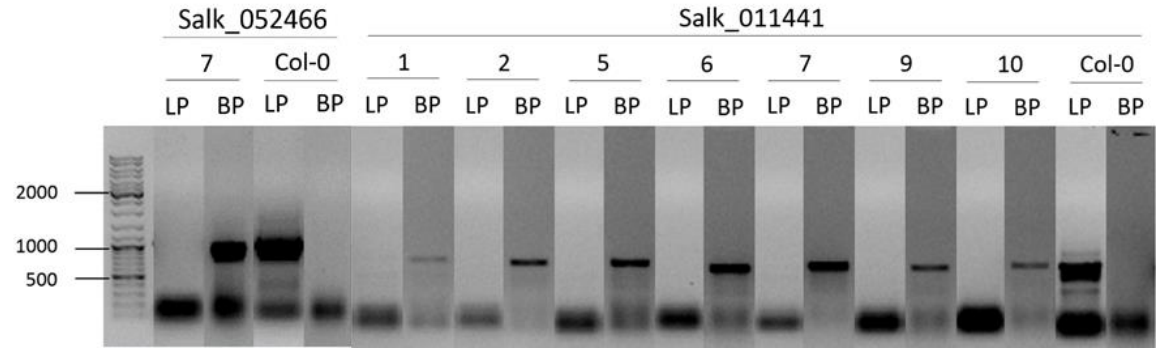
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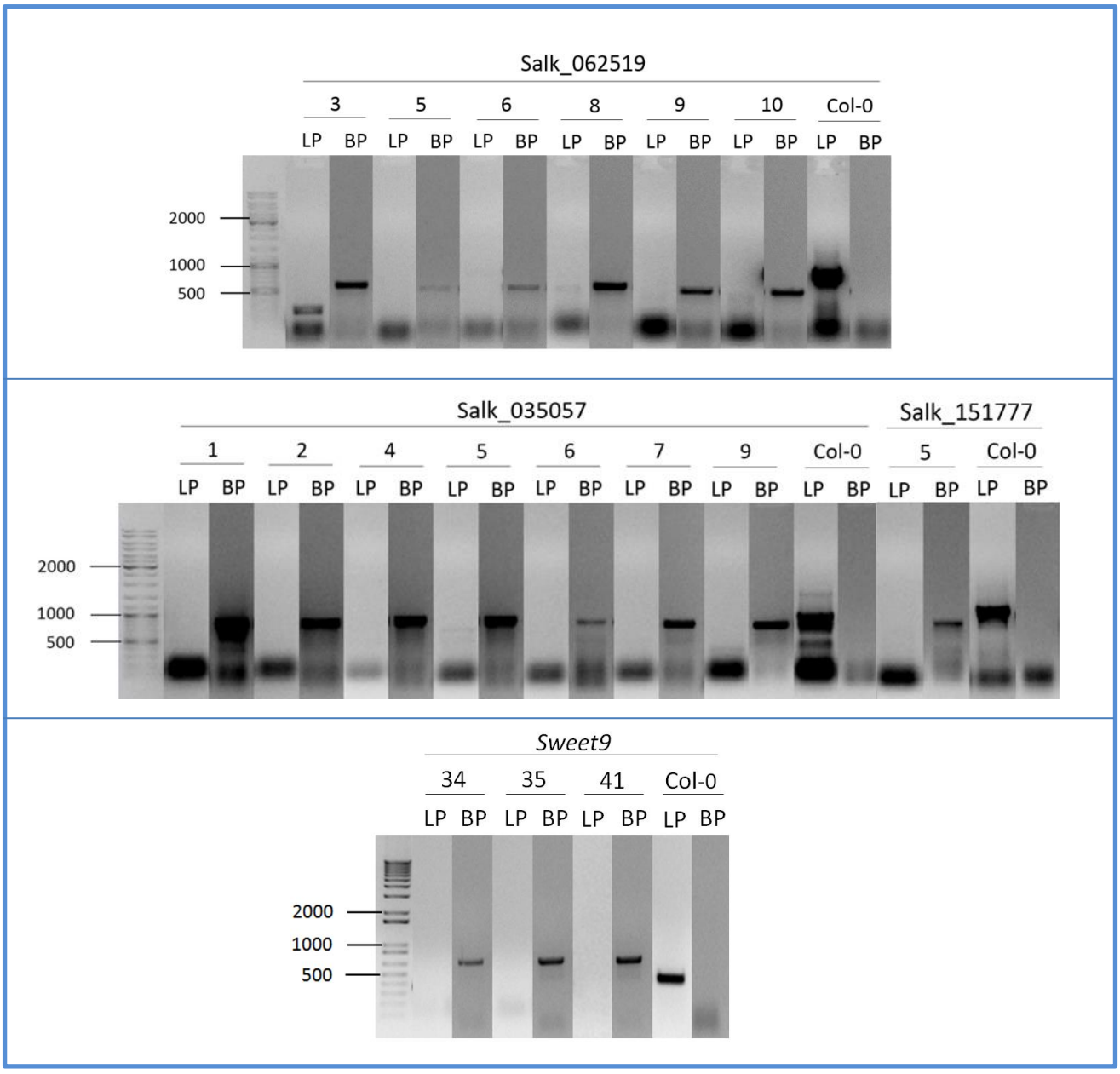

Figure S1. *Gel results of the genotyping. Only the homozygous plants are shown. The name of the Salk line and the plant number is depicted above the gel. LP stands for the LP+RP primer combination and BP stands for the LP+BP primer combination (see also Fig. 3 and Tab. 2).*

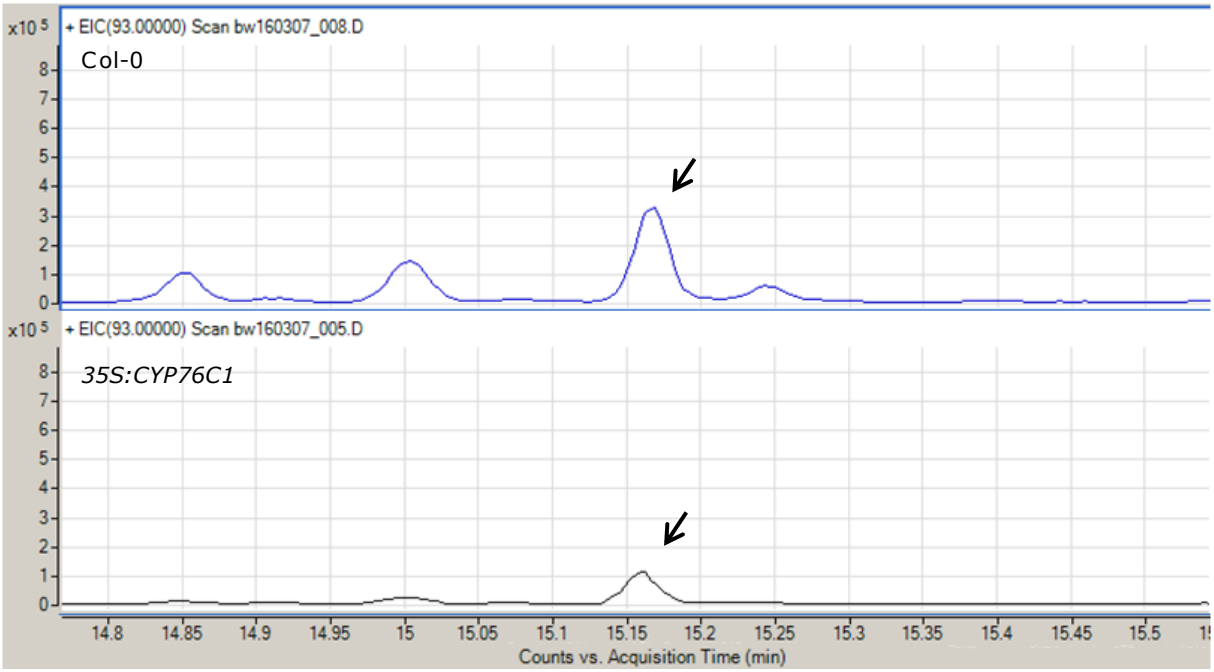

Figure S2. *Typical chromatogram of Col-0 and 35S:CYP76C1. The peak for lilac aldehyde is marked with an arrow.*

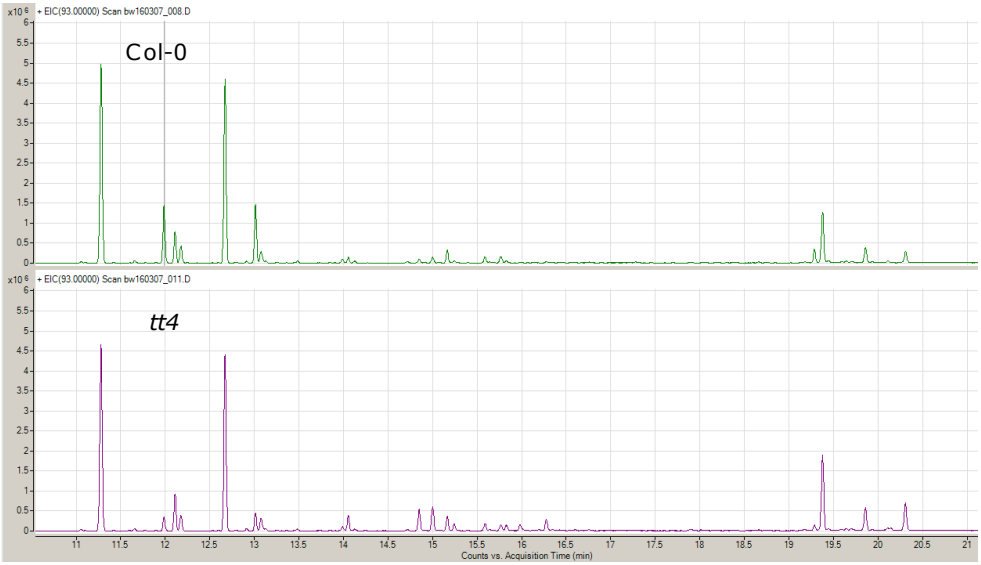

Figure S3. *Typical chromatogram of Col-0 and tt4. There are no significant differences between these two.*