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Do *Cotesia* parasitoid wasps discriminate cabbage plants infested by parasitized or unparasitized *Pieris* larvae?

Studies on parasitoid behavior and gene expressions

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Abstract

Plants have evolved various mechanisms to protect themselves against herbivorous insect attacks. After *Pieris* caterpillars attacked host plant *Brassica,* plants release a blend of volatiles as cues for oriented host searching of female parasitoids *Cotesia*. Parasitoids lay their eggs in larvae of *Pieris*, as a result the parasitized herbivores will die before their pupation. In order to understand molecular mechanism behind of plant-herbivore-parasitoid tritrophic system, windtunnel bioassay on parasitoid *Cotesia rubecula* behaviour and gene expression on *Brassica oleracea* were carried out. The results show that *C. rubecula* females are able to discriminate plants infested by unparasitized *P. rapae* larvae over parasitized ones (43.4 vs 27.2 % responding wasps), which is a significant difference. Specific cabbage genes from different signal transduction pathway (e.g. *BoLOX*, *BoPAL*, *BoDEF* and *BoPR1*) were investigated for the transcript levels in response to caterpillar feeding, regurgitant treatments derived from parasitized and unparasitized *Pieris* larvae, and mechanical damage by punching. Plants treated by regurgitant from parasitized larvae of *P. rapae* had a higher *BoLOX* transcript levels than unparasitized larvae treated. In contrast, *BoLOX* transcrips showed decreasing levels when plants were applied with regurgitant from parasitized larvae of *P. brassicae* compared with that from unparasitized larvae. The results shown there are differential and complex expression patterns of several cabbage genes using RT-PCR under the infestation from parasitized and unparasitized *Pieris* larvae. In conclusion, molecular tools can be applied here to understand the mechanism behind tritrophic system.

Keywords: Tritrophic interaction, *Cotesia* wasps, *Pieris* larvae, *Brassica oleracea*, RT-PCR

1. Introduction

1.1 Biointeractions of plants and insects

Plants provide food for animals, and ultimately, all animals rely on plants either directly or indirectly (Lack and Evans, 2001). Plants have been under attack of many insect species during the past 250 million years. These herbivores feed, reproduce, and shelter on plants (Blaakmeer, 1994).

Based on the mode of attackers, three categories of herbivores are recognized: sap-sucking insects, leaf-content feeder and chewing insects. Sap-sucking insects, aphids and thrips for instance, use their stylet to penetrate and drain sap from the phloem sieve elements of plant's vascular tissue. This kind of insects cause minimal direct plant damage, however, heavy infestation of sap-sucking insects results in shortage of plant nutrition and thus severely reduce growth of the plants. Cell-content feeding arthropods, like mites and thrips, use their stylets to pierce and empty mesophyll cells, leaving whitish spots of collapsed cell. Chewing insects, such as beetles and caterpillars, cause more spectacular plant tissue damage than sap-sucking insects (Buchanan *et al.,* 2000). The extent of damage frequently depends on the developmental stage of pest (Lack and Evans, 2001). For example, small cabbage white butterfly (*Pieris rapae*) larvae attack the leaves of cabbage. However, the butterflies are unharmful as they mature.

Feeding of herbivores not only damage plants directly but also facilitate infection by viral, bacterial and fungal pathogens. Many sap-sucking insect species are effective virus vectors. Sap-sucking insects can deliver virus directly into the plant vascular tissue, and then virus rapidly spreads throughout the whole plant by vascular system. Some virus species can even stay and replicate inside sap-sucking insects. Chewing insects rarely transmit viruses, but the tissue damage made by chewing insects always offers conveniences for colonization of fungal and bacterial pathogens (Lack and Evans, 2001).

The herbivores facilitate infection of pathogens. In return, are the microbes contributed to infestation of insects? In plant, plant hormone jasmonate and salicylate provide resistance signals to herbivorous insects and pathogens respectively. The aggression of biotrophic pathogens induces a series of specific plant defenses, mainly based on salicylic acid (SA) signaling pathway. SA pathway is considered to suppress octadecanoid pathway (produce Jasmonic acid, JA), i.e. SA and JA interact antagonistically. Because that plant responses towards insects are mainly JA signal based, the colonization of pathogens is thought to suppress octadecanoid pathway and therefore facilitate to insect herbivory. Though the experimental results can not support the hypothesis for a strict dichotomy of signaling by insects and pathogens, some experiments has provide evidences for this hypothesis. It has been shown that tomato plants expressing chemically-induced SAR have depressed polyphenol oxidase (PPO) activities, with a corresponding enhancement of insect herbivory (Thaler *et al*., 1999). Moreover, in cultivated tomato biochemical attenuation of the activity of PPO in dual-elicited plants resulted in increased of performance of cabbage looper caterpillars (*Trichoplusia ni*; Thaler *et al*., 2002).

1.2 Plant defenses against insects directly and indirectly

Plants evolve ingenious mechanisms to defense against insect herbivores, either constitutive or induced. The constitutive defense, including constitutive physical barriers such as a thick cuticle, gives plants the first protection against herbivores. However, production of resistance traits when they are not necessary is costly for plants. Therefore, induced defense is much more economic and efficient. Two broad categories of induced defense are recognized: direct defense and indirect defense (Kessler and Baldwin, 2002).

Plant direct defense is involved in several plant proteins and secondary metabolites. Proteinase inhibitors (PIs) are most well known antidigestive proteins which disturb proteolysis of the food by inhibiting proteinases in the herbivore gut. Disturbing of proteolysis leads to nutrition lack of the insects, and therefore inhibits their growth

and development (Baldwin *et al.*, 2001). Howe *et al.* (1996) obtained a tomato octadecanoid pathway mutant *defenseless 1* (*def1*), which is no longer able to induce PIs genes after herbivory. They demonstrated that the absence of PIs lead to increased performance of tobacco hornworm caterpillars (*Manduca sexta*) on tomato. Another well-known protein is polyphenol oxidases (PPOs), which are involved in catalyzing the biosynthesis of reactive quinones. Reactive quinones can be then polymerized into a glue to trap insects, or cross link proteins to reduce the nutritional quality of plants (Baldwin *et al.*, 2001).

Though many proteins, such as PIs and PPOs have been shown that play roles in deference towards insects, more important deterrents are considered as plant secondary compounds. First, many secondary metabolites that are induced by insects feeding are toxins or antifeedants to herbivores. Furthermore, it has been found that plants damaged by herbivores produce volatile components as synomones, which help parasitoids and predators of herbivore to find their herbivorous hosts. It is so called indirect defense of plant against herbivores.

Indirect plant defense in which plants produce volatiles to attract the natural enemies of herbivorous attackers was initially proved in 1988 by Marcel Dicke and associates. Dicke and coworkers demonstrated that herbivorous spider-attacked plants emit a blend of volatiles to help the prey searching of predatory mites (Dicke, 1988; Dicke *et al.*, 1990a; Dicke *et al.*, 1990b). Now it is clearly that plant volatiles induced by herbivore is different with volatiles from undamaged or mechanically damaged plants in both qualitative and quantitative, and these differences are detectable for some predatory mites or parasitoids (Agelopoulos *et al.*, 1994; Turlings *et al.*, 1995; Shiojiri *et al.*, 2000). Some researches also noticed that infochemicals for prey searching can be from herbivores themselves (Vinson, 1990). Since the discovery of indirect defense, the herbivore-induced volatiles and tritrophic system of plant-herbivore-natural enemies have been paid with great attentions. This “tritrophic” system has been found in many plant species belong to at least 12 plant families, in

combination with many species of herbivores and natural enemies (Van Poecke *et al.*, 2002).

1.3 Signal transduction pathways in plant defence

Herbivores wound the plant and apply regurgitant which contains elicitors to the plant. These elicitors may involve in digestion of food, inhibition of plant direct defence, or suppression of induced defences by influencing the signal pathways. Mechanical wounding in combination with elicitors activates various signal pathways. These signal pathways are mainly based on several hormones. Jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) have been reported to be induced by herbivore infestation in several plants (Dicke and Van Poecke, 2002), and thought as main players in defense signaling (De Vos *et al.*, 2006). These hormone-based signal cascades interact with each other (crosstalk) and lead to both direct and indirect defense against herbivore (Kessler and Baldwin, 2002).

JA is one of the most important herbivore-induced signal hormones. In 1971, JA was initially isolated from a fungus, *Botryodiploidi theobromae*, as an inhibitor of plant growth. It has been reported to inhibit the growth of rice, wheat, lettuce and so on (Buchanan *et al.*, 2000). From then on, JA and related compounds (Methy(-)-jasmonate, Isojasmonic acid and so on) have been proved play important roles in various physiology processes, including plant defense against herbivorous insects and necrotrophic pathogens (Thomma *et al.*, 2001; Dicke and Van Poecke, 2002; Poecke *et al.*, 2002).

When plants are wounded or attacked by microbes or infested by herbivore, accumulation of JA can be detected. JA and related compounds are able to activate expression of anti-fungal and anti-insectival proteins (Buchanan *et al.*, 2000). JA treated tomato increases its resistance against *Phytophthora infestans*, while *def1* mutant of tomato defective in JA biosynthesis are more susceptible (Cohen, 1993). This mutant is no longer able to induce PIs, therefore more susceptible to tobacco

hornworm (Howe *et al.*, 1996). JA and some related compounds have also been reported that able to induce plant volatiles that involved in indirect defense. For example, in Lima bean, maize and gerbera, treatment of JA and Methy (-)-jasmonate (MeJA) results in plants emission of herbivore-induced volatiles (Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002; Van Poecke and Dicke, 2004).

JA is the most important product of octadecanoid pathway (i.e. lipoxygenase pathway). Lipoxygenase is one of the first enzymes in the pathway, and mediate biosynthesis of JA. A Lipoxygenase gene, *BoLOX* has been cloned from the *Brassica oleracea*. It has been shown that that transcript levels of this *BoLOX* gene are strongly upregulated in response to feeding damage by locusts (*Schistocerca gregaria*), spider mites (*Tetranychus urticae*) and three species of caterpillars, including one generalist and two specialists (*Pieris rapae, P. brassicae, Mamestra brassicae*), and in response to infection with the pathogenic bacterium *Pseudomonas syringae* (Zheng *et al.*, 2007). Sequence analysis showed that *BoLOX* is closely related to *B. napus BnLOX2fl* and *Arabidopsis thaliana AtLOX2*, which mediates JA biosynthesis. Therefore, *BoLOX* gene can be used as a potential indicator of JA synthesis on *Brassica oleracea*.

Salicylic acid (SA) is mostly induced by biotrophic pathogens attraction (Thomma *et al.*, 2001; Dicke and Van Poecke, 2002). However it has also been reported involve in herbivores infestation. For example, an increase of SA has been detected in cotton under the herbivory by larva of *Helicoverpa zea*. Methyl SA (MeSA) accumulation is also detectable in several plant species under herbivore infestation. It is thought that SA blocks JA antagonistically, however, perhaps the production of SA and induction of plant volatiles by JA are separated spatially and/or temporally (Van Poecke, 2002; Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002).

Besides JA and SA, plant hormone ethylene has also reported play its roles in plant defense signaling, against both pathogens and herbivores (Dicke and Van Poecke, 2002; De Vos *et al.*, 2006). Ethylene and SA have been shown to act synergistically,

enhancing the accumulation of PR proteins (Buchanan *et al.*, 2000). For example, cooperate with SA, gaseous ethylene helps with the accumulation of *PR1* mRNA, but application of ethylene alone to tobacco leaves is not sufficient to induce the *PR1* gene. In Arabidopsis, induction of the defensin *PDF 1.2* gene transcript is regulated by defense signaling cascades that require ethylene. (Buchanan *et al.*, 2000). De Vos *et al* (2006) showed herbivore-induced ethylene primes the Arabidopsis leaf for augmented SA-dependent defenses, thereby providing an enhanced defensive capacity toward Turnip crinkle virus (TCV). Furthermore, acting in concert with JA, ethylene induces PI in tomato (O'Donnell *et al.*, 1996). Some experiments indicated ethylene action is downstream from JA in the wound response pathway, and they induce each others production (O'Donnell *et al.*, 1996; Dicke and Van Poecke, 2002).

In addition to plant hormones, a very unique signal molecule is systemin. Systemin is an 18-amino acid polypeptide processed from prosystemin, and it has been identified in tomato as potent inducer of PIs. Until now on, systemin and its homologues have only been found in Solanaceae plants, like tomato, potato, pepper and so on (Buchanan *et al.*, 2000). The exogenous application of systemin on tomato induced accumulation of PI proteins and mRNA, while knocking-down of prosystemin gene results in less PIs and more susceptible to *Manduca sexta* larvae. Wounding of plants induces the systemic accumulation of systemin in tomato, and it is transported from wound site throughout the plant within 90 minutes. However, recently grafting experiments using a JA biosynthesis mutant (*spr2*) and a JA signalling mutant (*jai1*) demonstrated that JA, rather than systemin is the long-distance mobile wound signal for systemic defence responses. Systemin is upstream component of an intercellular signaling cascade that requires the biosynthesis and action of JA (Howe, 2004).

1.4 Study system: herbivores, parasitoids and plants

Our model for the study of tritrophic system is consisting of *Cotesia* parasitoid (*C. rubecula* and *C. glomerata*), *Pieris* herbivore (*P. rapae* and *P. brassicae*) and host plant *Brassica oleracea*.

The small cabbage white butterfly (*Pieris rapae*) and large cabbage white butterfly (*P. brassicae*) are perhaps the most destructive of all butterflies (Chinery, 1993). Small cabbage white butterfly has pale green caterpillars, and caterpillar of the large cabbage white butterfly is yellow and black. Both two caterpillars destroy cabbages and other cruciferous plant. Small White distributes nearly all over the world, while Large White is less widely distributed (Feltwell, 1982; Chinery, 1993).

The genus *Cotesia*, belonging to the family of Braconidae, includes nearly 1000 species worldwide (Michel-Salzat and Whitfield, 2004). Many of these species, including *C. congregata*, *C. kariyai*, *C. rubecula* and *C. glomerata*, are frequently used as biological control agents in agricultural pest management because they are important parasitoids of numerous herbivorous insects. *C. glomerata* is a gregarious larval endoparasitoid of the small and large white butterflies, but prefers the latter. *C. rubecula* is a solitary parasitoid of small white butterflies (Agelopoulos *et al.*, 1994; Michel-Salzat and Whitfield, 2004).

When larvae of *P. rapae* or *P. brassicae* attack *Brassica oleracea* plants, plants release a blend of volatiles as cues for oriented host searching of female parasitoids, *C. glomerata* and *C. rubecula*. Parasitoids lay their eggs in herbivores; therefore the parasitized herbivores will die before their pupation (Agelopoulos *et al.*, 1994).

Superparasitism, *i.e.* oviposition on parasitized hosts (by a conspecific parasitoid), can result in nutrition lack of parasitoid's offspring. Hence the discrimination between parasitized and unparasitized host is in favor of the optimization of foraging efficiency (Fatouros *et al.*, 2005). Fatouros *et al* (2005) demonstrated that parasitoid *Cotesia* wasps are able to detect whether their host contains competitors (i.e. has been

parasitized by another *Cotesia*) or not based on the different plant volatiles induced by the parasitized and unparasitized larvae of *Pieris*. It is a crucial ability to enhance their reproductive success and parasitoids can save energy and time in finding suitable hosts. They showed according to the volatile cues, *C. rubecula* females are able to distinguish parasitized and unparasitized *P. rapae* (parasitized by *C. rubecula*), as well as parasitized and unparasitized *P. brassicae* (parasitized by *C. glomerata*). *C. glomerata* females prefer unparasitized *P. brassicae* than parasitized (parasitized by *C. glomerata*) ones. However, *C. glomerata* females are not able to distinguish parasitized or unparasitized *P. rapae* (parasitized by *C. rubecula*).

1.5 Interested genes selected for this study

Specific cabbage genes from different signal transduction pathway (e.g. *BoLOX*, *BoPAL*, *BoDEF* and *BoPR1*) were investigated for the transcript levels in response to caterpillar feeding, regurgitant treatments derived from parasitized and unparasitized *Pieris* larvae, and mechanical damage by punching.

BoLOX (lipoxygenase)

The *LOX* gene has been shown to be induced in response to wounding or herbivory in plants such as Arabidopsis and tomato (Bell *et al.*, 1995; Heitz *et al.*, 1997). It has been proved that antisense expression of *LOX* increases herbivore (*M. sexta*) performance in *Nicotiana attenuate* (Halitschke and Baldwin, 2003). In Arabidopsis, *LOX* co-suppression mutant *atlox2* results in reduction of indirect defence (Van Poecke and Dicke, 2002). Further more, *LOX* expression is inducible by exogenous application of JA on *B. oleracea, i.e.* JA treatment resulted in increased expression of *BoLOX* gene (Zheng *et al.*, 2007). Therefore, it and can be used as an indicator of JA accumulation on Brussels sprouts plants.

BoPAL (phenylalanine ammonia-lyase)

In plants, phenylalanine ammonia lyase (PAL) is a key enzyme and catalyzes the first step in the phenylpropanoid pathway. Phenylpropanoid pathway leads to the biosynthesis of a wide variety of natural products based on the phenylpropane skeleton. It has been indicated that this activity is often stimulated by microbial infection, resulting in the synthesis of wall-bound lignin- or suberin-like material and phenylpropanoid-derived phytoalexins. In addition, it has been proved that exogenous SA treatment resulted in increased expression of *PAL* gene that participates in resistance in plants (Lee *et al.*, 1992; Galis *et al.*, 2004). Therefore, *PAL* gene can be used as an indicator of SA accumulation in plant.

BoDEF (plant defensin)

Plant defensins are small, cysteine-rich peptides that accumulate at the periphery of plant plasma membrane and are frequently found in dry plant seeds. They are induced during the defense response in plants (Buchanan *et al.*, 2000). An Arabidopsis defensin gene (*PDF1.2*) encodes a plant defensin and protects plant against fungal pathogens. By studying the effect of exogenously applied plant defense regulators and through the use of plant mutants in defense signaling pathways, it has been established that induction of the *PDF1.2* gene in Arabidopsis by fungal pathogens is independent of SA. Instead, induction of the *PDF* gene most probably involves components of MeJA and ethylene response pathways (Mitter *et al.*, 1998). We would like to investigate expression patterns of cabbage defensin gene *BoDEF* (BoDEF encoded a predicted protein of 79 amino acids and showed 87 % amino acid sequence identity with Arabidopsis PDF1.2; S-J. Zheng and M. Dicke, unpublished data) under feeding of *P. rapae* and *P. brassicae*.

BoPR1 (pathogenesis-related protein1)

PR1 is pathogen-related (PR) protein which inducible by various plant pathogens in many plant species. However detailed roles of PR1 in plant defense are still

unknown. The accumulation of PR1 is involved in SA signal. Exogenous application of SA resulted in increase in PR-1 transcript levels (De Vos *et al.*, 2006). Therefore *PR1* gene is frequently used as an indicator of SA. Ethylene also helps with the accumulation of *PR1* mRNA, cooperating with SA. However, exogenous application of ethylene alone to tobacco and Arabidopsis leaves is not sufficient to induce the *PR1* gene (Buchanan *et al.*, 2000; De Vos *et al.*, 2006).

1.7 Questions addressed in this thesis

In the Arabidopsis study, it has been argued that both the SA and the octadecanoid pathways are probably involved in the herbivore-specific responses (Van Poecke *et al.*, 2003). Several genes induced by *P. rapae* infesting have been documented in Arabidopsis (e.g. *AtLOX;* van Poecke *et al.*, 2001); it suggests that their orthologous genes in *B. oleracea* could play the same role. Furthermore, it has been demonstrated that *C. rubecula* and *C. glomerata* are able to avoid superparasitism and discriminated their suitable (unparasitized) host, by the cue of cabbage plant volatiles (Fatouros *et al.*, 2005).

Based on the results mentioned above, we are interested in the molecular aspect of tritrophic interactions among parasitoid *Cotesia*, herbivore *Pieris* and host plant *Brassica oleracea* (Brussels sprouts), especially the differential expression pattern of plant genes under the disoperation from parasitized and unparasitized *Pieris* larvae. For these purposes, 1) a two-choice bioassay was performed by behaviour test of *C. rubecula* in a wind tunnel set-up, to prove whether *C. rubecula* could really discriminated parasitized and unparasitized hosts by the different plant volatiles; 2) differential expression patterns of several genes involved in different signal transduction pathways (e.g. *BoLOX, BoPAL, BoDEF, BoPR1*) were investigated by RT-PCR.

2 Material and methods

2.1 Plants and insects

Brussels sprouts plants (*Brassica oleracea* var. gemmifera cv. Cyrus) were grown in a greenhouse Unifarm of Wageningen University (20-30 ºC, 50-70% RH, 16L: 8D). Plants of 5 to 6 weeks old were used for the experiments.

For behavior test, *P. rapae* and *P. brassicae* caterpillars (both parasitized and unparasitized) were reared on *B. oleracea* plants in a climate room (21±1ºC, 50-70% RH, 16L: 8D). For the molecular analysis caterpillars were reared on Brussels sprouts plants under greenhouse conditions (20-30 ºC, 50-70% RH, 16L: 8D), except the *P. rapae* parasitized by *C. glomerata* (and its corresponding unparasitized contrast). They were reared in climate room condition.

The parasitoids *C. rubecula* and *C. glomerata* were reproduced through parasitization of *P. rapae* and *P. brassicae* larvae, respectively. The *P. rapae* and *P. brassicae* larvae for wasps' reproduction were reared on Brussels sprouts plants under greenhouse conditions (20-30 ºC, 50-70% RH, 16L: 8D).

Cocoons of *C. rubecula* and *C. glomerata* were reared in cages in a climatic chamber (23±1ºC, 50 – 70% RH, 16L: 8D). Once eclosion, the wasps were provided with water and honey. The wasps are not contacted with either plants or larvae before bioassays. They are referred to as naive wasps.

2.2 Plants for parasitoid behaviour assays

For the two-choice bioassay, 20 L1 *P. rapae* caterpillars were parasitized by 3~7 days-old *C. rubecula* females, and subsequently was reared on cabbage plants for 48 hours in a climate room (21±1ºC, 50-70% RH, 16L: 8D). To increase the ratio of parasitization, each caterpillar was observed to be stung by a wasp. Individual wasp is

used to parasitize no more than 10 *P. rapae* larvae. At the same time, 10 unparasitized L1 caterpillars were reared on cabbage under identical circumstances.

After parasitized larvae feeding plants for 2 d, 10 potential parasitized larvae were transferred into 5-6 weeks old Brussels sprouts plant for infestation. The larvae can move through the whole plants freely, without any limitations. Another 10 unparasitized larvae were placed on other plant with the same age. After 48 h, the infested plants were used in the bioassay to investigate volatiles preference of parasitoids in wind tunnel.

2.3 Windtunnel bioassays

Windtunnel bioassays were based on the method described by Geervliet *et al.* (1994). Experiments were performed in a wind tunnel set-up (25±5ºC, 50-70% RH, 0.7 kLUX), with a wind speed of 0.2 m/s. Two plants, infested by parasitized and unparasitized *P.rapae* larvae respectively, are used as odor sources. The plants were placed at the upwind end of the wind tunnel. Plant positions were changed from left to right after 5 wasps tested, to compensate for unforeseen asymmetric effects. Naive wasps were transferred to the centre of the release cylinder in the wind tunnel, which was 60 cm downwind of the odour sources. Wasp transport was taken place by infested plant leaves, parasitized and unparasitized alternately.

After release, the flying of the wasp was observed. If wasp landing on one of the odor sources in 10 minutes, and stood on the plant for more than 30 seconds, it was recorded as response. Two different responses were discriminated: “local” and “systemic”. Local choice was refer to wasp landing on the infested leaf and systemic refer to a landing of uninfested leaf. If wasp did not landings one of the odor sources in 10 minutes, it was recorded as no response. χ^2 test (chi-square test) was used in data analysis. No more than 20 wasps were released in an experimental day.

After wind tunnel test, parasitized caterpillars were reared on cabbage plants until the forth instar, and dissected by needles to examine ratio of parasitization.

2.4 Plants for gene expression studies

Based on the fact that the amount of feeding could not be under control, in the gene expression analysis we used leaves in which a standardized amount of artificial damage and regurgitant were applied. The way of regurgitant collection is the same as described by Fatouros *et al.*(2005). Briefly, regurgitant was collected from forth instar larvae of *P. rapae* and fifth instar larvae of *P. brassicae*, both parasitized and unparasitized larvae. According to my observation, 1st instar *P. rapae* larvae take 7 to 8 days to be forth instar after *C. rubecula* parasitizing, while 1st instar larvae of *P. brassicae* take 11 to 12 days to grow to fifth instar after *C. glomerata* parasitizing. 1st instar *P. rapae* larvae take about 13 to 14 days to grow to fourth instar after parasitizing by *C. glomerata*. Regurgitant droplets were collected by 5 µl glass capillary tube, and immediately put in separate vials on ice. All larvae were then dissected by needles before regurgitant was pooled to ensure that they were indeed parasitized.

Three tiny punches (ca. 0.5 mm^2) were made on the youngest expanded leaf of the 5-6 weeks old plant by a pin needle within the area of 2.5 cm in diameter (indication in the Table 1). After punching, 3 µl of collected regurgitant droplets were applied on these mechanical damaged leaves (1 µl of regurgitant for each punched hole). Three different pairs of larvae were used to collect regurgitant. They were *P. rapae* parasitized or unparasitized by *C. rubecula*; *P. brassicae* parasitized or unparasitized by *C. glomerata* and *P. rapae* parasitized or unparasitized by *C. glomerata.* The other treatments were included in the experiments and listed in the Tab. 1. They were leaves from intact plants without any treatment (-); 1st instar larva of *P. rapae* feeding for 24 h (+); mechanical damage by needle punching and 1 hour feeding by a 1st instar larva of *P. rapae* (the larva removed after 1 h feeding). After 2, 6, 24 h of different treatments, leaf disks with size of 2.5 cm in diameter were harvested by punching, and freezed in liquid nitrogen immediately for later RNA isolation.

Table. 1 Lists of different treatments in gene expression studies

-	+	Punching			1st larva feeding for 1 hour			3µl regurgitant from unparasitized larva			3µl regurgitant from parasitized larva		
1	2	3	4	5	6	7	8	9	10	11	12	13	14
0h	24h	2h	6h	24h	2h	6h	24h	2h	6h	24h	2h	6h	24h
													

2.5 RNA isolation from cabbage leaves

100-200 mg of plant material was harvested by punching, and freezed in liquid nitrogen immediately. The freezing samples were grinded into a fine powder in a 2 ml Eppendoff tube. 750 µl of RNA extraction buffer (100 mM Tris pH8.5, 100 mM NaCl, 20 mM EDTA, 1% Sarkosyl) with ß-mercaptoethanol (7 µl per 1ml extraction buffer) was then added. Following vigorously vortexing, 750 µl of buffer-saturated phenol was applied and samples were vortexed again. After centrifuge at 14000 rpm for 15 min, aqueous phase (about 600 µl) was transferred to a fresh 1.5 ml Eppendoff tube. 500 µl of phenol/chloroform (1:1) was added into aqueous phase, and simples were centrifuged again at 14000 rpm for 10 min. Aqueous phase was then transferred again into a fresh 1.5 ml Eppendoff tube, afterwards 700 µl RNAase free 2-propanol was added and mixed well. The mixed samples were kept at -80 °C for 15 minutes and then centrifuged at 14,000 rpm for 10 minutes at 4 °C. The supernatant was removed and pellet was dissolved in 500 µl of DNase / RNase-free DEPC-treated water. Then 500 µl 4 M LiCl was added and the samples were kept on ice for overnight at 4°C cold room. Overnight samples were spun down at 14,000 rpm for 10 minutes at 4 °C. The supernatant was discarded and RNA pellet was dissolved in 400

µl of DNase / RNase-free DEPC-treated water. And then RNA was precipitated by adding 40 µl of 3 M NaAc and 1 ml 96% ethanol. The tubes were kept at -80 °C for 10 minutes and centrifuged at 14,000 rpm for 5 minutes at 4 °C. Finally, RNA pellets were dried on air, and then dissolve in DNase / RNase-free DEPC-treated water. The concentration of RNA was measured with a photometer BIO-RAD SmartSpec™3000. DNA samples can be stored at -80°C or converted into cDNA immediately.

2.5 cDNA synthesis and RT-PCR

5 µg mRNA from treated plant leaves were used to synthesize first-strand cDNA. 20 µl (total 5 µg) mRNA was incubated with 2 µl oligo-dT primer and 2 µl dNTP for 5 min in a 65°C thermo-cycler. Then, the sample was chill on the ice and spin down by centrifuge. Afterwards, 8 µl first-strand buffer primer, 4 µl 0.1 M DTT, 2 µl RNase OUT (Invitrogen) and 2 µl M-MLV reverse transcriptase (Invitrogen) were added into mixture into a total volume of 40 µl. The mixture was incubated at 25 °C for 10 minutes and 37 °C for 50 minutes. Finally, reaction was terminated by incubating at 70 °C for 15 minutes.

PCR reaction was performed in a total volume of 25 µl with 18.8 µl water, 2.5 µl 10x PCR buffer (Invitrogen), 0.5 µl dNTPs, 0.5 µl of each primer and 0.2 µl Super *Taq* polymerase (Invitrogen) and 1 µl of first-strand cDNA. The PCR products were evaluated by agarose gel electrophoresis. As loading control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used a housekeeping gene. The prime pairs of selected genes in RT-PCR analysis was listed in the Tab. 2.
The touchdown PCR program used for *BoLOX*, *BoPAL* and *BoPR1* gene expressions was 2 min at 94°C; 5 cycles of 30 sec at 94°C, 3 min at 72°C; 5 cycles of 30 sec at 94°C, 30 sec at 70°C, 3 min at 72°C; 15 cycles of 30 sec at 94°C, 30 sec at 68°C, 3 min at 72°C. The gene-specific primers designed here produced overlapping products 1030 bp, 639 bp, and 150 bp for *BoLOX*, *BoPAL* and *BoPR1*, respectively. Touchdown PCR was also used for the housekeeping gene *GAPDH* and target gene *BoDEF*. The PCR program was 2 min at 94°C; 5 cycles of 30 sec at 94°C, 3 min at

72°C; 5 cycles of 30 sec at 94°C, 30 sec at 70°C, 3 min at 72°C; 10 cycles of 30 sec at 94°C, 30 sec at 68°C, 3 min at 72°C. The gene-specific primers produced overlapping products 299 bp, 156 bp, for *GAPDH* and *BoDEF*, respectively.

Table 2. Lists of primers used in RT-PCR

Gene	Forward (from 5' to 3')	Reverse (from 5' to 3')
BoDEF	CACCCTTCTCTTCGCTGCTCTTGTTG	TGTGCTCCTTCAAGTCGAATGCACTG
BoLOX	GGAGTGGTCCACAGTCAAGGGCACTG	CCCCCTGCTGATGAGGTCTGCAGGTA
GAPDH	CACTGACAAGGACAAGGCTGCTGCT	CGGCTCTTCCACCTCTCCAGTCCTTC
BoPAL	ATGGCTCGGCCCTCAGATCGAAGTG	TCAACGACCTTAAGCAAGTC
BoPR1	TCATTTACTGTTCTCGACTTC	CGTCCCACTGCACGGGACCTACG

3 Results

3.1 The preference of *C. rubeculla* parasitoids

Windtunnel bioassays were carried out in 8 independent experiments for overtime period of 3 months. There was a clear trend that *C. rubecula* females preferred plants infested by unparasitized *P. rapae* caterpillars over the plants infested by parasitized ones in independent experiments although the size of feeding by different state of larvae were not always the same. The parasitization rate of *P. rapae* larvae varied from 50 to 90 % depending on individual experiment (Tab. 3). Our accumulated data show that much more *C. rubecula* females (43.4 vs 27.2 % wasps) landed on plants infested by unparasitized *P. rapae* caterpillars than the plants infested by parasitized ones (Fig. 4; P=0.02). The total responses of wasps (70.6 %) to infested plants were quite high compared to 29.4 % wasps with no response (Tab. 3).

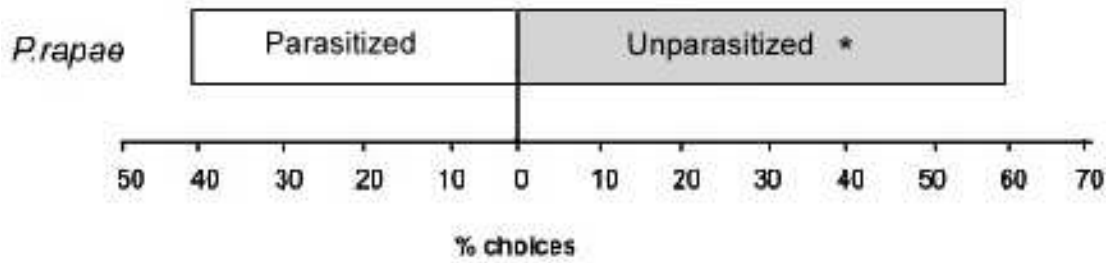

Fig. 1. Response of *C. rubecula* female wasps to cabbage plants infested with unparasitized and parasitized *P. rapae* larvae.

Table 3. The behaviour performance of *C. rubecula* and parasitization rate of *P. rapae* larvae in 8 independent experiments. The numbers in brackets were the percentage of wasps' response or no response to different treated plants.

Exp.	Total tested wasps	Number of no responding wasps	Number of wasps landing on plants infested with unparasitized larvae	Number of wasps landing on plants infested with parasitized larvae	Percentage of parasitization
1	20	6	9	5	85
2	20	6	9	5	60
3	20	3	9	8	90
4	20	5	9	6	50
5	13	4	6	3	50
6	12	2	5	5	50
7	20	9	8	3	70
8	11	5	4	2	60
Total	136	40 (29.4 %)	59 (43.4 %)	37 (27.2 %)	

3.2 Analysis of gene transcript patterns

RT-PCR analysis shows that accumulation of *BoLOX* transcript levels were most abundant in one larva feeding for 24 h both by *P. rapae* or *P. brassicae* (lane 2 in Fig. 2A, 2B and 2C). There are no obvious accumulations of *BoLOX* transcripts in intact leaf (lane 1 in Fig. 2A, 2B and 2C). Herbivorous *Pieris* larva initial feeding for 1 h and regurgitant treatments both from parasitized and unparasitized larva also induced the accumulations of *BoLOX* transcripts although to a lesser content compared to larva feeding for 24 h (lanes 8, 11 and 14 in Fig. 2A, 2B and 2C). Surprisingly, tiny mechanical damage by 3 punching also showed the lower level accumulations of *BoLOX* transcripts compared to two types of regurgitant treatments (lane 5 in Fig. 2A,

2B and 2C). Furthermore, plants treated by regurgitant from parasitized larvae of *P. rapae*, had a higher *BoLOX* transcript levels and also faster accumulations of *BoLOX* RNA than unparasitized larvae in some cases (lanes 9-11 and 12-14, Fig. 2A). In contrast, *BoLOX* transcripts showed a lower levels when plants were applied with regurgitant from parasitized larvae of *P. brassicae*, compared with regurgitant treatments by unparasitized larvae (lanes 9-11 and 12-14, Fig. 2B). The third experimental series was made by *P. rapae* parasitized or unparasitized by *C. glomerata*. In this experimental series, *BoLOX* transcripts did not show a differential accumulation patterns (lanes 9-11 and 12-14, Fig. 2C). In general, *BoLOX* transcripts are at higher level on 24 h post treatments (in lanes 5, 8, 11 and 14, Fig. 2). All these 3 experimental series were repeated at least 4 times, and similar results were given for *BoLOX* transcript patterns.

Besides *BoLOX* transcript levels were analyzed, the accumulation patterns of *BoPAL* transcripts were also investigated in detailed because phenylalanine ammonialyase (PAL) catalyzes the first step in the biosynthesis of phenylpropanoids. The accumulation of *BoPAL* transcripts has a higher abundant at 6 h post treatments after punching damage, initial larva feeding for 1 h and regurgitant treatments both from parasitized and unparasitized larva (Lanes 4, 7, 10 and 13, Fig. 2). Compared to having a higher levels of *BoLOX* transcripts at 24 h post treatments (lanes 5, 8, 11 and 14, Fig. 2), *BoPAL* transcript levels were usually decreased with time from 6 h to 24 h. These results indicate that *BoPAL* expression patterns are quite different from that of *BoLOX*. In conclusion, the results from RT-PCR analysis suggested *BoLOX* and *BoPAL* interact antagonistically to the treatments mentioned above.

In addition, *BoDEF* and *BoPR1* transcript levels were also investigated by RT-PCR analysis. Although these genes were also upregulated in some treatments, in general, there were no consistent patterns of *BoDEF* and *BoPR1* transcripts in these treatments (Fig. 2A, 2B and 2C). Quantified gene expressions are needed before a solid conclusion can be drawled.

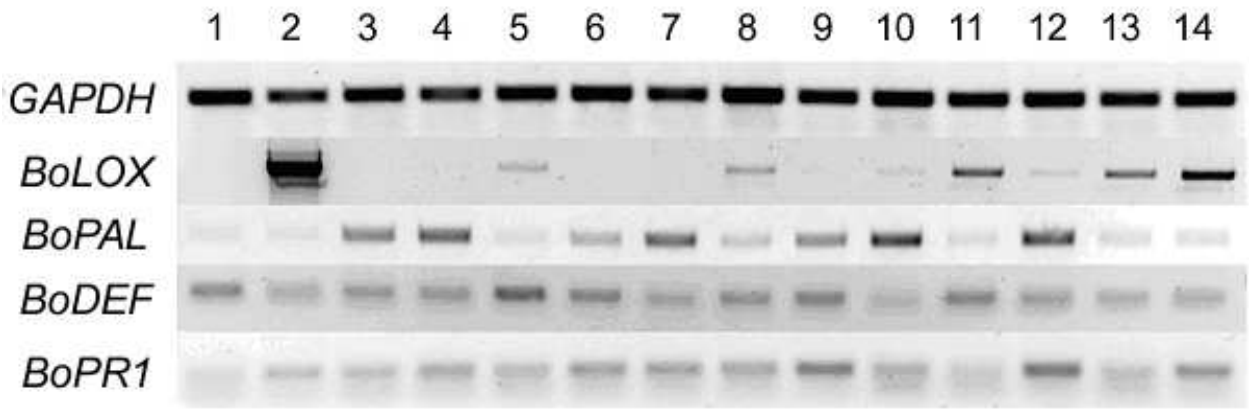

Fig. 2A *P. rapae* parasitized by *C. rubecula*

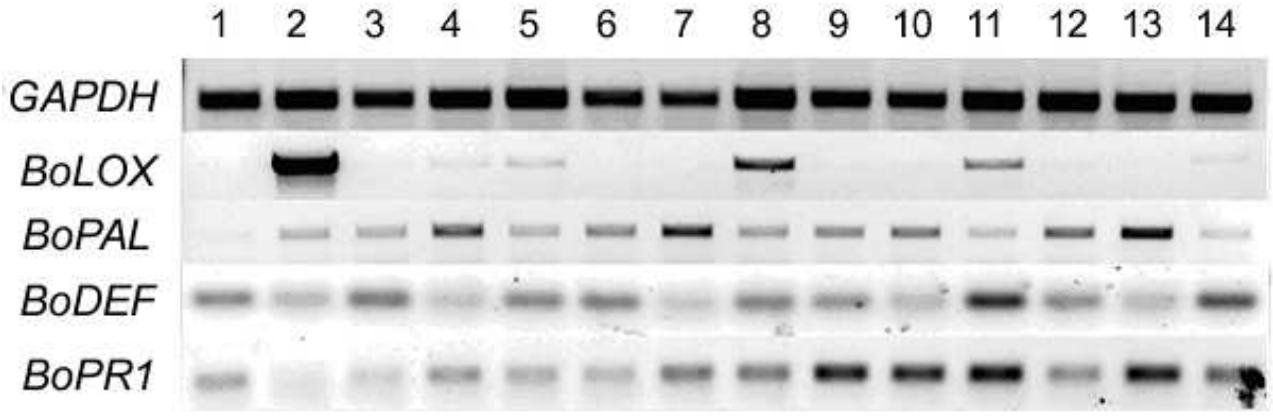

Fig. 2B *P. brassicae* parasitized by *C. glomerata*

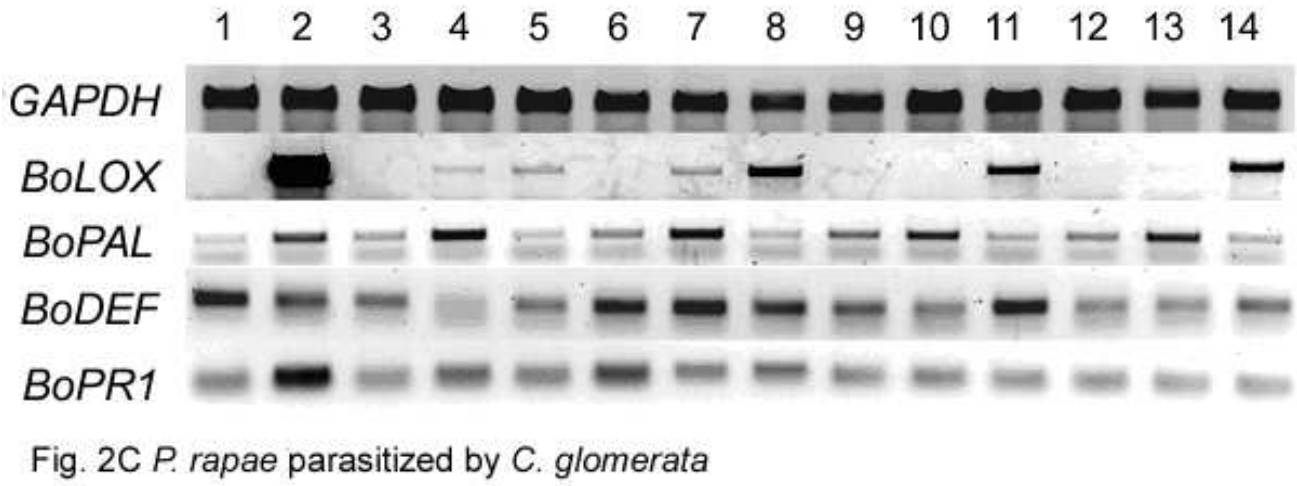

Fig. 2. Gene expression patterns of *BoLOX*, *BoPAL*, *BoDEF* and *BoPR1* upon herbivous feeding, punching damage and regurgitant treatments from both parasitized and unparasitized herbivorous larvae. Lane 1 is control from intact plant leaf; Lane 2 is from plant leaf under 24 h feeding of one 1st instar herbivous larva, *P. rapae* (A and C) or *P. brassicae* (B); Lanes 3-5 represent 2, 6, 24 h after damaging leaf by punching; Lanes 6-8 represent 1 h initial feeding by one 1st instar herbivous larva (*P. rapae* or *P. brassicae*) and 2, 6, 24 h after removing larva; Lanes 9-11 represent 2, 6, 24 h after application of regurgitant from unparasitized larvae; Lanes 12-14 represent 2, 6, 24 h after application of regurgitant from parasitized larvae. A, B and C denote 3 types of experimental series, i.e. *P. rapae* parasitized by *C. rubeculla*; *P. brassicae* parasitized by *C. glomerata* and *P. rapae* parasitized by *C. glomerata*, respectively. Each series consists of at least 4 independent experiments.

4 Discussion

4.1 *C. rubeculla* wasps can discriminate different state of infested plants

The results from this study clearly show that *C. rubecula* females without oviposition experience could discriminate suitable hosts; unparasitized over parasitized (Tab. 2 and Fig. 1). The outcome consists with the results of Fatouros *et al.* (2005). They infested plants by the larvae after 4 days parasitization, while larvae after 2 days parasitization were used in this study. In this way, the difference in feeding damage between unparasitized and parasitized larvae could be reduced. It is critical for *C. rubecula* females as solitary endoparasitoid to have host discrimination because supernumerary larvae compete to the extent that only a single parasitoid can emerge (Salt, 1961). The possible reason for *C. rubecula* discriminating unparasitized over parasitized host is due to the volatiles cues emitted by different states of plants response to herbivorous larvae feeding. Fatouros *et al.* (2005) demonstrated that cabbage plants treated with regurgitant of parasitized *P. brassicae* caterpillars emitted lower amounts of volatiles than plants treated with unparasitized caterpillars by gas chromatography-mass spectrometry (GC/MS) analysis of headspace odors. The odor differences between plants treated with regurgitant of parasitized *P. rapae* caterpillars and unparasitized caterpillars in this study need to be investigated in the future.

4.2 *BoLOX* transcripts are accumulated by herbivores and mechanical damages

The dynamic expression patterns of *BoLOX* under punching mechanical damage and herbivory infestation were investigated with RT-PCR. Plant materials were harvested on different time points, i.e. 2, 6 and 24 h after treatments. The results of RT-PCR show that transcript levels of *BoLOX* were significantly upregulated from undetectable levels to very high levels in 24 h. These upregulations can be triggered by feeding of two different caterpillar species (*P. rapae* and *P. brassicae*), regurgitant treatment (both parasitized and unparasitized caterpillar of two species), as well as mechanical damages (needle punching). The accumulation of *BoLOX* transcript levels

by two caterpillar feeding (*P. rapae* and *P. brassicae*), was similar to the results of Zheng *et al.* (2007). Based on these results, *BoLOX* and its final product JA in the jasmonate pathway may play important roles in regulation of plant defenses against both herbivores and mechanical wounding.

However, induction levels of *BoLOX* transcripts by mechanical damage were much lowered than by herbivory or regurgitant treatment. This finding suggests that elicitors in herbivore regurgitant are very important for *B. oleracea* plants to detect caterpillar attacks. In the phytopathology research, people found the fungal, bacterial and oomycetes pathogens secrete elicitors (avirulence proteins, AVRs and extracellular proteins, ECPs), interacting with plant resistance (R) proteins, triggering downstream signaling and leading to resistance. In plant-herbivore interactions, similar things could happen too. Herbivore elicitors have their receptors in plants. These receptors act upstream of *BoLOX*, interacting with insect elicitors, triggering downstream JA / ethylene pathways and resulting both direct and indirect defenses. Nowadays, a number of insect elicitors have been isolated and characterized, e.g. β-glucosidase from *P. brassicae* (Mattiacci *et al.*, 1995). It will be a very interesting topic to find such receptors in plants.

4.3 *BoLOX* gene could be employed as an indicator in mutant selection

To investigate the receptors, a reverse genetics strategy can be employed, i.e. making mutants and finding interesting phenotypes. Mutants can be random by EMS, T-DNA or transposon, or targeted towards selected interesting genes by RNAi. Interested “phenotypes” mean plant lost (partly) its capability of direct and / or indirect defense against herbivores. The capability of direct defense can be evaluated by expression levels of systemic wound response protein (SWRP) genes, e.g. *PIN* gene (proteinase inhibitor). However, evaluation of indirect defense can not be performed by behavior analysis in wind tunnel, because that is too much works for mutant selection. Therefore, *BoLOX* can be employed as an excellent indicator for mutant selection. Selection of interested mutants can be depended on expression

pattern of *BoLOX* after herbivory treatment. Based on the fact that *BoLOX* act downstream of receptor genes and inducible by herbivory, we expect knocking out/down of upstream receptor or intermediate genes can result in an "uninducible" of *BoLOX* under herbivory. "Uninducible" means in mutant lines, induction levels of *BoLOX* by herbivory were as same as by mechanical damage or even undamaged plants. Once some mutant lines are obtained by *BoLOX* expression analysis, the further works can be done to exam their capability of direct and / or indirect defenses.

Comparing with random mutagenesis, goal-directed mutagenesis targeting towards selected interesting genes will increase efficiency of finding interesting mutants. Recently, Stulemeijer *et al.* (2007) showed that tomato *MPKs* play roles with regard to hypersensitive response (HR) and resistance against *Cladosprium*; meanwhile Kandoth *et al.* (2007) demonstrated the same genes are also required for defense against herbivorous insects *M. sexta*. These results imply that some genes may have similar functions in defense against both pathogens and insects. Leucine-rich repeat (LRR) receptor-like proteins (RLPs) and LRR receptor-like kinases (RLKs) are two big gene families in plant genome. In plant, both RLPs and RLKs located on the membrane of cell and some of them have been shown to play a role in pathogen resistance as *R* genes, e.g. *Cf* (*Cladosprium fulvum disease resistance*) and *Ve* (*Verticillium wilt resistance*) genes in tomato, *Xa21* (*Xanthomonas oryzae resistance*) gene in rice (Kruijt *et al.*, 2005). They may play similar roles in plant-herbivore interaction. Moreover, several intermediate genes have been found in *R* gene mediated defense signaling in plant-microbe interactions, e.g. *EDS1* (*Enhanced disease susceptibility 1*; Aarts *et al.*, 1998), *NDR1* (*Non-race-specific disease resistance 1*; Century *et al.*, 1995), *RAR1* (*Required for Mla12 resistance 1*; Azevedo *et al.*, 2002; Muskett and Parker, 2003), *SGT1*(*Suppressor of the G2 allele of SKP1*, a protein first identified in yeast; Peart *et al.*, 2002; Muskett and Parker, 2003), *NRC1* (*NB-LRR protein required for HR-associated cell death 1*; Gabriëls *et al.*, 2007), *MPKs* (*Mitogen-activated protein kinases* Stulemeijer *et al.*, 2007) and *MEK* (*Mitogen-activated protein kinase kinase*, MAPKK; Gabriëls *et al.*, 2007). It will be

interesting to investigate their roles in plant defense against insects by reverse genetics strategy.

In addition, virus induced gene silencing (VIGS) has been employed as an efficient tools for rapid and high-throughput analyzing of genes involved in plant defense against herbivores. For example, using VIGS, Kandoth *et al.* (2007) showed tomato *MPKs* are required for successful defenses against *M. sexta.* Though VIGS is often preformed on Solanum plants, it is also efficient on cruciferous plants. Cabbage leaf curl virus (CbLCV; Turnage *et al.*, 2002) and tobacco rattle virus (TRV; Burch-Smith *et al.*, 2006) have been successfully used in silencing of the target genes in Arabidopsis. If these viral vectors also initiate the silencing of target genes in *B. oleracea*, it will be a much more rapid method (about 2 to 3 weeks) than RNAi (more than 6 months) for the further studies.

4.4 Differential expression of *BoLOX* between plants treated by parasitized and unparasitized caterpillars

Parasitism can cause a number of changes in the host, particularly in its development, either delaying or accelerating. For example, it can induce precocious development and moulting, increase or reduce host growth rate, terminate host growth, and retard host development. Some parasitoids may even alter a host's behaviour to benefit the wasp themselves (Quicke, 1997). Many of these changes are involved in overcoming host defenses. Parasitism of hosts leads to a reduction in their immune response against foreign objects (Vinson, 1990). Moreover, Fatouros *et al.* (2005) showed that plants reduce the production of specific herbivore-induced volatiles after a successful recruitment of their bodyguards (parasitoids). By the cue of changed plant volatiles, parasitoids can detect whether their hosts contain competitors. This result suggested that physiological changes of host caused by parasitism can also help with host searching of parasitoid.

GC/MS analysis was showed that *B. oleracea* plants treated with regurgitant of parasitized *P. brassicae* caterpillars emitted lower amounts of volatiles than plants treated with unparasitized caterpillars (Fatouros *et al*., 2005). This result clearly implied the parasitism cause some changes in the host, and may result in altering of elicitors in host regurgitant, quantitatively and / or qualitatively. Changed elicitors may influence in plant defense signaling and lead to differential volatiles.

To reveal differential plant defense signaling under treatments of parasitized and unparasitized *Pieris* caterpillars, expression patterns of *BoLOX* were used as indicator of JA and analyzed by RT-PCR. Plants treated by regurgitant from *C. rubecula*-parasitized *P. rapae*, had a higher (and faster in some cases) expression of *BoLOX* than unparasitized treatments. However, regurgitant from *P. rapae* which parasitized by *C. glomerata* did not give these differences. As we know, parasitism of *C. rubecula* leads to significant reducing growth of host herbivore *P. rapae*, while *P. rapae* parasitized by *C. glomerata* is less influent and as similar size as unparasitized one. Furthermore, *C. glomerata* also do not reduce growth of *P. brassicae* larvae compared to unparasitized one. Combining these herbivorous larvae phenotypes with expression pattern of *BoLOX*, it is concluded that parasitism by *C. rubecula* effects drastically on the *P. rapae* caterpillars' physiology, and may result in more concentrated elicitors in the regurgitant of caterpillars. *C. glomerata* can not give same effect on both *P. rapae* and *P. brassicae*. In contrast, parasitism by *C. glomerata* reduces concentration of elicitors in regurgitant of *P. brassicae* in some way. Therefore, this parasitism results lower expression of *BoLOX*, comparing with unparasitized one. Recently, *C. rubecula* has been found also able to parasitize *P. brassicae.* It will be interesting to check *BoLOX* expression pattern under treatment of regurgitant from *P. brassicae*, parasitized or unparasitized by *C. rubecula*. Results from this experiment may offer some new ideas / evidences for above hypotheses.

4.5 Herbivore-induced volatile blends may also involved in other signal molecules

Comparing with regurgitant treatments by unparasitized larvae, *BoLOX* showed a lower expression when plants were applied with regurgitant from *P. brassicae* parasitized by *C. glomerata*. GC/MS analysis also showed that *B. oleracea* treated with regurgitant of parasitized *P. brassicae* caterpillars emitted lower amounts of volatiles than plants treated with unparasitized caterpillars (Fatouros *et al*., 2005). Furthermore, the results from this study and combined from Fatouros *et al*. (2005), collectively show that *C. rubecula* and *C. glomerata* females prefer for landing on leaves treated with regurgitant of unparasitized larvae in the wind tunnel tests. All these seem very logically.

However, *BoLOX* transcript levels showed a higher accumulations when plants were applied with regurgitant from parasitized *P. rapae* by *C. rubecula*. It implied there should be more accumulation of JA and leading to higher amounts of volatiles. But in the wind tunnel tests, *C. rubecula* females still preferred for landing on leaves treated with regurgitant of unparasitized larvae. Unfortunately, GC/MS data of plants treated with regurgitant of unparasitized or parasitized *P. rapae* is not available in this study. Nevertheless, it seems that JA is not the sole signal molecule which involved in this indirect defense. There should be some other signal molecules play roles in the induction of *B. oleracea* volatiles.

Together with JA, ethylene has been proved that is involved in plant defense against necrotropic pathogens, wounding and herbivores (De Vos *et al.*, 2006; Thomma *et al.*, 2001). It is required for accumulation of *PR1, PDF1.2* as well as *PI*. Therefore, it maybe also involve in indirect defenses, i.e. herbivore infestation stimulate accumulation of ethylene in plants. Ethylene, in combination with JA, triggers downstream genes expression, leading to emitting of volatiles and attraction of bodyguards. Examination of expression patterns of genes involved in ethylene production may help us to understand ethylene's roles better. These genes include

ACC synthase gene (*ACS* in Arabidopsis, responsible for the early step in ethylene production; Sato and Theologis, 1989) and ACC oxidase gene (*ACO2* in Arabidopsis, responsible for the last step in ethylene formation; Rodrigues-Pousada *et al.*, 1993). Moreover, GC/MS analysis and parasitoid behavior studies on ethylene mutants, e.g. *eer1* and *eer2* (*enhanced ethylene-response* in Arabidopsis; Larsen *et al*., 2001; De Paepe *et al.,* 2005) and *etr1* (ethylene insensitive mutant of Arabidopsis; Bleecker *et al.*, 1988) may give some evidences for above hypotheses.

In addition, reactive oxygen species (ROS) and Nitric oxide (NO) are considered playing some signaling roles in plant defense against pathogens. Moreover, NO is also a signal molecule used by mammals to regulate various biological processes of immune, nervous, and vascular system (Buchanan *et al*., 2000). Therefore, they may have similar function in plant defense against herbivore, either direct or indirect. SA, ABA and electrical signals are also factors should be considered.

4.6 *BoLOX* and *BoPAL* interact antagonistically

BoPAL transcripts were significantly upregulated at 6 h post treatments, either mechanical damages or herbivory. It was decreased at 24 h post treatments (Fig. 2). Furthermore, *BoLOX* which involved synthesis of JA was significantly upregulated on 24 h post treatments. These results suggested *BoLOX* and *BoPAL* interact antagonistically. Because *PAL* is mainly regulated by SA, it may involve in the negative cross-talking between JA and SA signaling. However, another SA-induced gene, *BoPR1* did not show this antagonistical interaction with *BoLOX*. This maybe involve in cross talking with other signaling molecules, e.g. ethylene (Kato *et al.*, 2000; Buchanan *et al*., 2000). There is no differences of *BoPAL* expression between mechanical damages and herbivory. This result implied that herbivore elicitor may not involve in induction of SA, in case of *Pieris-Brassica* interactions. Furthermore, to be more precisely quantify transcript levels of different genes involved this tritrophic interactions, qRT-PCR is needed in the future study.

5 Summary

This study shows that *C. rubecula* can use *P. rapae*-induced *B. oleracea* volatiles for host discrimination between unparasitized and parasitized in flight. This is a crucial capability for parasitoid to save energy and time for finding suitable hosts, and enhance successful ratio of reproduction. By RT-PCR analysis, molecular mechanism behind plant-herbivore-parasitoid tritrophic interactions was investigated. Due to complex patterns of *BoLOX* and *BoPAL* transcripts, it is concluded that other signal pathways, and cross-talking among these pathways are also involved in induced plant defense. The findings in this study add new level of intricacy to herbivore-plant–parasitoid interactions, as well as defense signal regulation / transduction network in plants. Apparently, signal molecules such as JA and ethylene play an important role in the primary response of plants against insect attack, but the final outcome of the indirect defense reaction is shaped by so far indistinct cross talking and / or unidentified additional factors.

At present, comparing with studies of plant-microbe interactions, our knowledge of herbivore defense is still incomplete. A better understanding of the factors / processes involved in plant defense is needed. The factors include herbivores, plants, and parasitoids, pathogenic and non-pathogenic microbes in plants and even microbes in regurgitant of herbivores. The processes include reorganization of herbivore elicitors, different signaling pathways and their cross talking. High throughput analysis of differential gene expression is helpful to accelerate of our understanding of these complex interactions.

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7 References

Aarts N, Metz M, Holub E, Staskawicz BJ, Daniels MJ and Parker JE. 1998. Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. Proc. Natl Acad. Sci. USA, 95: 10306–10311.

Agelopoulos NG and Keller MA. 1994. Plant-natural enemy association in the tritrophic system, *Cotesia rubecula-Pieris rapae-Brassicaceae* (Cruciferae): II. Preference of *C. rubecula* for landing and searching. J. Chem. Ecol. 20:1735-1748.

Azevedo C, Sadanandom A, Kitagawa K, Freialdenhoven A, Shirasu K and Schulze-Lefert P. 2002. The RAR1 interactor SGT1, an essential component of R gene-triggered disease resistance. Science 295: 2073–2076.

Baldwin IT, Halitschke R, Kessler A and Schittko U. 2001. Merging molecular and ecological approaches in plant-insect interactions. Current Opinion in Plant Biology 4: 351-358.

Bell E, Creelman RA, Mullet JE. 1995. A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proc. Natl. Acad. Sci. USA 92: 8675-8679

Blaakmeer A. 1994. Infochemicals in a tritrophic system, interactions between Brassica, Pieris and Cotesia. PHD thesis.

Bleecker AB, Estelle MA, Somerville C and Kende H. 1988. Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science 241: 1086–1089.

Buchanan BB, Gruissem W and Jones RL. 2000. Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville.

Burch-Smith TM, Schiff M, Liu Y, Dinesh-Kumar SP. 2006. Efficient virus-induced gene silencing in Arabidopsis. Plant Physiol. 142: 21-27.

Century KS, Holub EB and Staskawicz BJ. 1995. *NDR1*, a locus of Arabidopsis thaliana that is required for disease resistance to both a bacterial and a fungal pathogen. Proc. Natl Acad. Sci. USA, 92: 6597–6601.

Chinery M. 1993: Collins Field Guide, Insects of Britain and Northern Europe, 3rd Edition. Harper Collins Publishers, London.

Cohen Y, Gisi U and Niderman T. 1993. Local and Systemic Protection Against Phytophthora infestans Induced in Potato and Tomato Plants by Jasmonic Acid and Jasmonic Methyl Ester. Phytopathology 83:1054-1062

De Paepe A, De Grauwe L, Bertrand S, Smalle J and Van der Straeten D. 2005: The Arabidopsis mutant *eer2* has enhanced ethylene responses in the light. J Exp. Bot. 56(419):2409-20.

De Vos M, Van Zaanen W, Koornneef A, Korzelius JP, Dicke M, Van Loon LC, Pieterse CM. 2006. Herbivore-induced resistance against microbial pathogens in Arabidopsis. Plant Physiol. 142: 352-363.

Dicke M. 1988. Prey preference of the phytoseiid mite *Typhlodromus pyri*: Response to volatile kairomones. Experimental and Applied Acarology 4:1 -13

Dicke M, Van Beek TA, Posthumus MA, Ben Dom N, Van Bokhoven H and De Groot AE. 1990a. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions: involvement of host plant in its production. Journal of Chemical Ecology. 16:381–398.

Dicke M, Sabelis MW, Takabayashi J, Bruin J and Posthumus MA. 1990b. Plant strategies of manipulating predatorprey interactions through allelochemicals: Prospects for application in pest control. Journal of Chemical Ecology 16: 3091-3118.

Dicke M. and Van Poecke RMP. 2002. Signalling in plant-insect interactions: signal transduction in direct and indirect plant defence. In Plant Signal Transduction, eds. D. Scheel & C. Wasternack, pp. 289-316. Oxford University Press, Oxford.

Fatouros NE, Van Loon JJA, Hordijk KA, Smid HM and Dicke M. 2005. Herbivore-induced plant volatiles mediate in-flight host discrimination by parasitoids. Journal of Chemical Ecology 31:2033-2047.

Feltwell J. 1982. Large white butterfly: the biology, biochemistry and physiology of *Pieris brassicae* (Linnaeus), 1st Edition. Series Entomologica (vol. 18). Kluwer Academic Publishers, the Hague.

Gabriëls SHEJ, Vossen JH, Ekengren SK, van Ooijen G, Abd-El-Haliem AM, van den Berg GC, Rainey DY, Martin GB, Takken FL, de Wit PJ and Joosten MH. 2007. An NB-LRR protein required for HR signalling mediated by both extra- and intracellular resistance proteins. Plant J: 50:14-28.

Galis I, Smith JL, Jameson PE. 2004. Salicylic acid-, but not cytokinin-induced, resistance to WClMV is associated with increased expression of SA-dependent resistance genes in *Phaseolus vulgaris*. J Plant Physiol. 161: 459-466.

Halitschke R and Baldwin IT. 2003. Antisense LOX expression increases herbivore performance by decreasing defense responses and inhibiting growth-related transcriptional reorganization in *Nicotiana attenuate*. The Plant Journal 36: 794–807.

Heitz T, Bergey DR, Ryan CA. 1997. A gene encoding a chloroplast-targeted lipoxygenase in tomato leaves is transiently induced by wounding, systemin, and methyl jasmonate. Plant Physiol. 114: 1085-1093

Howe GA. 2004. Jasmonates as Signals in the Wound Response. Journal of Plant Growth Regulation 23: 223-237.

Howe GA, Lightner J, Browse J and Ryan CA. 1996. An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. Plant Cell 8: 2067-2077.

Kandoth PK, Ranf S, Pancholi SS, Jayanty S, Walla MD, Miller W, Howe GA, Lincoln DE, Stratmann JW. 2007. Tomato MAPKs LeMPK1, LeMPK2, and LeMPK3 function in the systemin-mediated defense response against herbivorous insects. Proc. Natl. Acad. Sci. USA. 104: 12205-12210.

Kato M, Hayakawa Y, Hyodo H, Ikoma Y, Yano M. 2000. Wound-induced ethylene synthesis and expression and formation of 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACC oxidase, phenylalanine ammonia-lyase, and peroxidase in wounded mesocarp tissue of *Cucurbita maxima*. Plant Cell Physiol. 41:440-447.

Kessler A and Baldwin IT. 2002. Plant responses to insect herbivory: The emerging molecular analysis. Annual Reviews Plant Biology 53: 299-328.

Kruijt M, Kock M, Wit P. 2005. Receptor-like proteins involved in plant disease resistance. Mol. Plant Pathology 6: 85-97.

Lack AJ and Evans DE. 2001. Plant Biology. BIOS Scientific Publishers Ltd., Oxford.

Larsen PB and Chang C. 2001. The Arabidopsis *eer1* Mutant Has Enhanced Ethylene Responses in the Hypocotyl and Stem1. Plant Physiol. 125: 1061–1073.

Lee SW, Nazar RN, Powell DA and Robb J. 1992. Reduced *PAL* gene suppression in *Verticillium*-infected resistant tomatoes. Plant Molecular Biology 18: 345-352.

Mattiacci L, Dicke M and Posthumus MA. 1995. β-Glucosidase: An elicitor of the herbivore-induced plant odor that attracts host-searching parasitic wasps. Proc. Natl. Acad. Sci. USA 92: 2036-2040

Michel-Salzat A and Whitfield JB. 2004. Preliminary evolutionary relationships within the parasitoid wasp genus *Cotesia* (Hymenoptera: *Braconidae*: *Microgastrinae*): combined analyses of four genes. Syst Entomol 29: 371–382.

Mitter N, Kazan K, Way HM, Broekaert WF and Manners JM. 1998. Systemic induction of an Arabidopsis plant defensin gene promoter by tobacco mosaic virus and jasmonic acid in transgenic tobacco. Plant Science 136: 169-180.

Muskett P and Parker J. 2003. Role of SGT1 in the regulation of plant R gene signalling. Microbes Infect. 5: 969–976.

O'Donnell PJ, Calvert C, Atzorn R, Wasternack C, Leyser HMO, Bowles DJ. 1996. Ethylene as a signal mediating the wound response of tomato plants. Science 274: 1914-1917.

Peart JR, Lu R, Sadanandom A, Malcuit I, Moffett P, Brice DC, Schauser L, Jaggard DA, Xiao S, Coleman MJ, Dow M, Jones JD, Shirasu K, Baulcombe DC. 2002. Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. Proc. Natl Acad. Sci. USA. 99: 10865–10869.

Quicke DLJ. 1997. Parasitic Wasps. Pp. 221. Chapman & Hall Press, London.

Rodrigues-Pousada RA, De Rycke R, Dedonder A, Van Caeneghem W, Engler G, Van Montagu M, Van Der Straeten D. 1993. The Arabidopsis 1-Aminocyclopropane-1-Carboxylate Synthase Gene 1 Is Expressed during Early Development. Plant Cell. 5: 897-911.

Salt G. 1961. Competition among insect parasitoids. Symp. Soc. Exp. Biol. 15: 96-119

Sato T and Theologis A. 1989. Cloning the mRNA encoding 1-aminocyclopropane-1-carboxylate synthase, the key enzyme for ethylene biosynthesis in plants. Proc. Natl. Acad. Sci. USA. 86: 6621-6625.

Shiojiri K, Takabayashi J, Yano S and Takafuji A. 2000. Herbivore-species-specific interactions between crucifer plants and parasitic wasps (Hymenoptera: *Braconidae*) that are mediated by infochemicals present in areas damaged by herbivores. Appl. Entomol. Zool 35:519-524.

Stulemeijer IJ, Stratmann JW and Joosten MH. 2007. Tomato Mitogen-Activated Protein Kinases LeMPK1, LeMPK2, and LeMPK3 are activated during the Cf-4/Avr4-Induced hypersensitive response and have distinct phosphorylation specificities. Plant Physiol. 144: 1481-1494.

Thaler JS, Fidantsef AL, Duffey SS and Bostock RM. 1999. Trade-offs in plant defense against pathogens and herbivores: A field demonstration of chemical elicitors of induced resistance. J. Chem. Ecol. 25: 1597-1609.

Thaler JS, Karban R, Ullman DE, Boege K and Bostock RM. 2002. Cross-talk between jasmonate and salicylate plant defense pathways: effects on several plant parasites. Oecologia 131:227–235.

Thomma BPHJ, Penninckx IAMA, Broekaert WF, Cammue BPA. 2001. The complexity of disease signaling in Arabidopsis. Curr. Opin. Immunol. 13: 63-68.

Turnage MA, Muangsan N, Peele CG and Robertson D. 2002. Geminivirus-based vectors for gene silencing in Arabidopsis. Plant J. 30: 107-114.

Turlings TCJ, Loughrin JH, Mccall PJ, Röse, USR, Lewis WJ and Tumlinson JH. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. Proc. Natl. Acad. Sci. USA 92: 4169-4174

Van Poecke RMP. 2002. Indirect defence of Arabidopsis against herbivorous insects, combining parasitoid behaviour and chemical analyses with a molecular genetic approach. PHD Thesis.

Van Poecke RMP and Dicke M. 2004. Indirect defence of plant against herbivores: Using *Arabidopsis thaliana* as a Model plant. Plant Biology 6: 381-401.

Van Poecke RMP, Posthumus MA, Dicke M. 2001. Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: chemical, behavioral, and gene-expression analysis. J. Chem. Ecol. 27:1911-1928

Van Poecke RMP, Roosjen M, Pumarino L and Dicke M. 2003. Attraction of the specialist parasitoid *Cotesia rubecula* to *Arabidopsis thaliana* infested by host or non-host herbivore species. *Entomologia Experimentalis et Applicata* 107: 229-236.

Vinson SB. 1990. How parasitoids deal with the immune system of their host: an overview. Arch. Insect Bioch. Physio. 13:3-27

Zheng SJ, Van Dijk JP, Bruinsma M and Dicke M. 2007. Sensitivity and speed of induced defense of cabbage (*Brassica oleracea* L.): dynamics of *BoLOX* expression patterns during insect and pathogen attack. Molecular Plant-Microbe Interactions (In press)

Appendix

1. Unstandardized behavior experimental results:

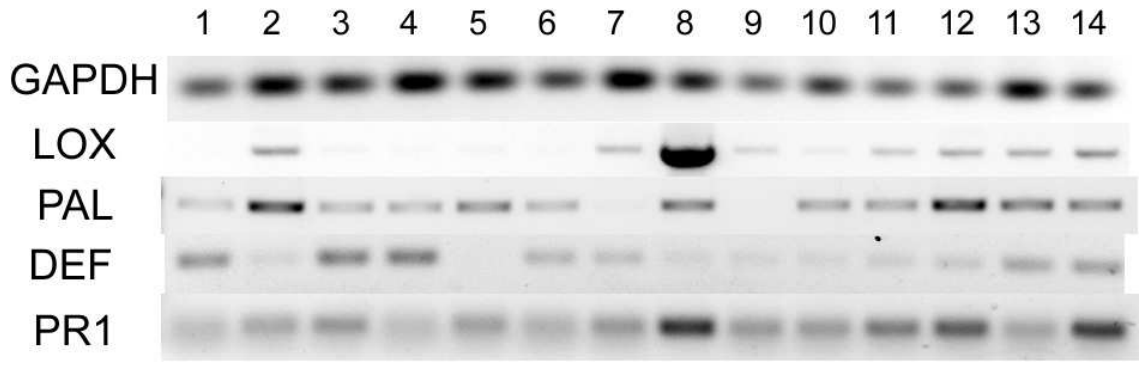
Total	No Choice	Unpara	Para	Remark
21	16	4	1	8 Days para+2~3 days plant treatment, 4 para p.rapae/plant
20	6	7	7	2 Days para+2~3 days plant treatment, 7 para p.rapae/plant (2 of them unparaed)
20	4	9	7	4 Days para+2 days plant treatment,
12	6	2	4	5 Days para+1 day plant treatment
10	2	3	5	0 Days para+2 Days plant treatment
10	4	2	4	5 Days para+1 Days plant treatment
8	4	3	1	1 Days para+1 Days plant treatment
20	4	5	11	1 days para+3 days plant treatment
21	7	10	4	Single leaf used, 1 Days para+3 days plant treatment
10	4	1	5	Single leaf used, 5 Days para+1 Days plant treatment
				Following experiments are done by regurgitant treatment
10	10	0	0	10 days para+24 hours plant treatment
8	8	0	0	24 hours plant treatment
10	7	2	1	5 days para+24hours plant treatment
10	7	1	2	Single leaf used, 5 days para+24hours plant treatment
Total	NoChoice	Unpara	Para	
190	**89**	**49**	**52**	

Statistics:

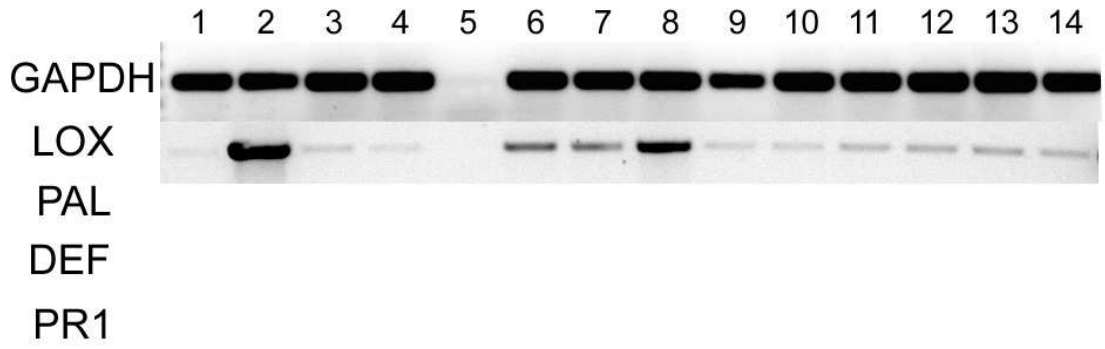
Total	No Choice	Unpara	Para	
79	23	28	28	Plant treatment after 3 days (or longer) parasitization
111	66	21	24	Plant treatment after 2 days (or shorter) parasitization
41	18	12	11	Single leaf experiments
38	32	3	3	Regurgitant treatment

2. All repeats of RT-PCR analysis

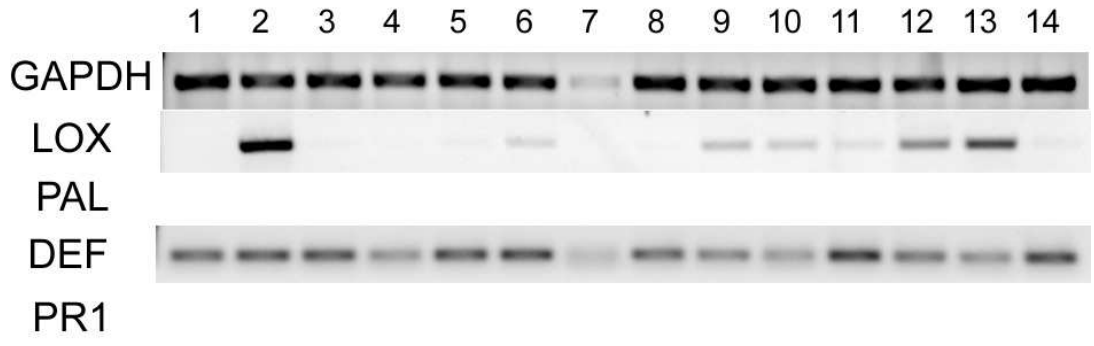
2.1 Series_1 *P. rapae* parasitized by *C. rubecula*



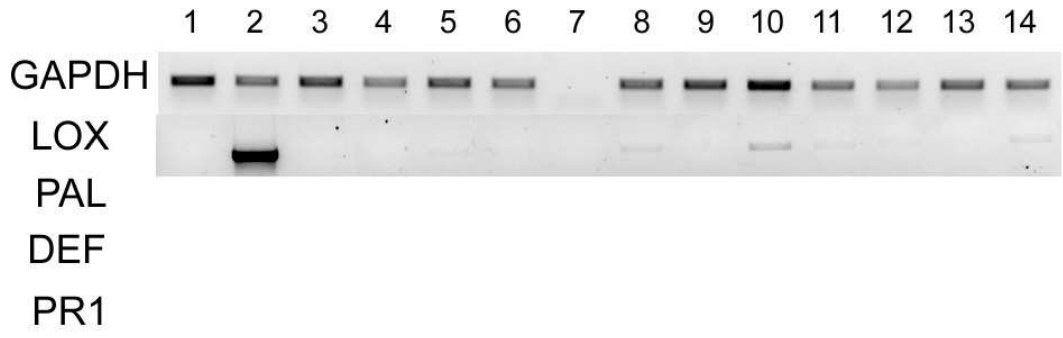
series 1_1th: *P.rapae* parasitized by *C.rubecula*



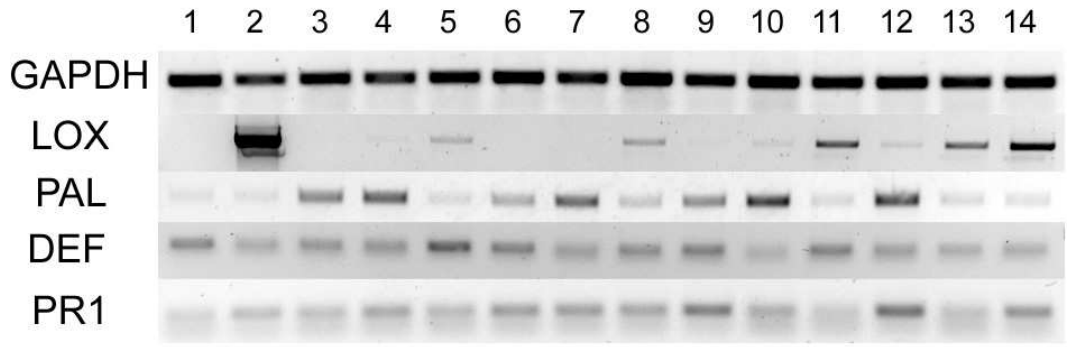
series 1_2th: *P.rapae* parasitized by *C.rubecula*



series 1_3th: *P.rapae* parasitized by *C.rubecula*

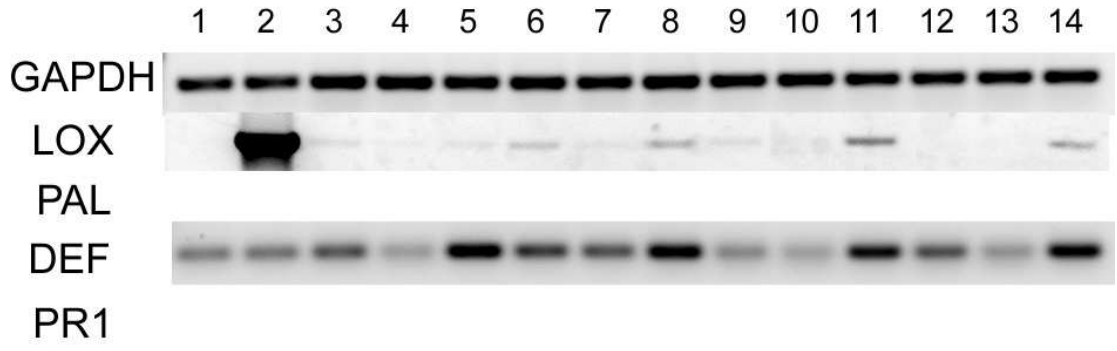


series 1_7th: *P.rapae* parasitized by *C.rubecula*

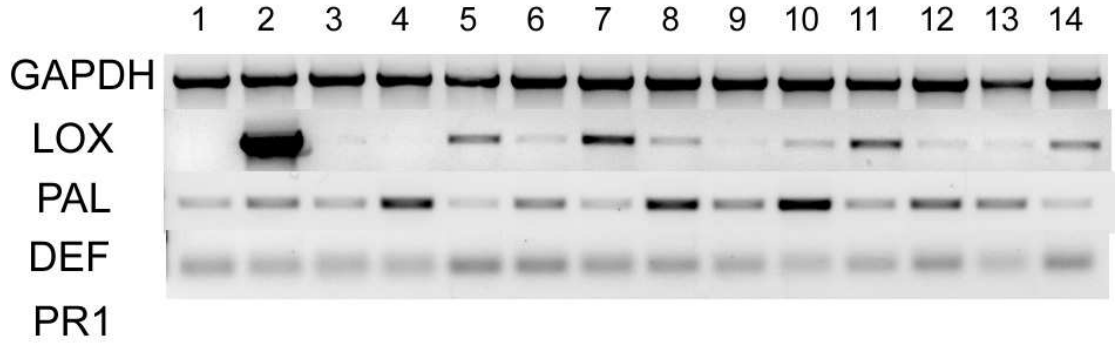


series 1: *P.rapae* parasitized by *C.rubecula*

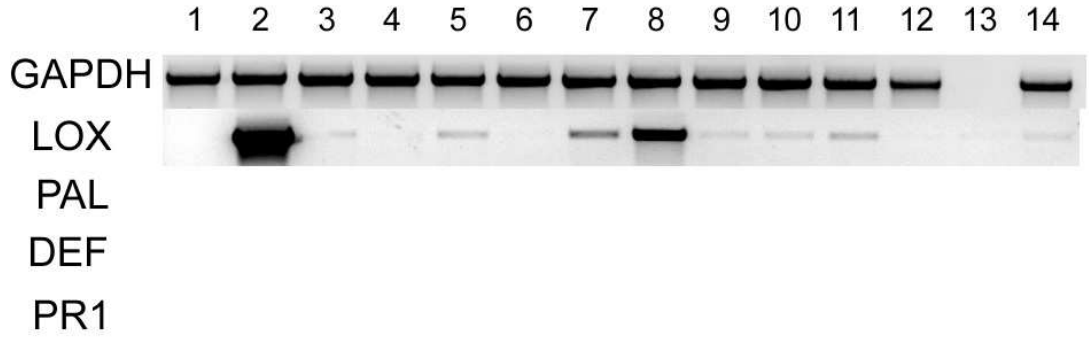
2.2 Series_2 *P. brassicae* parasitized by *C. glomerata*



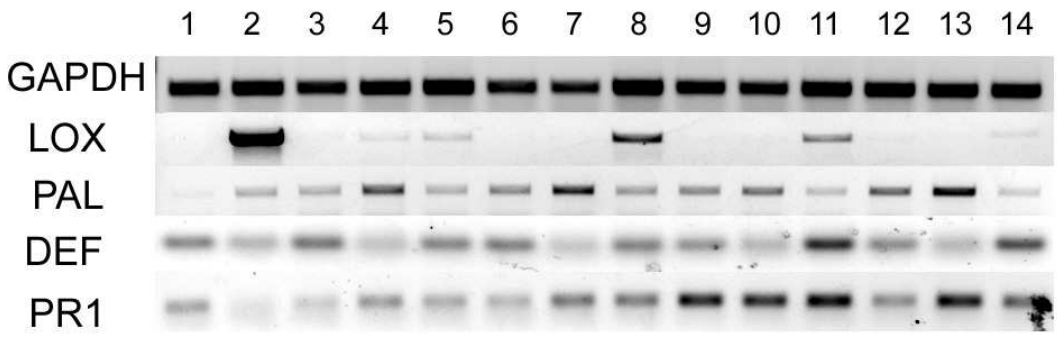
series 2_4th: *P.brassicae* parasitized by *C.glomerata*



series 2_5th: *P.brassicae* parasitized by *C.glomerata*

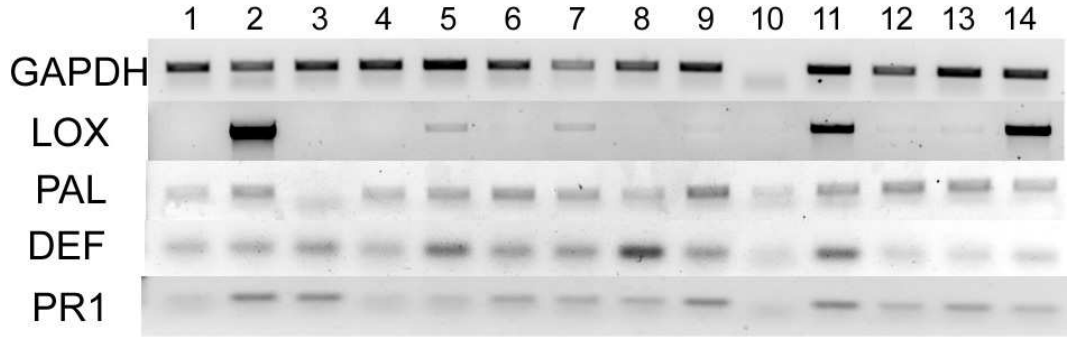


series 2_6th: *P.brassicae* parasitized by *C.glomerata*

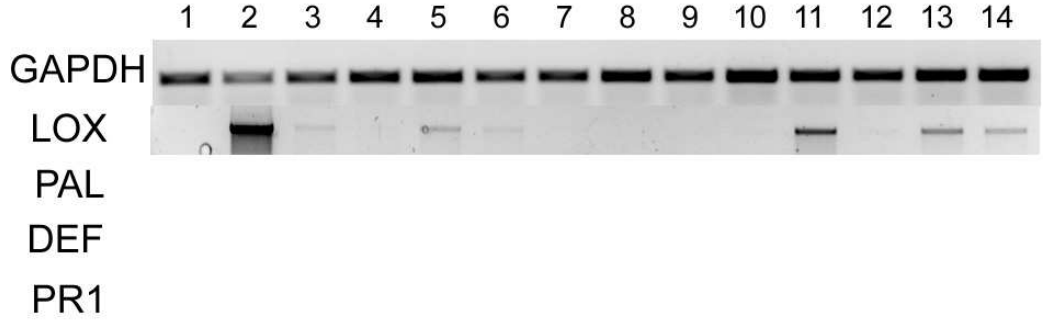


series 2_8th: *P.brassicae* parasitized by *C.glomerata*

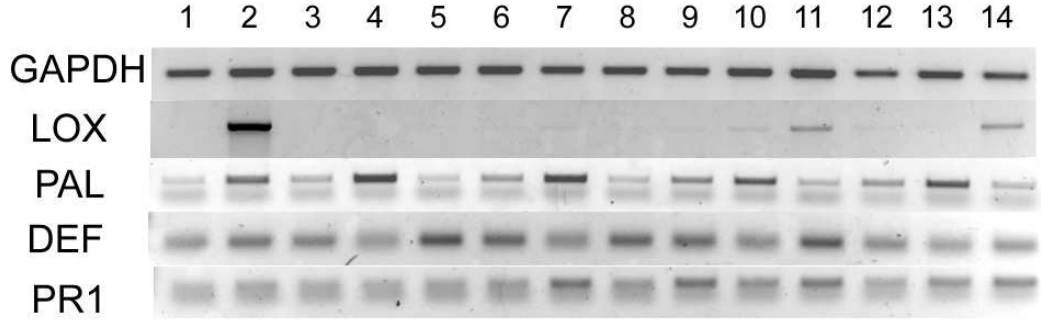
2.3 Series_3 *P. rapae* parasitized by *C. glomerata*



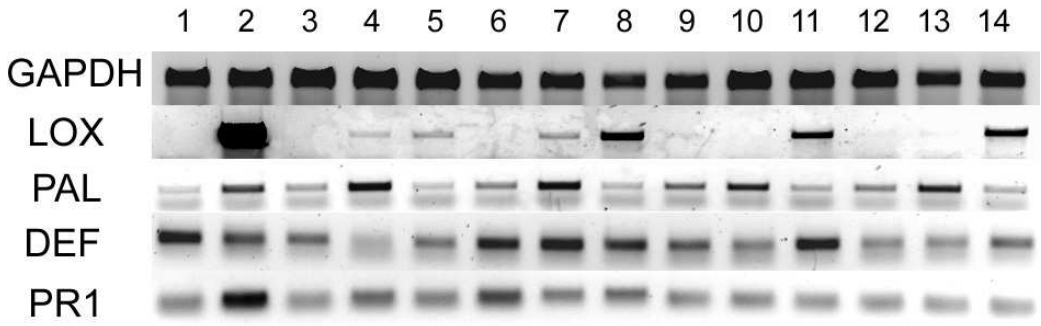
series 3_9th: *P.rapae* parasitized by *C.glomerata*



series 3_11th: *P.rapae* parasitized by *C.glomerata*



series 3_12th: *P.rapae* parasitized by *C.glomerata*



series 3_13th: *P.rapae* parasitized by *C.glomerata*

3. RNA samples for all molecular experiments

Series	Exp	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14
1	1st	Used Left Conc	Used Left Conc	Used Left Conc	Used Left Conc	Used Left Conc	Used Left Conc	Used Left Conc	Used Left Conc	Used Left Conc	Used Left Conc	Used Left Conc	Used Left Conc	Used Left Conc	Used Left Conc
	2nd	Used 3.64 Left 14.36 Conc 1375	Used 5.97 Left 12.03 Conc 837	Used 10.72 Left 7.28 Conc 466	Used 9.45 Left 8.55 Conc 529	Used 11.91 Left 6.09 Conc 420	Used 7.28 Left 10.72 Conc 687	Used 4.02 Left 13.98 Conc 1244	Used 4.44 Left 13.56 Conc 1127	Used 5.87 Left 12.13 Conc 852	Used 8.22 Left 9.78 Conc 608	Used 5.45 Left 12.55 Conc 918	Used 6.26 Left 11.74 Conc 799	Used 5.79 Left 12.21 Conc 863	Used 7.16 Left 10.84 Conc 698
	3rd	Used 2.98 Left 25.02 Conc 1678	Used 4.36 Left 23.64 Conc 1148	Used 4.39 Left 23.61 Conc 1141	Used 2.87 Left 25.13 Conc 1737	Used 4.69 Left 23.31 Conc 1066	Used 5.22 Left 22.78 Conc 957	Used 2.96 Left 25.04 Conc 1691	Used 5.03 Left 22.97 Conc 994	Used 2.83 Left 25.17 Conc 1767	Used 2.99 Left 25.01 Conc 1674	Used 3.94 Left 24.06 Conc 1270	Used 3.44 Left 24.56 Conc 1453	Used 3.49 Left 24.51 Conc 1434	Used 3.85 Left 24.15 Conc 1300
	7th	Used 4.84 Left 23.16 Conc 1033	Used 2.51 Left 25.49 Conc 1991	Used 2.54 Left 25.56 Conc 1965	Used 6.29 Left 21.71 Conc 795	Used 5.25 Left 22.75 Conc 953	Used 4.18 Left 23.82 Conc 1195	Used 0 Left 0 Conc 0	Used 2.6 Left 25.4 Conc 1923	Used 4.19 Left 23.81 Conc 1194	Used 5.48 Left 22.52 Conc 912	Used 3.63 Left 24.37 Conc 1377	Used 5.92 Left 22.08 Conc 844	Used 5.91 Left 22.09 Conc 846	Used 3.62 Left 24.38 Conc 1381
	10th	Used 2.32 Left 25.68 Conc 2159	Used 4.67 Left 23.33 Conc 1070	Used 3.76 Left 24.24 Conc 1331	Used 6.05 Left 21.95 Conc 827	Used 3.01 Left 24.99 Conc 1661	Used 3.01 Left 24.99 Conc1662	Used 3.05 Left 24.95 Conc 1643	Used 5.95 Left 22.05 Conc 839	Used 2.91 Left 25.09 Conc 1715	Used 2.45 Left 25.55 Conc 2036	Used 3.85 Left 24.15 Conc 1298	Used 3.45 Left 24.55 Conc 1451	Used 5.81 Left 22.19 Conc 859	Used 4 Left 24 Conc 1249
2	4th	Used 13.19 Left 14.81 Conc 379	Used 13.05 Left 14.95 Conc 383	Used 8.09 Left 19.91 Conc 618	Used 6.81 Left 21.19 Conc 734	Used 7.70 Left 20.3 Conc 649	Used 6.63 Left 21.37 Conc 754	Used 7.35 Left 20.65 Conc 680	Used 8.36 Left 19.64 Conc 598	Used 4.65 Left 23.35 Conc 1076	Used 6.52 Left 21.48 Conc 767	Used 5.79 Left 22.21 Conc 864	Used 8.96 Left 19.04 Conc 558	Used 9.45 Left 18.55 Conc 529	Used 9.65 Left 18.35 Conc 518
	5th	Used 3.53 Left 24.47 Conc 1417	Used 6.35 Left 21.65 Conc 787	Used 4.13 Left 23.87 Conc 1212	Used 2.99 Left 25.01 Conc 1670	Used 6.07 Left 21.93 Conc 824	Used 7.96 Left 20.04 Conc 628	Used 5.08 Left 22.92 Conc 984	Used 2.98 Left 25.02 Conc 1677	Used 4.83 Left 23.17 Conc 1036	Used 4.41 Left 23.59 Conc 1133	Used 3.72 Left 24.28 Conc 1345	Used 3.22 Left 24.78 Conc 1551	Used 3.52 Left 24.48 Conc 1420	Used 4.86 Left 23.14 Conc 1028
	6th	Used 9.78 Left 18.22 Conc 511	Used 10.78 Left 17.22 Conc 464	Used 4.48 Left 23.52 Conc 1117	Used 3.01 Left 24.99 Conc 1659	Used 6.28 Left 21.72 Conc 796	Used 6.22 Left 21.78 Conc 804	Used 5.03 Left 22.97 Conc 994	Used 4.91 Left 23.09 Conc 1018	Used 3.74 Left 24.26 Conc 1620	Used 4.38 Left 23.62 Conc 1558	Used 8.18 Left 19.82 Conc 1117	Used 3.86 Left 24.14 Conc 1297	Used 3.87 Left 24.13 Conc 1293	Used 6.34 Left 21.66 Conc 789
	8th	Used 4.1 Left 23.9 Conc 1219	Used 4.24 Left 23.76 Conc 1179	Used 2.1 Left 25.9 Conc 2378	Used 4.01 Left 23.99 Conc 1246	Used 3.18 Left 24.82 Conc 1572	Used 3.93 Left 24.07 Conc 1273	Used 3.1 Left 24.9 Conc 1611	Used 2.32 Left 25.68 Conc 2155	Used 11.47 Left 16.53 Conc 436	Used 4.98 Left 23.02 Conc 1003	Used 3.9 Left 24.1 Conc 1281	Used 3.51 Left 24.49 Conc 1426	Used 4.17 Left 23.83 Conc 1198	Used 3.77 Left 24.23 Conc 1326

Concentration unit：ug/ml
Left=Total-used-2ul (for checking concentration)
1st and 9th 20ul, others 30ul.

Series	Exp	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14
3	9th	Used 1.39 Left 16.61 Conc 3588	Used 2.13 Left 15.87 Conc 2358	Used 1.98 Left 16.02 Conc 2531	Used 2.25 Left 15.75 Conc 2225	Used 2.3 Left 15.7 Conc 2177	Used 1.68 Left 16.32 Conc 2976	Used 1.92 Left 16.08 Conc 2609	Used 2.49 Left 15.51 Conc 2005	Used 1.99 Left 16.01 Conc 2515	Used 2.37 Left 15.63 Conc 2110	Used 2.97 Left 15.03 Conc 1686	Used 1.92 Left 16.08 Conc 2604	Used 1.87 Left 16.13 Conc 2675	Used 2.12 Left 15.88 Conc 2365
	11th	Used 1.95 Left 26.05 Conc 2567	Used 5.77 Left 22.23 Conc 866	Used 2.58 Left 25.42 Conc 1944	Used 2.91 Left 25.09 Conc 1715	Used 2.59 Left 25.41 Conc 1932	Used 3.27 Left 24.73 Conc 1532	Used 1.9 Left 26.1 Conc 2626	Used 2.98 Left 25.02 Conc 1678	Used 2.39 Left 15.61 Conc 2086	Used 2.27 Left 25.73 Conc 2200	Used 2.65 Left 25.35 Conc 1895	Used 2.18 Left 25.82 Conc 2288	Used 2.79 Left 25.21 Conc 1786	Used 2.94 Left 25.06 Conc 1705
	12th	Used 2.68 Left 25.32 Conc 1868	Used 2.82 Left 25.18 Conc 1774	Used 3.16 Left 24.84 Conc 1580	Used 6.9 Left 21.1 Conc 724	Used 4.66 Left 23.34 Conc 1074	Used 6.65 Left 21.35 Conc 752	Used 2.9 Left 25.1 Conc 1726	Used 4.14 Left 23.86 Conc 1207	Used 4.0 Left 24 Conc 1251	Used 4.9 Left 23.1 Conc 1020	Used 4.59 Left 23.41 Conc 1089	Used 4.87 Left 23.13 Conc 1026	Used 4.66 Left 23.34 Conc 1074	Used 4.35 Left 23.65 Conc 1149
	13th	Used 6.76 Left 21.24 Conc 738	Used 2.63 Left 25.37 Conc 1899	Used 4.8 Left 23.2 Conc 1042	Used 5.68 Left 22.32 Conc 877	Used 3.65 Left 24.35 Conc 1371	Used 6.28 Left 21.72 Conc 796	Used 5.08 Left 22.92 Conc 984	Used 4.92 Left 23.08 Conc 1017	Used 3.36 Left 24.64 Conc 1487	Used 3.76 Left 24.24 Conc 1330	Used 4.06 Left 23.94 Conc 1231	Used 4.39 Left 23.61 Conc 1141	Used 2.65 Left 25.35 Conc 1889	Used 4.14 Left 23.86 Conc 1207

Concentration unit：ug/ml
Left=Total-used-2ul (for checking concentration)
1st and 9th 20ul, others 30ul.