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RESEARCH PAPER

Timing of induced resistance in a clonal plant network

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ABSTRACT

After local herbivory, plants can activate defense traits both at the damaged site and in undamaged plant parts such as in connected ramets of clonal plants. Since defense induction has costs, a mismatch in time and space between defense activation and herbivore feeding might result in negative consequences for plant fitness. A short time lag between attack and defense activation is important to ensure efficient protection of the plant. Additionally, the duration of induced defense production once the attack has stopped is also relevant in assessing the cost–benefit balance of inducible defenses, which will depend on the absence or presence of subsequent attacks. In this study we quantified the timing of induced responses in ramet networks of the stoloniferous herb *Trifolium repens* after local damage by *Mamestra brassicae* larvae. We studied the activation time of systemic defense induction in undamaged ramets and the decay time of the response after local attack. Undamaged ramets became defense-induced 38–51 h after the initial attack. Defense induction was measured as a reduction in leaf palatability. Defense induction lasted at least 28 days, and there was strong genotypic variation in the duration of this response. Ramets formed after the initial attack were also defense-induced, implying that induced defense can extend to new ramet generations, thereby contributing to protection of plant tissue that is both very vulnerable to herbivores and most valuable in terms of future plant growth and fitness.

INTRODUCTION

Plant defense against herbivores is a costly process (Gershenzon 1994; Sagers & Coley 1995; Elle *et al.* 1999). Defense costs can be a direct consequence of diverting resources from growth and reproduction, and can indirectly stem from ecological phenomena such as reduced competitive ability, autotoxicity, and possible decreases in pollinator visits (Heil & Baldwin 2002; Strauss *et al.* 2002). Inducible defenses may have evolved to reduce the costs of constitutive defense expression by reducing the time during which defense is active (Herms & Mattson 1992; Karban & Baldwin 1997; Agrawal *et al.* 1999). In spite of its obvious importance for the costs and benefits of inducible defenses, very little is known about the duration of the induction status after herbivory.

After local herbivore damage, many plants increase the production of defense compounds to deter or discourage attackers from continued feeding. Induced defense in plants is based on phenotypic plasticity in chemical, nutritional, and mechanical traits (Gómez *et al.* 2008). Defense levels show bell-shaped intensity curves over time: after an initial build-up phase, inducible defense levels reach a peak, and then gradually decrease to base levels (Schultz 1988; but see Underwood 1998). The duration of the different phases in relation to the dynamics of herbivore feeding determines the effectiveness of induced defenses and should therefore be subject to selection under natural field conditions.

Two key aspects, subsequently called activation and decay time, should be considered in the study of temporal dynamics of systemically inducible defense responses. The *systemic activation time* is the time required for producing a defense signal at the site of damage, transporting the signal, and activating defense mechanisms in undamaged plant parts. The *decay time* of an induced response refers to the period of time during which plastically enhanced defense traits are expressed in the plant.

In order to optimize resource investment in induced defense and reduce allocation costs, the temporal expression of defense should match the average time span of transitory herbivore attacks. This can only be achieved by fine-tuning defense activation and decay times. The plastic induction of defense implies an unavoidable time lag between information acquisition (*i.e.*, initial herbivore damage) and plant response (*i.e.*, activation of defense), potentially resulting in ineffective protection of the plant under attack (DeWitt *et al.* 1998). Depending on the study system, this time lag can range from hours (Baldwin *et al.* 1994; Alborn *et al.* 1996) and days (McAuslane *et al.* 1997; Underwood 1998; Agrell *et al.* 2003) to whole seasons (Zvereva *et al.* 1997). Nevertheless, initial damage is usually a reliable cue for future herbivory risk (Karban & Adler 1996; Karban *et al.* 1999), and hence can be used as predictive signal for subsequent damage.

Clonal plant networks are formed from assemblages of interconnected individuals (ramets) that share vascular connections through which resources and defense signals can be

transported (Stuefer *et al.* 2004). In the stoloniferous herb *Trifolium repens,* defense is systemically induced after local herbivore attacks (Gómez *et al.* 2008). This response is based on the internal transmission of unknown signals through stolon connections between members of the ramet network (Gómez & Stuefer 2006). In spite of clear benefits of having private network channels for information transfer, the systemic induction of resistance in a clonal plant network also has costs, which become apparent if defense activation does not match the spatio-temporal patterns of herbivore attack (Gómez *et al.* 2007). Consequently, selection should act to fine-tune defense expression times and match activation and decay periods to long-term patterns of herbivore threats in natural habitats.

Despite the importance of systemic activation and decay time for the short-term temporal dynamics of the induced responses and for longer-term micro-evolutionary processes, few ecological studies have so far investigated the timing of up- and down-regulation of plastic defense in the same system. In this study, we aim to quantify both of these temporal aspects of plastic defense expression within clonal networks of *Trifolium repens*. To assess genetic variability in the timing of the induced responses, four wild *T. repens* genotypes were used in our study. We used *Mamestra brassicae* larvae as herbivores. The following specific research questions were addressed: (i) How long does it take for a ramet to become systemically induced after localized damage to an adjacent ramet (*systemic activation time*); (ii) how long does a clonal plant network remain defense-induced after systemic induction by herbivore attacks (*decay time*); and (iii) do natural genotypes differ in systemic activation and decay times?

We answered these questions by applying localized herbivore attacks on ramets of *T. repens* (white clover) networks, and by measuring systemic defense induction through time. The latter was done by means of preference tests of generalist insect larvae for undamaged sibling ramets connected to damaged ramets.

MATERIALS AND METHODS

Study system

We used four genotypes of the stoloniferous herb *Trifolium repens* (white clover), originally collected from a natural population along the river Waal in Ewijk, The Netherlands. The same genotypes (A13, A23, B11, and D28) were previously used in other studies (Gómez & Stuefer 2006; Gómez *et al.* 2007, 2008), and were used in these experiments because of their ability to become defense-induced after herbivore damage. *M. brassicae* larvae (cabbage army moth) were used as herbivores. This common European species was chosen because it is a polyphagous herbivore known to feed on a wide range of plants including over 70 species in 22 families (Rojas *et al.* 2000). Egg batches were obtained from the Entomology Laboratory at Wageningen University. The colony was reared on biologically grown cabbage and maintained at 23 °C and a 16 h/ 8 h photoperiod. All the experiments were performed in the greenhouse complex of the Radboud University Nijmegen in the spring and fall of 2006.

Pre-growth conditions

Apical cuttings consisting of three unrooted ramets on a single stolon were placed in trays with wet potting soil and covered with plastic foil for 5 days to promote rooting. Thereafter, they were individually transplanted into plastic trays (16 cm × 12 cm × 5 cm) filled with a mixture of sand and potting soil (volume ratio 1:3). Twenty microliters of a solution containing nitrogen-fixing root bacteria (*Rhizobium trifolii;* obtained from the Animal Ecology Department at the Free University of Amsterdam) were added to each tray to promote nodulation. The experiment started 1 week after cuttings had been transplanted.

Defense induction

Systemic defense induction was achieved through controlled herbivore attacks on the second and third youngest ramets of the main stolon of each cutting (Fig. 1). To apply localized herbivory we confined either two 4-week-old (systemic activation time experiment) or three 1-week-old caterpillars (decay time experiment) together with the two target leaves in a Petri dish mounted on the plant. An empty Petri dish was placed on control plants. The larvae were starved during the night preceding the start of the experiment to promote immediate consumption of leaf tissue. The voracious nature of *M. brassicae* larvae, together with the fact that they were starved overnight before the defense induction treatment started, make it highly likely that the larvae started to eat as soon as they were placed on the plants. Thus, most of the amount of leaf tissue offered to the larvae in the defense induction treatment (two leaves; approx. 5–6 cm^2 leaf tissue) was probably consumed rather rapidly. In the experiment on decay time of induced defense, the herbivores were left on the plants for 2 days. The herbivores fed for the same amount of time as on plants during the induction treatment to avoid potential differences in the duration of the stimulus and thus the induction of defense in the plant over time.

Choice tests

We used dual-choice tests to measure the presence or absence of systemically induced defense in undamaged ramets. This approach was used both for assessment of the time required for systemic activation and decay of induced defense (see below). These behavioral tests have proven sensitive and accurate in detecting systemic defense induction in our system (Gómez & Stuefer 2006; Gómez *et al.* 2007, 2008). We performed choice tests between the youngest fully expanded leaf of the control and corresponding defense-induced plant. Both leaves were excised from the plant and placed on moist filter paper in a Petri dish. A 3–4 week-old caterpillar was released in the middle of the Petri dish and allowed to feed until approximately 30% of one of the leaves was consumed, to avoid a decrease in selectivity by the larva due to food shortage (Akhtar & Isman 2004). All choice tests were stopped after 48 h, irrespective of the amount consumed. At the end of each choice test, digital pictures were taken and the consumed leaf area was measured using the image analysis software IMAGE PRO PLUS, version 1.1 (Media Cybernetics, Bethesda, MD, USA). A reduced herbivore preference for induced as

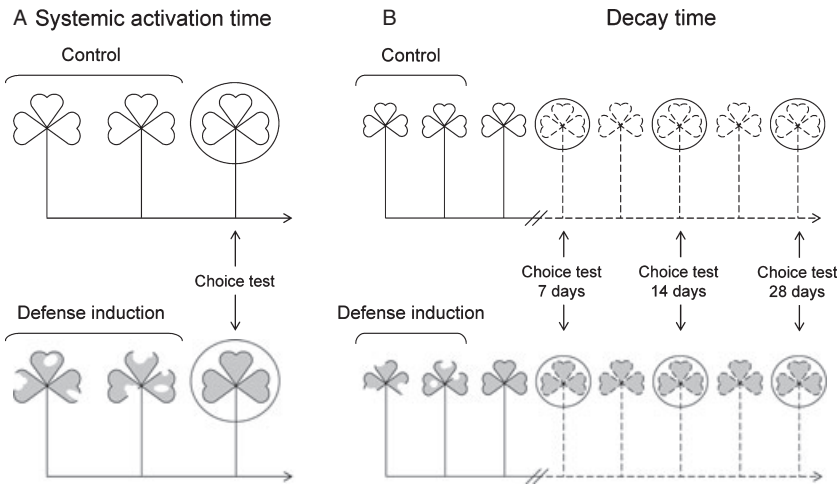

Fig. 1. Schematic representation of the experimental set-up. Pairs of plants assigned to control (white) and defense-induction treatments (gray) were used to study (A) the *systemic activation time* of defense after local damage (left drawing), and (B) the *decay time* of defense expression measured 7, 14, and 28 days after induction (right drawing). Dashed lines represent ramets that were not present at the time of the defense induction treatment.

compared to uninduced plants can be seen as a sign of defense induction (*sensu* Karban & Baldwin 1997), defined as a decrease in the herbivore's preference or performance when feeding on a plant that has been previously damaged.

During the systemic activation time experiment, the choice tests in the different induction treatments partly overlapped in time. In the decay time experiment, it was not feasible to perform choice tests from different time points simultaneously because the time points were too far apart. To minimize the possibility that an unknown factor rather than the defense induction treatment might influence herbivore selectivity on different days, an identical experimental protocol was followed. The herbivores were handled and reared in identical conditions and all the choice tests were conducted in the same controlled environment at the three time points.

T. repens can show strong sectoriality (Lötscher & Hay 1996; Marshall & Price 1997), which results in limited vascular contacts between adjacent ramets belonging to different stem orthostichies. For this reason all choice tests were carried out with the youngest fully expanded leaves belonging to the same orthostichy as the most heavily damaged ramet used for experimental defense induction. A comparable leaf age was selected from the control plant.

Systemic activation time

To determine the period of time necessary to induce defense responses in undamaged ramets, we carried out three herbivory treatments differing in the duration of the controlled herbivore attack. Larvae were allowed to feed for 24, 38, and 51 h. We used 240 cuttings (four genotypes × two treatments × 10 replicates × three time points). At each time point, 40 cuttings were randomly assigned to the control group and 40 cuttings to the defense induction group. At the end of each time point, we performed 10 dual-choice tests per genotype, matching undamaged ramets of control and putatively defense-induced plants as described above. Each cutting was only used in one choice test. Figure 1 schematically shows the experimental set-up.

Decay time

To monitor the maintenance of induced defense expression over time, we tested for the presence or absence of defense induction 7, 14, and 28 days after the controlled herbivore attack had taken place. We used 80 cuttings (four genotypes × two treatments × 10 replicates). Half of the 80 cuttings were randomly assigned to the control and half to the defense induction group. To test whether the plants were defense-induced, 10 dual-choice tests per genotype were conducted. At each time point, the youngest fully expanded ramets from induced and control plants were collected for the choice tests. The same plants were sampled for the choice tests at the three different time points. The excision of ramets does not induce a decrease in leaf palatability (Gómez *et al.* 2007). Due to the high modular growth rate of *T. repens*, ramets used for the choice tests at 7, 14, and 28 days after induction were not present at the time of controlled defense induction.

Statistical analysis

All dual-choice test data were analyzed with repeated measures ANOVA to account for the interdependence of leaves within choice tests. Defense induction and genotype were used as within- and between-subject factors, respectively. The four genotypes were chosen for these experiments because in an original screening they showed the ability to become defense induced after herbivore damage (Gómez & Stuefer 2006). For this reason, *i.e.*, the genotypes used in this study do not represent a random sample of all genotypes, the factor 'genotype' was considered fixed in all analyses. The decay of induced resistance was analyzed by double-repeated measures ANOVA, considering census dates as second repeated factor. This was necessary because the same plants were used for choice tests on the three sampling dates. All analyses were conducted with SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Systemic activation time

Herbivores displayed no measurable preference for control or defense-induced leaves after 24 and 38 h following the start of the defense induction treatment (Table 1). After 51 h, however, defense induction became apparent as a 52% reduction in palatability of leaves originating from induced as

Table 1. Systemic activation time. Repeated measures ANOVA to test for effects of genotype (between-subject effect) and defense induction (within-subject effect) on leaf damage by feeding caterpillars in dual-choice tests at three different time points from the beginning of the defense induction treatment.

	24 h			38 h		51 h	
source	df	MS	F	MS	F	MS	F
between subjects effects							
genotype (gen)	3	1.84	4.69**	1.32	0.88	0.39	1.63
error	36	0.39		1.50 (35)		0.24	
within subjects effects							
induction (ind)	1	0.11	0.47	0.95	1.76	3.21	6.26*
ind × gen	3	0.13	0.52	0.65	1.2	0.92	1.79
error(Ind)	36	0.24		0.54 (35)		0.51	

Values in brackets indicate degrees of freedom. Statistically significant effects are marked with asterisks. *P < 0.05, **P < 0.01.

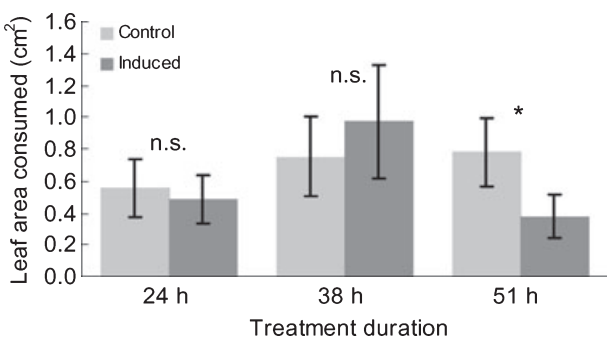

Fig. 2. Average (±SE) of all genotypes for leaf area consumed from control (light gray) and defense-induced (dark gray) ramets in choice tests performed 24, 38, and 51 h after the start of the defense induction treatment. Symbols above the bars report the results of significance tests for differences between treatments (n.s. = not significant, *P < 0.05)

compared to control plants (Fig. 2). There was a significant genotype effect after 24 h, which disappeared in the 38- and 51-h test. We found no significant genotype by defense induction interaction, indicating that there was no genotypic difference in the dynamics of systemic defense activation after a local herbivore attack.

Decay time

Our dual-choice tests indicated that defense induction persisted for at least 28 days (Table 2). Nevertheless, the strength of the defense expression decreased significantly with time (time effect in Table 2; Fig. 3). Seven days after the end of the defense induction treatment, control ramets suffered six-times more damage than defense-induced ramets. After 28 days, the overall effect of induction was still significant, but the difference in damage between control and induced ramets was reduced. After 28 days, control ramets were consumed only twice as much compared to defense-induced ramets. After 28 days, control and induced ramets of three out of the four genotypes were about equally damaged in the dual-choice tests, while herbivores continued to exhibit a strong preference for control ramets in the case of the fourth genotype (Fig. 4; significant genotype-by-defense induction interaction in Table 2).

Table 2. Double-repeated measures ANOVA to test for effects of genotype (between-subject effect), induction (first within-subject effect), and decay time (second within-subject effect) on leaf damage caused by feeding caterpillars in dual-choice tests.

source	df	MS	F
between subjects effects			
genotype (gen)	3	0.40	1.77
error	36	0.23	
within subjects effects			
induction (ind)	1	9.08	37.75***
ind × gen	3	0.87	3.61*
error (ind)	36	0.24	
time	2	0.93	10.04***
time × gen	6	0.11	1.18
error (time)	72	0.09	
ind × time	2	0.23	1.09
ind × time × gen	6	0.36	1.72
error (ind × time)	72	0.21	

Statistically significant effects are marked with asterisks. *P < 0.05, ***P < 0.001.

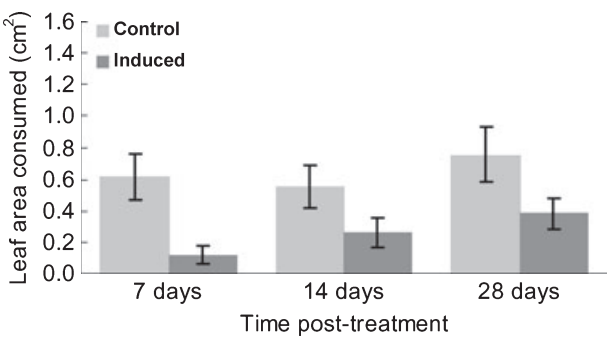

Fig. 3. Average (±SE) of all genotypes for leaf area consumed from control (light gray) and defense-induced (dark gray) ramets in choice tests performed 7, 14, and 28 days after the end of the defense induction treatment.

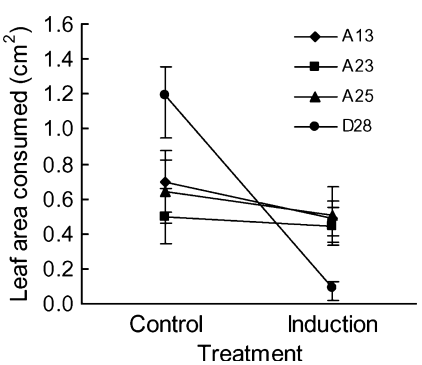

Fig. 4. Leaf area consumed by herbivores in dual-choice tests (control *versus* defense-induced ramets) performed 28 days after the defense induction treatment.

The ramets used for testing herbivore preferences 7, 14, and 28 days after defense induction were not present at the point in time when defense induction took place.

DISCUSSION

Our data show a significant decrease in palatability of undamaged ramets of *T. repens* within 51 h after a localized herbivore attack on adjacent ramets. This inducible change in leaf palatability is interpreted as a sign of systemic defense activation. *T. repens* showed a prolonged decay of defense expression, and remarkable genotypic variation for this trait. Four weeks after herbivore attack, an overall difference in palatability between control and systemically induced ramets was still present. This study also shows that systemic defense induction can be passed on internally to newly formed ramets, suggesting improved protection of future clonal offspring.

Costs and benefits associated with the activation and maintenance of defensive traits are likely to exert selection pressure on the timing of defense activation and decay after induction. On a local scale, rapid defense activation reduces the chance of spatio-temporal mismatches between defense expression and actual herbivore threat, thereby optimizing defense investment. However, considerable costs may be incurred by clonal plant networks if defense traits are activated beyond the dispersal area of feeding herbivores, or if initial damage is not a reliable cue for subsequent herbivore attacks (Stuefer *et al.* 2004). If there is strong selection pressure acting on the timing of induced resistance, all genotypes are expected to show a similar response. However, our results demonstrated that this is only true for the time needed to activate defense systemically, while substantial genotypic differences were observed in the decay time. This may imply that stronger selection pressures act on activation than on decay times, or that the costs involved in maintaining the induction status for prolonged periods of time constrain the evolution of long-term defense induction. In general, genetic variation for functional traits is often upheld by trade-offs with other environment-dependent, fitness-relevant traits.

The time lag between localized herbivore damage and the systemic activation of defense may be viewed mainly as a function of three processes: (i) within-leaf processes such as phloem loading, which affects signal transport out of the damaged leaf (Babst *et al.* 2005), (ii) long-distance trafficking of resources and signals between sites of damage and sites of defense activation, which is driven by source–sink relationships (Gómez & Stuefer 2006), and (iii) induced expression of defense traits in undamaged leaf tissues (Baldwin *et al.* 1994). Since phloem sap moves at an average of 50 to 100 $mm \cdot hr^{-1}$ (Cronshaw 1981), the initial and final phases can be held responsible for the prolonged defense activation time, such as that reported in this study. The approach used in this study, however, does not allow investigating whether the activation time was spent mainly on production of the inducing signal at the site of damage or on the build-up of defenses in undamaged leaves. The dynamics of these processes may constrain adaptive responses to mobile herbivores by hindering rapid and efficient spread of induction signals within clonal plant networks. External communication pathways using volatile compounds may provide an alternative strategy to avoid vascular and timing constraints (Heil & Silva Bueno 2007).

The strength of induced resistance decreased gradually with time in our system, which corroborates earlier studies on non-clonal plants (Stout *et al.* 1996; Anderson *et al.* 2001; Agrell *et al.* 2003; Alves *et al.* 2007). One of the four genotypes used in this study, however, showed a prolonged strong decrease in palatability after defense induction, indicating differences in defense timing between the selected genotypes in this study. In the case of prolonged herbivore attacks, genotypes with long decay times are likely to gain relative advantages over genets with short decay times, because of the selectivity of foraging herbivores. We have recently shown for the same genotypes used in this study (Gómez *et al.* 2007) that maintaining systemically induced defense over a period of 3 weeks has rather low costs in terms of plant performance, while prolonged defense expression confers marked benefits in case of subsequent herbivore attack. These results are indicative of thus far unexplored links between genetic variation in temporal defense expression and the feeding dynamics of insect herbivores and their impact on competitive interactions between genotypes in natural populations of clonal plants.

The current study points to an additional benefit resulting from prolonged defense induction. Ramets developed *after* the end of the defense induction treatment showed increased protection against herbivory. This finding implies that induced defense can be extended to new ramet generations, thereby contributing the protection of plant tissue that is both very vulnerable to herbivores (Bråthen *et al.* 2004; Gómez *et al.* 2007) and very valuable in terms of future plant growth and fitness (Beinhart 1963). Protection of developing ramets through plastic defense induction may be a crucial element in the defense strategy of *T. repens* and similar clonal plant species.

Since network-wide induction of defense *via* vascular signaling follows source–sink gradients (Gómez & Stuefer 2006), systemically induced defense traits are likely to be unidirectionally expressed in clonal plants. Consequently, plastic defenses are mainly expressed in sink regions for carbohydrates, but cannot easily reach source regions within the network. Because young ramets represent strong sinks and are usually preferred by herbivores, systemic defense signaling may often be ineffective in preventing damage to older (source) ramets (Gómez *et al.* 2008). However, the enhanced protection of newly formed ramets, as shown here, may last long enough to bridge the period of time during which the ramets are especially vulnerable to herbivory. Protection during the early stage of development can be crucial for reducing herbivore damage (Aide & Londoño 1989). An early warning system such as that present in clonal plant networks will increase their chance of establishment and survival in the presence of future herbivore attacks.

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