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ORIGINAL PAPER

Plant volatile-induced aphid resistance in barley cultivars is related to cultivar age

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Abstract Recent studies have shown that volatile chemical interaction between certain barley (*Hordeum vulgare*) cultivars can cause reduced host plant acceptance by the aphid *Rhopalosiphum padi*, and that certain cultivars can induce this effect while others can respond. In this study, we tested whether inducing and responding capabilities are linked to year of release in Swedish two-rowed spring barley. Eighteen cultivars released between 1897 and 1992 were tested in randomly selected subsets with pairwise combinations of volatile emitters and receivers. Significantly reduced aphid acceptance as a result of exposure to volatiles from plants of a different cultivar were found in 24% of the cultivar combinations. In general, older cultivars had a higher degree of aphid resistance after barley volatile treatment than did younger cultivars. The inducing effect of the emitter was also related to date of emitter cultivar release but the time relationship was reversed. Combinations with a younger volatile emitter and an older volatile receiver gave the strongest reduction in aphid acceptance of treated plants. Linear relationships between microsatellite diversity of emitting cultivars and their efficiency as inducers indicated that younger cultivars might have a more unique odour, whereas older cultivars may be more sensitive to induction.

Introduction

Evidence that volatile substances emitted by plants can induce responses in other plants has been steadily increasing. Volatiles emitted by a plant attacked by herbivores can activate defence mechanisms in neighbouring plants (Engelberth et al. 2004) or in neighbouring parts of the same plant (Frost et al. 2007), making them less suitable to herbivores. There are also examples of induced indirect defence, for example a plant responding to volatiles from herbivore-damaged plant can produce extrafloral nectar that attracts herbivore natural enemies (Heil and Silva Bueno 2007). The interactions between plants, herbivores and natural enemies can be very complex (e.g. Viswanathan et al. 2005; Soler et al. 2007) and the full functional range of plant volatiles in tritrophic systems is probably yet to be discovered.

Most studies have involved damage to the volatile-emitting plants, either artificial or by herbivores. Over the last 10 years, however, evidence has been mounting that undamaged plants also emit volatiles that affect other plants, and that the responses can affect higher trophic levels such as aphids (Pettersson et al. 1999; Ninkovic et al. 2002; Glinwood et al. 2004) and their predators (Ninkovic and Pettersson 2003). This type of multitrophic interaction has been termed allelobiosis (Pettersson et al. 2003; Ninkovic et al. 2006). The phenomenon was first reported

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by Pettersson et al. (1999) who showed reduced acceptance of barley plants by bird cherry-oat aphid (*Rhopalosiphum padi* L.) after volatile interaction between different cultivars. Reduction in aphid acceptance only occurred in certain cultivar combinations, suggesting that the effect depends both on the composition of volatile emissions from the inducing plant and on the ability of the receiving plant to respond to that particular composition. The effect of allelobiotic interactions in barley on *R. padi* was later observed under field conditions (Ninkovic et al. 2002). Recently, a positive relationship has been found between plant volatile-induced reduction in *R. padi* host acceptance and reduced growth of the aphid in barley resistance screening tests (Ninkovic and Åhman 2009). In the current study, we extend the analysis of allelobiosis in barley to a broader selection of genotypes, 18 two-rowed Swedish cultivars representing almost a century of barley breeding.

Generally, plant traits not prioritised in a certain cultivation system will not be selected for by plant breeding and may disappear with time. Because knowledge about allelobiosis is recent, barley cultivars have not been bred specifically for allelobiotic abilities. Furthermore, selections for yield mainly take place in later generations with genetically homogenous plant material sown in plots, which would not favour unconscious selection for allelobiosis. Therefore, allelobiotic responses might have diminished with time and if these traits are not taken into consideration when new cultivars are bred, they will continue to do so.

In 2004, Bertholdsson reported growth-repressing allelopathic activity against ryegrass (*Lolium perenne* L.) in barley to have declined with breeding. This inspired us to make a screening of allelobiotic properties in barley cultivars representing the twentieth century barley breeding. We chose to use cultivars for which genetic changes over time had previously been analysed using molecular markers (Kolodinska Brantestam et al. 2007). In the current study, these same microsatellite data were analysed for intra-cultivar genetic variation in relation to allelobiotic effects on *R. padi*. We suspected there might be a similar decline in allelobiotic abilities as found for allelopathic traits by Bertholdsson (2004), and the first aim of this study was to test whether the allelobiotic inducing and responding capacity of barley cultivars (measured by aphid host acceptance) have changed with breeding. The second aim was to study how widespread the allelobiotic traits are, and investigate the connection between genetic diversity and allelobiotic capacity. We hypothesised that older and more recent cultivars differ genetically in two ways relevant for allelobiosis: (1) gene sets related to allelobiotic traits might have been altered by breeding and (2) genetic homogeneity of seed lots might differ with cultivar age.

Materials and methods

Plant material

Eighteen two-rowed Swedish spring barley cultivars from the Nordic Genetic Resource Center were tested. Those cultivars first appeared on the Swedish market between 1897 and 1992 (Kolodinska Brantestam 2005b) (Supplementary Appendix). Gene bank-stored seed lots, of which all, but one (Ingrid) had previously been analysed for genetic diversity (Kolodinska Brantestam 2005a, 2007) were propagated in two generations before use in the aphid tests.

Aphids

Test aphids were taken from a multi-clonal stock of *R. padi* continuously reared on oats under summer conditions in the greenhouse facilities of the Swedish University of Agricultural Sciences, Uppsala, Sweden.

Exposure to plant volatiles

Six barley seeds were sown in each pot (size 8 × 8 × 6 cm) filled with Hasselfors Garden Special soil and allowed to develop for a week in a greenhouse chamber at 18–22°C and a minimum 16-h photo period. Natural light was supplemented by light from HQIE lamps. Plants of different cultivars were sown in separate pots, and cultivars were kept at least 50 cm apart at all times to avoid effects of volatile exposure from other cultivars prior to the onset of the experiments.

Plants of one barley genotype were exposed to volatiles from another in a series of two-chamber cages consisting of connected inducing and responding chambers (IC and RC) (each chamber 10 × 10 × 40 cm) (Pettersson et al. 1999; Ninkovic et al. 2002) connected to a vacuum pump. Airflow through the system was 1.3 l/min. Air passed over a pot containing plants of the first barley genotype in the IC and then over a pot with barley plants of the second genotype in the RC before being vented outside the greenhouse. Each pot was placed in a Petri dish to prevent plant interaction via root exudates. Individual pots were watered using an automated drop system (DGT Volmatic) and no additional fertiliser was given.

On each test occasion, plants of the same cultivar were placed in the RC of all cages, and plants of between 1 and 3 different cultivars were placed in the IC, one particular cultivar in each chamber. Thus, in each test, one receiving cultivar was tested against between 1 and 3 volatile-emitting cultivars. Each combination of cultivars was arranged in four blocks, with each block consisting of between 1 and 3 different cultivar combinations and a control (with IC empty). Tests were carried out in a greenhouse chamber with the same temperature and light conditions as for plant cultures.

Because it was impossible to test all cultivar combinations, subsets of cultivars were selected at random to be tested together as volatile emitters and receivers. The subsets normally consisted of four cultivars. However, due to poor germination in some cultivars, there were not always 12 combinations of the 4. In total, 18 cultivars were tested as emitters and 15 as receivers in 37 combinations (Supplementary Appendix).

Aphid host plant acceptance

After 5 days of exposure to plant volatiles from the emitting cultivar, plants of the receiving cultivar were tested for aphid acceptance under greenhouse conditions using a no-choice test (Ninkovic et al. 2002). A polystyrene tube (122 × 30 mm) was placed over the second leaf of the barley plant and 10 bird cherry-oat aphids of mixed ages were released into the tube. The upper end of the tube was covered by a fine-meshed net and the lower end was plugged with a piece of plastic foam with a slit for the leaf. A wooden stick was used to support the tube. After 2 h, the aphids settled (not walking) on the tested leaf were counted. This time is sufficient for aphids to settle and reach the phloem with their stylet (Prado and Tjallingii 1997). In each pot of receiving plants, five individual plants were tested, so the dataset for each combination of cultivars consisted of 20 plants (5 × 4 pots). In some cases, the number of germinated seeds was not sufficient to reach this figure, but tests were included in the analysis when the number of tested plants was 15 or more.

Aphid acceptance of plants may differ between tests, for example, due to small variations in conditions between different test occasions. This was corrected for by dividing A_t with A_0, where A_t is the mean number of aphids accepting the volatile-treated plants and A_0 is the mean number of aphids accepting the control plants of a certain cultivar in a test. Thus, reduced acceptance gives a ratio of <1 while a ratio of 1 indicates that no allelobiotic induction of relevance for aphids occurred.

Genetic diversity

In a previous study (Kolodinska Brantestam et al. 2007), inter-cultivar variation in microsatellite loci over time was studied in cultivars released between 1897 and 1998. Microsatellite loci in 4–8 individual plants (only 2 plants in two particular cultivars) had been analysed in all, but one of the old cultivars, with 21 primer pairs from the Scottish Crop Research Institute. Information on primers, method of DNA extraction, PCR reaction for microsatellite (simple sequence repeats, SSR) and product analysis is given by Kolodinska Brantestam et al. (2007).

Statistical analyses

Differences in aphid acceptance between control and treated plants were tested by two-factor ANOVA, where the factors were "Volatile treatment" (between 1 and 3 cultivars and control) and "Block". Significance level was set to $p < 0.05$. If the F value for the treatment was significant, the ANOVA was followed by Tukey's HSD test at $p < 0.05$.

Aphid responses to volatile-treated and non-treated control plants were tested in simple linear regression analyses in which age or genetic diversity of the cultivars serving as volatile receivers or emitters were treated as independent variables. Data for all dependent variables were normally distributed according to Shapiro–Wilk's test. All analyses were performed with the Statistica software (Statsoft Inc., 2005).

Genetic distances (Rogers 1972) between the pairs of volatile emitters and receivers were calculated using the NTSYS-pc statistical package (Rohlf 1998). SSR diversity (h) at each locus was calculated using the gene diversity index of Nei (1973): $h = 1 - [\text{SUMpi(Square)}]$, where pi is the frequency of the ith allele of the locus and average value of SSR diversity was calculated over all 21 loci.

Results

Changes in allelobiotic relationships with time of cultivar release

Barley volatile induction resulting in significantly reduced aphid acceptance appeared in 9 out of 37 tested cultivar combinations, amounting to 24.3% (Supplementary Appendix). In no case was there a significant increase in aphid acceptance of plants that had been exposed to volatiles from another cultivar.

There was a statistically significant relationship between aphid acceptance of volatile-treated plants as compared to control plants and the release year of the receiver cultivar ($F_{(1,35)} = 6.1$, $p < 0.019$, $r^2 = 0.148$). Younger cultivars generally had a weaker allelobiotic response (Fig. 1). Aphid response to the treatment was also significantly related to age of the volatile emitter, but the age dependency was reversed ($F_{(1,35)} = 4.3$, $p < 0.046$, $r^2 = 0.109$) (Fig. 2). The younger the volatile-emitting cultivar, the greater was the reduction in aphid acceptance of the volatile-treated plants.

Combining this into one variable describing the age difference (release year emitter − release year receiver)

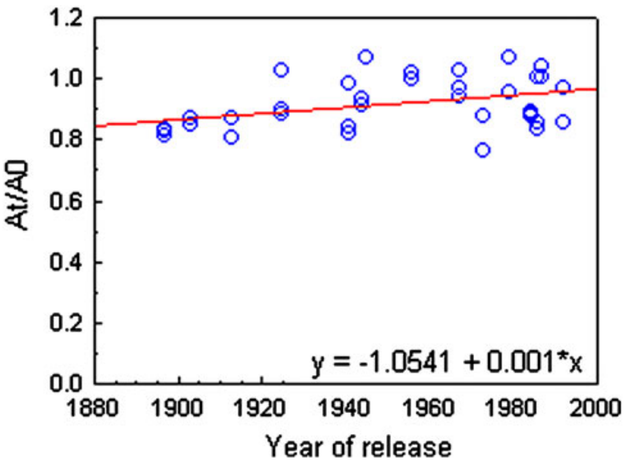

Fig. 1 Relationship between allelobiotic effect on *R. padi* acceptance of volatile-exposed barley cultivars and year of receiver cultivar release onto the market. A_t acceptance of plants exposed to volatiles from another cultivar, A_0 acceptance of plants exposed to air alone (control)

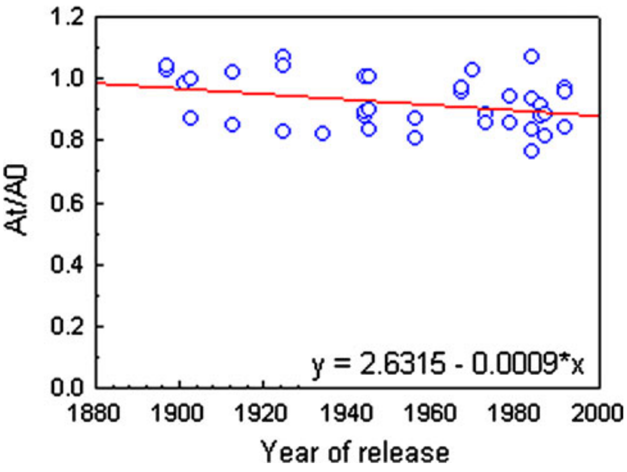

Fig. 2 Relationship between allelobiotic effect on *R. padi* acceptance of volatile-exposed barley cultivars and year of emitter cultivar release onto the market. A_t acceptance of plants exposed to volatiles from another cultivar, A_0 acceptance of plants exposed to air alone (control)

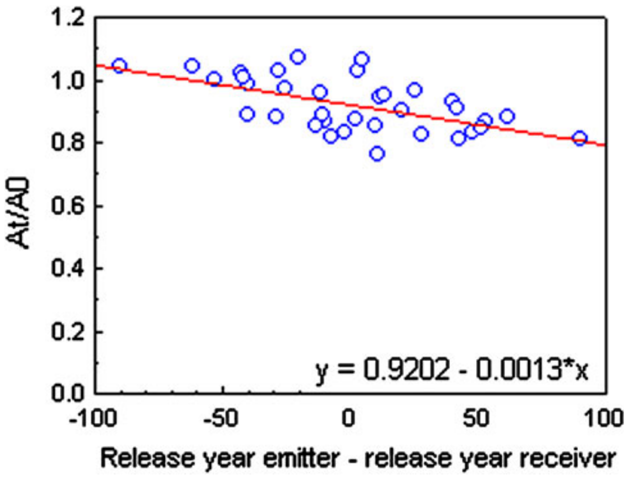

Fig. 3 Relationship between allelobiotic effect on *R. padi* acceptance of volatile-exposed barley cultivars and difference in year of release onto the market between emitter and receiver cultivar (year emitter release − year receiver release). A_t acceptance of plants exposed to volatiles from another cultivar, A_0 acceptance of plants exposed to air alone (control)

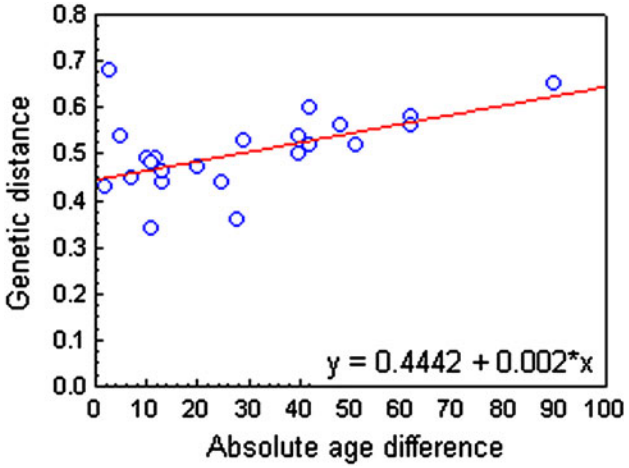

Fig. 4 Relationship between genetic distance (Rogers 1972; Rohlf 1998) as determined by SSR markers and absolute age difference in pairs of barley volatile emitter and receiver cultivars

showed an even stronger relationship ($F_{(1,35)} = 18.4$, $p < 0.0001$, $r^2 = 0.344$) (Fig. 3). When age difference regardless of which cultivar was the emitter and which was the receiver (i.e. absolute age difference) was related to aphid response, there was no longer a significant relationship ($F_{(1,35)} = 0.42$, $p < 0.52$). Thus, the strongest induction effects were obtained between a younger volatile emitter and an older volatile receiver.

Genetic distance between cultivars based on SSR marker data were significantly positively related to absolute age difference between the two cultivars combined ($F_{(1,31)} = 15.5$, $p < 0.0004$, $r^2 = 0.333$) (Fig. 4), i.e. in general terms, the larger the age difference, the larger the genetic distance between the two. However, genetic distance and aphid acceptance of volatile-exposed plants relative to control were not significantly related ($F_{(1,31)} = 3.2$, $p < 0.08$), and there was a trend for reduction in the inducing effect with greater genetic distance. Thus, genetic distance as measured here did not explain why there was an age-dependent relationship in inducer and volatile-responding effect.

Variation in microsatellite loci and aphid responses

Average gene diversity and number of polymorphic alleles of the volatile emitter were significantly linearly related to

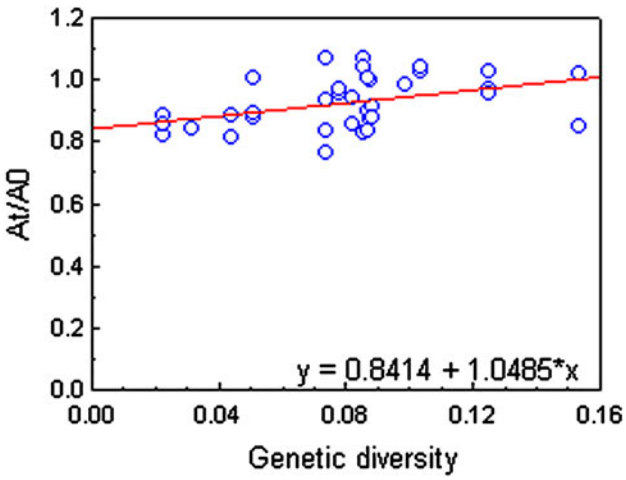

Fig. 5 Relationship between allelobiotic effect on *R. padi* acceptance of volatile-exposed barley cultivars and average genetic diversity (Nei 1973) of volatile-emitting cultivars as based on SSR markers. A_t acceptance of plants exposed to volatiles of another cultivar, A_0 acceptance of plants exposed to air alone (control)

the strength of the induced reduction in aphid acceptance ($F_{(1,33)} = 6.7$, $p < 0.015$, $r^2 = 0.168$ (Fig. 5) and $F_{(1,33)} = 5.4$, $p < 0.027$, $r^2 = 0.140$, respectively). Generally, the less diverse the emitting cultivar, the stronger was the induction effect. There was no corresponding relationship between diversity of SSR loci of the volatile-receiving cultivar and strength of induction based on aphid responses ($F_{(1,33)} = 2.4$, $p < 0.13$ and $F_{(1,33)} = 2.8$, $p < 0.11$, respectively).

There was no significant linear relationship between cultivar age and genetic variation within cultivars based on average gene diversity of SSR loci or number of polymorphic alleles ($F_{(1,15)} = 1.5$, $p < 0.24$ and $F_{(1,15)} = 2.5$, $p < 0.13$, respectively), meaning that the loci tested were no more diverse in older cultivars than in younger ones.

Discussion

The strength of the aphid response to allelobiosis in receiving plants decreased over time in cultivars released during the twentieth century while the inducing ability increased. The greatest reduction in aphid acceptance therefore occurs when an older cultivar is exposed to volatiles from a younger cultivar. We discuss two possible explanations for this pattern: (1) allelobiotic traits have been altered by plant breeding and (2) the expression of allelobiotic traits is related to genetic homogeneity of the cultivars.

In the current study, we found that there was a statistically significant time trend for older cultivars to respond more strongly to volatile induction. Older, so-called land races are known to harbour more resistance to aphids, e.g. to Russian wheat aphid (Bonman et al. 2005). Some of the oldest cultivars in our test set are selections from land races (Bertholdsson 2004). Land races are also known to have a high genetic diversity (Maciel et al. 2003; Ram et al. 2007), but the magnitude of the allelobiotic response was not significantly related to the SSR diversity of the volatile-receiving cultivars. This indicates that the volatile-responding capacity may have been reduced with breeding.

Plant genotype and volatile profile have previously been shown to be closely linked (e.g. Degen et al. 2004; Keszei et al. 2008). To explain why barley-inducing capacity increased over the years, we tried to distinguish whether this is due to a change in volatile emission caused by breeding, or if it is a result of younger cultivars being more genetically homogenous than older cultivars. This could make them more distinct in their volatile profile and therefore give them a high-inducing ability. Current rules for UPOV variety approval (UPOV document TGP/1) put a high demand on genetic uniformity, while older cultivars were often not so uniform even at release and can therefore be expected to have a more diverse volatile profile when compared with more recently released cultivars. In addition, genetic diversity within cultivars might have increased since cultivar release, due to mutations and inadvertent gene introductions via crossings and seed mixtures over years of propagation. This is likely to have happened to a larger extent in older cultivars, which have been propagated for more generations. In our test for increase in intra-cultivar genetic diversity with time since release, we found a trend in the expected direction, but it was not statistically significant. We did, however, find a significant relationship between emitter-inducing capacity and SSR diversity. The lower the diversity of the emitter, the higher was the induced resistance effect in the receiver. Currently, we do not know which of the two explanations for the higher induction by younger cultivars is most likely, i.e. whether younger cultivars, being more genetically homogenous than older cultivars, emit a more narrow volatile spectrum and hence a clearer signal, or if volatile emission from barley have been altered by breeding to become more conspicuous to other plants. However, comparing conditions for plants growing in the wild and plants bred for monoculture might give some clues.

In nature, plants compete with each other for space, light, nutrients and water. Thus, it should be favourable for a plant to be able to detect and respond to competitors, but also remain cryptic to them. Ninkovic (2003) showed that barley volatiles can cause changes in the shoot/root biomass ratio in a responding cultivar, suggesting that the primary role of allelobiosis in nature may not be defence against aphids, but rather competition with other plants for resources. Because barley is bred to grow in monoculture with few weeds, plant competition in cultivated fields occurs mainly between genetically identical or almost identical plant individuals, at the same developmental stage (cf. Körner et al. 2008). This may mean that plants have very similar volatile profiles, making it difficult for them to distinguish self from close neighbour volatile emissions. This in turn, would relax the need for being cryptic by restricted release of volatiles, and result in plants that are weak responders, but strong inducers.

Whether there was significant reduction in aphid acceptance of the volatile receiver was dependent on both cultivars in the pair, but genetic distances between emitter and receiver did not relate to the strength of the allelobiotic response. Several studies have explored effects on inter-cultivar genetic diversity of cultivated barley caused by breeding, but the results are contradictory regarding whether there is genetic erosion over time (e.g. Kolodinska Brantestam et al. 2007; Malysheva-Otto et al. 2007, and references therein). We found that the genetic difference was greater the bigger the age difference between the cultivars, but this did not explain the patterns of induction.

Allelobiosis provides the potential for a new cultivation system in which plant volatile-induced resistance allows reduced inputs of insecticides. Allelobiotic interactions may be exploited as cultivar mixtures of inducing and responding cultivars, or in monoculture with inducing chemicals applied directly to a responding cultivar. Mixtures of different genotypes have been found to give higher yields than monocultures in cereals (Gustafsson 1953; Szumigalski and van Acker 2006; Tratwal et al. 2007), although this is not always the case (Jokinen 1991; Mercer 2006). It was recognised at an early stage that the outcome depended on which cultivars were combined (Gustafsson 1953). Our findings suggest that allelobiosis may contribute to the effects of barley cultivar mixing on aphid pests. Therefore, we suggest that allelobiotic properties should be taken into consideration when new cultivars are bred for use in mixtures.

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