Stronger induction of callose deposition in barley by Russian wheat aphid than bird cherry-oat aphid is not associated with differences in callose synthase or β-1,3-glucanase transcript abundance

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Abstract

The effects of infestation by the bird cherry-oat aphid (BCA), (*Rhopalosiphum padi* L) and the Russian wheat aphid (RWA) (*Diuraphis noxia* Mordvilko) on callose deposition and transcription of genes related to callose accumulation were investigated in barley (*Hordeum vulgare* L. cv. Clipper). The BCA, which gives no visible symptoms, induced very limited callose deposition, even after 14 days of infestation. In contrast, RWA, which causes chlorosis, white and yellow streaking and leaf rolling, induced callose accumulation already after 24 h in longitudinal leaf veins. The deposition was pronounced after 72 h, progressing during 7 and 14 days of infestation. In RWA-infested source leaves, callose was also induced in longitudinal veins basipetal to the aphid-infested tissue, whereas in sink leaves, more callose deposition was found above the feeding sites. Eight putative callose synthase genes were identified in a database search, of which seven were expressed in the leaves, but with similar transcript accumulation in control and aphid-infested tissue. Five out of 12 examined β-1,3-glucanases were expressed in the leaves. All five were upregulated in RWA-infested tissue, but only two in BCA-infested tissue, and to a lesser extent than by RWA. The results suggest that callose accumulation may be partly responsible for the symptoms resulting from RWA infestation and that a callose-inducing signal may be transported in the phloem. Furthermore, it is concluded that the absence of callose deposition in BCA-infested leaves is not because of a stronger upregulation of callose-degrading β-1,3-glucanases in this tissue, as compared to RWA-infested leaves.

Introduction

Callose, a β-1,3-glucan, is an important component in plant tissue. It is formed as part of basic developmental processes at the cell plate during the formation of new walls in dividing cells and it is found around pollen mother cells, in pollen grains and in pollen tubes. Callose is also present in plasmodesmatal canals, root hairs and in spiral thickenings in tracheids (Stone and Clarke 1992). In addition, callose is locally deposited in sieve pores and plasmodesmata in response to wounding and other physiological stress and in plugs or plates (normally referred to as papillae) in response to pathogen infection (Donofrio and Delaney 2001, Stone and Clarke 1992). Wound callose is formed rapidly, mostly within minutes of wound initiation (Nakashima et al. 2003, Radford et al. 1998) and is deposited between the plasma membrane and the cell wall. It is electron-lucent in electron micrographs and forms an intense yellow, UV light-induced fluorescence with the aniline blue fluorochrome (Stone and Clarke 1992).

The rapid deposition of callose in sieve pores has long since been viewed as an efficient wound response that seals off the pores in damaged phloem to prevent assimilate loss (Sjölund 1997). Nevertheless, aphids (Homoptera: Aphididae), which damage the sieve elements when penetrating with their stylets, manage to feed from phloem elements during long periods. It has been proposed that aphids prevent

wounding reactions, such as callose formation and protein plugging, by sealing the stylet puncture site with sheath saliva. In addition, components in the watery saliva injected into the sieve elements may interact with sap ingredients (Will and van Bel 2006). However, recent studies indicate that some aphid species do cause callose formation (Botha and Matsiliza 2004, Kusnierczyk et al. 2008). Thus, we have found that the Russian wheat aphid (RWA) (*Diuraphis noxia* Mordvilko), an aphid that causes leaf rolling and other symptoms, causes callose deposition in cereals. Callose was associated with sieve plates (SP) and pore plasmodesmata between the companion cells and their associated sieve tubes, and the phloem transport rate was reduced in the damaged phloem tubes in wheat (Botha and Matsiliza 2004). This is suggestive evidence that callose formation may at least partly explain the severe symptoms caused by RWA infestation. This idea was supported by results from a recent ultrastructural study in barley, where effects of RWA and the bird cherry-oat aphid (BCA) (*Rhopalosiphum padi*, L) were compared (Saheed et al. 2007). BCA co-occurs with RWA on wheat and other cereals, but usually does not cause any visible damage symptoms (Messina et al. 2002). Besides differences in the salivary secretions in the xylem elements because of these two aphids, the effects on phloem tissue were different. In RWA-infested tissue, there was deposition of callose in sieve pores and also in plasmodesmata and pore plasmodesmata between companion cells and sieve tubes, whereas in BCA-infested tissue only sieve pores appeared to contain callose (Saheed et al. 2007).

The present investigation was set up to further examine the earlier reported difference between the effects of RWA and BCA with regard to the deposition of callose. Structural studies were complemented with studies of transcript abundance of genes potentially involved in callose metabolism. As a difference in the deposition of callose may be the result of differences in the rate of biosynthesis or in degradation of the compound, both processes were considered. Callose synthesis is carried out by callose synthase complexes (Verma and Hong 2001). A plant gene family with sequences similar to *FKS1*, a β-1,3-glucan synthase in yeast, has been identified, containing 12 glucan synthase-like (*GSL)*-related genes in *Arabidopsis* (Richmond and Somerville 2000, Verma and Hong 2001). In rice, 10 *GSL* sequences were annotated in the genome and put in phylogenetic relationship with the *Arabidopsis GSL*s (Yamaguchi et al. 2006). In barley, a gene homologous to the yeast *FKS* gene has been identified –*HvGSL1*. It is a member of a family of at least six genes and was linked by biochemical evidence to callose synthesis activity (Li et al. 2003).

Callose degradation has been less studied. Earlier studies reported that wound callose disappears over the course of days (Currier and Webster 1964). The enzymes involved would be β-1,3-glucanases. Indeed, a tobacco mutant deficient for a class I β-1,3-glucanase showed a reduced plasmodesmatal size exclusion limit and enhanced callose deposition (Iglesias and Meins 2000). In cotton, it was shown that increased transcript accumulation of a fiber-specific β-1,3-glucanase gene, *GhGluc1*, became evident at the time of callose degradation coinciding with plasmodesmata reopening during cotton fiber elongation (Ruan et al. 2004). The β-1,3-glucanases belong to a group of pathogenesis-related proteins that has been widely studied in connection with stress-related conditions (Muthukrishnan et al. 2001, Van Loon and van Strien 1999). RWA has been found to induce β-1,3-glucanases in wheat (van der Westhuizen et al. 1998, 2002). The β-1,3-glucanase protein amounts and enzyme activity levels were higher in varieties containing a resistance gene against RWA, than in isogenic lines without this gene (van der Westhuizen et al. 1998, 2002). The same resistance gene was found to correlate to absence of callose formation in wheat induced by another aphid, *Sitobion yakini* (de Wet and Botha 2007), suggesting that high β-1,3-glucanase levels might regulate callose accumulation. In the present work, the regulation of individual β-1,3-glucanase and callose synthase genes was analyzed at transcript level in one barley cultivar infested by either RWA or BCA in order to investigate whether the difference in callose accumulation caused by these two aphid species were caused by differential transcript accumulation.

Materials and Methods

Plant material, aphid colony maintenance and treatments

Barley (*Hordeum vulgare* L. cv. Clipper) seeds were pre-germinated in Petri dishes and sown in potting soil (60:40; peat : vermiculite mixture) in plastic pots. They were watered twice a week with Long-Ashton nutrient solution (Hewitt 1966) and grown in a controlled environment (Conviron S10H Controlled Environments Limited, Winnipeg, Manitoba, Canada) at 24°C, 66% relative humidity (RH) day and 22°C, 60% RH night, 14 h photoperiod. The colonies of RWA *D. noxia* (Mordvilko) and the BCA *R. padi* (L.) were obtained from the ARC-Small Grain Institute, Bethlehem, South Africa. Aphid colonies were maintained on young barley plants for at least three generations to avoid any effects carried over from previous hosts (Shufran et al. 1992) and kept in insect cages in separate growth cabinets, maintained at 18°C, 66% RH day and 15.5°C, 66% RH night, 14 h photoperiod. Illuminations in the two cabinets were achieved using a combination of fluorescent tubes (F48T12.CW/VHO1500, Sylvania, Danvers, MA) and frosted incandescent 60 W bulbs (Philips, Eindhoven, the Netherlands) and the irradiation level was 250 μmol m^{-2} s^{-1}.

For fluorescence microscopic investigations, clip cages that were 2 cm in diameter, as previously described by Noble (1958), were used to enclose 10 adult aphids to the mid-length of a fully expanded leaf for each of the aphid feeding treatments. Leaves of control plants carried an empty cage and 10 replicate plants were set up per treatment. A mature leaf (second leaf above coleoptile) and newly expanded leaf (fourth or fifth leaves above coleoptiles) were selected as the source and sink leaf, respectively, for short-term feeding responses, while the second or third leaf above the coleoptiles were selected for long-term responses. The aphids were allowed to feed for 24, 48 and 72 h (short-term feeding responses) and 7 and 14 days (long-term feeding responses) after which the leaves were selected for the study of feeding-related callose deposition using the fluorescence microscope and aniline blue fluorochrome to visualize the callose.

For investigations of gene regulation, 10-day-old plants were infested with aphids by placing the clip cages around the second leaf above coleoptiles and placing 20 aphids per plant within the clip cages and 24 replicate plants were set up for each treatment. At time zero (same day when putting aphid cages on) plant tissues equivalent to the tissue caged-in were harvested and frozen immediately in liquid nitrogen and stored at −80°C. After 24, 48 and 72 h, aphids were brushed off the infested leaves and the plant tissue within the clip cages was harvested as described above and stored at −80°C until further use. Controls were treated the same way except that no aphids were present. Eight plants per treatment were pooled together and immediately frozen in liquid nitrogen and then kept at −80°C.

Fluorescence microscopy

Whole leaves were cut from the plants after gently removing clip cages. The areas to which aphid feeding had been confined were marked for each treatment as defined above, including the aphid-free control leaves. Leaves were transferred immediately into a Ca^{2+}-free buffer (10 m*M* 2-[morpholino] ethanesulfonic acid (MES), 0.5 m*M* $MgCl_2$, 0.5 m*M* KCl and 125 m*M* mannitol, adjusted to pH 7.2) and the abaxial surface gently scraped on a glass plate with a single edge razor blade under the buffer. This was carried out to remove the cuticle and the underlying epidermal tissue in order to expose 'windows' into the underlying mesophyll and vascular tissues. A solution of aniline blue fluorochrome (4',4-[carbonyl bis(benzene 4,1-diyl) bis(imino)] bis(benzene sulfonic) acid (Biosupplies, Parkville, Australia) (427 μ*M* in distilled water, kept foil-wrapped at 4°C until needed) was applied to the leaf strips on glass slides and then covered with cover slips. The tissue was incubated in the fluorochrome solution for 30 min at 20°C and then washed in a fresh Ca^{+}-free MES buffer (see above). Examination of callose fluorescence was carried out under UV light, using an Olympus BX61 wide-field fluorescence digital imaging microscope (Olympus, Tokyo, Japan; Wirsam Scientific, Johannesburg, South Africa), fitted

with an aniline blue-specific filter cube (with an excitation of 425–444 nm and emission of 475 nm). Images were saved in a database using the program ANALYSIS (Soft Imaging System GmbH, Münster, Germany), and imported as bitmaps to Corel Draw 12 (Corel Corporation Ottawa, Canada 2003) for presentation.

RNA extraction

Plant material from barley cv. Clipper was harvested in three biological replicates with eight individual plants in each. Total RNA was isolated from 100 mg frozen plant powder using Total RNA Purification from Plant (Macherey–Nagel GmbH, Düren, Gemany), according to the kit protocol. DNA was digested during the purification and purified RNA was eluted in RNase-free water.

Reverse transcriptase polymerase chain reaction and primer design

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed by using SuperScript™ One-Step RT-PCR System with Platinum®*Taq* DNA polymerase (Invitrogen, Paisley, UK). Thirty nanograms of total RNA was used as template. To identify putative callose synthase and β-1,3-glucanase genes we used builds #51 and 52 for *H. vulgare* in the unigene database at *National Center for Biotechnology Information* (NCBI) (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene; Wheeler et al. 2003). For callose synthases, specific primers were designed for eight sequences potentially coding for callose synthases identified in database searches (Basic Local Alignment Search Tool, BLAST; searches at ncbi.nlm.nih.gov). The searches were carried out on barley expressed sequence tags (ESTs) with *HvGSL1* as starting point. From the result list, sequences representing the different unigenes were selected by following the link to the unigene database. Sequences representing different unigenes were in their turn used as query in further BLAST searches to identify additional unigenes. Doing so we identified seven unigenes, of which four are annotated on the Barley1 GeneChip (Close et al. 2004). These seven sequences plus one EST (BU982241), which is not assigned any unigene and is included in the alignment presented in Li et al. (2003), were chosen for primer design. For β-1,3-glucanases, 16 unigenes were identified in database searches (unigene builds #51 and #52 at ncbi.nlm.nih.gov). Six of them are annotated on the Barley1 GeneChip, the other 10 have significant similarities to Barley1 contigs as determined by BLAST searches using sequences representing the unigenes against the Barley1 exemplar sequences. Specific primers were designed for 12 unigenes, the remaining 4 being too similar to other sequences to allow for specific primer design. Primer sequences are given in Table 1. Primers were purchased from Eurofins MWG Operon, Germany, and designed using the web-based primer design tool at the Eurofins website (www.eurofinsdna.com). Primers were then used as query in BLAST searches against barley EST sequences at ncbi.nlm.nih.gov and only primer pairs where at least one primer had two mismatches to sequences belonging to other unigenes and the other one at least one mismatch were chosen. Cycling conditions on Programmable Thermal Controller PTC-100™, MJ Research, were 45°C for 30 min and 94°C for 2 min, 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min, and finally 72°C for 5 min. Products were run on 2% agarose gels run in 1× Tris/Borate/EDTA (ethylenediaminetraacetic acid) (TBE) (Sambrook et al. 1989) and visualized by ethidium bromide.

Table 1. Sequences of primers specific for callose synthases and β-1,3-glucanases used for RT-PCR. Contig numbers are in bold when the unigenes are annotated on Barley1. In all other cases, the Barley1 contigs with highest similarity to the unigenes are indicated. Although Hv.10307 and Hv.27045 both have highest similarity to contig13350_at, they are different from each other.

	Unigene or EST	Contig no. on Barley1	Primer sequences
Callose synthases	Hv.4615	**4949**	F1 5'-CCACCATCGTGATCCTTATCGTGAT-3'
			R1 5'-CATGATCGCCGGCTTCAGTGCCTGA-3'
	Hv.20627	**8428**	F1 5'-GTGGAAAACAGTGCGCTCGTTGGCT-3'
			R1 5'-GCCTGGTTGAACAGTAGCCGTGTCT-3'
	Hv.17716	**13152**	F1 5'-AGTGCACTAGCATTCTTAGCAACTG-3'
			R1 5'-CAGCGTCGTACATCCGAGAGAT-3'
	Hv.17389	**19065**	F1 5'-TCGGTGGTCAGAAGAAGGAACG-3'
			R1 5'-ATGGCAGTATCCGACGACTACG-3'
	Hv.19863		F1 5'-CTCCACATGGGCCTTTATCTGC-3'
			R1 5'-TTTCCCGCTCCTTTCAGCTTC-3'
	Hv.22049		F1 5'-TGGAGGAGCTGAATACAGAGCG-3'
			R1 5'-TGAACGGGAAGGGAGCAAAGAG-3'
	Hv.11694		F1 5'-CGTAGCTTGCTCTTGGAGCTTG-3'
			R1 5'-AGAACGATGCCAATAACCAGCC-3'
	BU982241		F1 5'-GTTGCTGCTTGTCCTCCCTATG-3'
			R1 5'-TCTGTATTTAGCGCCACCATGC-3'-
β-1,3-glucanases	Hv.24036	**1636**	F1 5'-GTTCCTGAGGCCCATCCTTAACTT-3'
			R1 5'-CGTCGAAGAGGTTGGTGTATGTCAA-3
	Hv.18110	**1637_s**	F2 5'-TCGCCATGTTCAACGAGAACC-3'
			R3 5'-TGCTTGGTTGCACTCTTCC-3'
	Hv.26605	**1639**	F2 5'-ACGCGCAGGCGTACAACCAGGGATT-3'
			R2 5'-ACGACGCAGCTTATTCGACCACGAA-3'
	Hv.19837	10477	F1 5'-TCCGGCTCCTACTGAGCACTGAAAG-3'
			R1 5'-ATGCACTACGCCTGTACAGAGCTGC-3'
	Hv.60	**11921**	F1 5'-AAGCAACCTGTCTACCCGACA-3'
			R1 5'-ACATTGTTTCAAGCACAAATATATT-3'
	Hv.10048	**8262**	F1 5'-GTCCCGTCTATAATTCCTTTGG-3'

		R1 5'-GAAGAAGGTGAGTGACGATG-3'
Hv.21394	1632	F1 5'-GCCATGTTCAAGGAGAACTTC-3'
		R1 5'-TCCACGTTACCCTTCACTCC-3'
Hv.27045	13550	F1 5'-CAAGAGGTCTGGTGCAATCGAG-3'
		R1 5'-TTCTTCACCATCCATGCAAAGC-3'
Hv.8964	11289	F1 5'-ACGAGAACAAAAAGGAAGGG-3'
		R3 5'-ACAAAATTGATAGCTCTTAGT-3'
Hv.79	M96940	F1 5'-TGTTCAACGAGAACCAGAAGCC-3'
		R1 5'-GTATCAATCTAGTGTCCACCAAACC-3'
Hv.24396	**11289**	F1 5'-CATGTTCAACGAGAACCAAAAG-3'
		R1 5'-TGAGATCGACCGAAGTAGTAG-3'
Hv.10307	13350	F1 5'-TCTTCCCCAACAAGCAACC-3'
		R1 5'-GTTAGAGCATTCACAAGCCC-3'

Real-time RT-PCR

Real-time RT-PCR was carried out as a two-step procedure. Reverse transcription was carried out using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions, with 30 ng of total RNA as template in a total reaction volume of 20 µl. The PCR step was accomplished with iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions, with 1 µl of the cDNA reaction as template. Three biological replicates (consisting of eight individuals each) were analyzed for each treatment. All reactions were prepared as duplicates in each run. A no-template control was run for each primer pair. Actin was used as reference gene for normalization (primers; Actin F 5'-TTCTCGACTCTGGTGATGGTGT-3' and Actin R 5'-CAAGCTTCTCCTTGATGTCCCT-3'). Cycling conditions on MyIQ™ Single-Color Real-Time PCR Detection System (Bio-Rad) were: 50°C for 10 min, 95°C for 5 min and 45 cycles of 95°C for 10 s and 59°C for 30 s. A melting curve was run after the PCR starting at 95°C for 1 min, 55°C for 1 min and then 80 cycles, increasing each cycle by 0.5°C, starting at 55°C for 10 s.
For calculating relative transcription ratio between sample and control compared to reference gene, a formula by Pfaffl (2001) was used (ratio = $(E_{target})^{\Delta CPtarget\ (control\text{-}sample)}/(E_{ref})^{\Delta CPref\ (control\text{-}sample)}$). Results were correlated with primer efficiency by using LINREGPCR software (Ramakers et al. 2003).
The significance of changes in transcript accumulation was analyzed by performing *t*-test on log2-transformed data, comparing samples from BCA- and RWA-infested plants with control plants from the same time point.

Results

Callose distribution in control tissues

The development of wound callose, associated with the damage caused by scraping the leaves, was effectively minimized by scraping under Ca^{2+}-free MES buffer pH 7.2. The principal results for the fluorescence study are illustrated in Figs 1–3. In control leaf tissue, there was little if any callose, except that associated with SPs and pore-plasmodesmal units (PPUs) in small (SV), intermediate (IV) vascular bundles or in cross veins (XV) after 72 h (Fig. 1). A similar response was observed after 24 and 48 h (data not shown). There was no obvious difference in callose deposition between source or sink leaves (Fig 1A, C, E compared with Fig. 1B, D, F), or depending on the presence of an (empty) aphid cage (Fig. 1C, D) as compared with sections above (Fig 1A, B) or below (Fig. 1E, F) the cage.

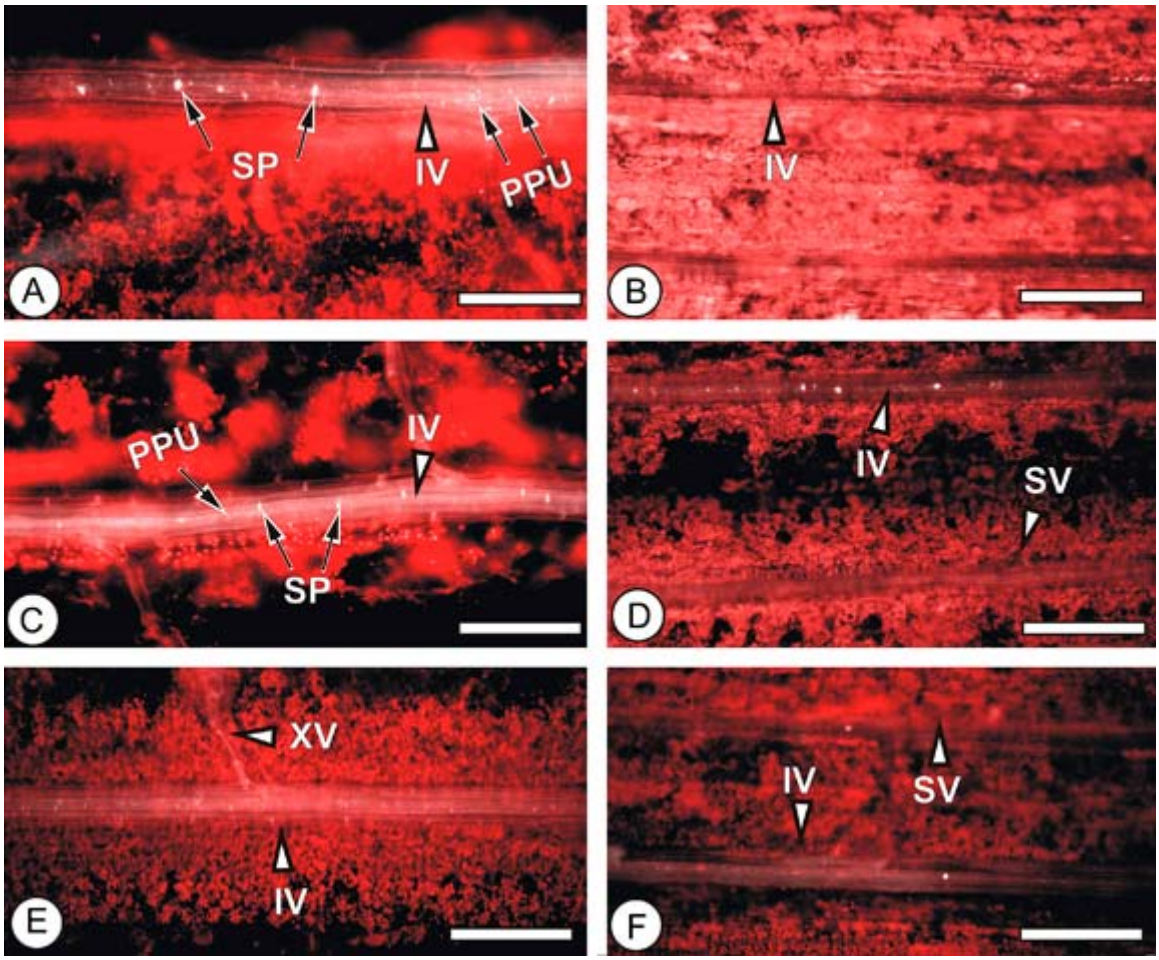

Fig. 1. Longitudinal sections of control, uninfested barley leaves stained for callose distribution and viewed using a wide-field fluorescence microscope. (A, B) Part of leaf blade above an empty aphid cage. (C, D) Section from leaf portion within an empty aphid cage. (E, F) Part of leaf blade below an empty aphid cage. (A, C, E) Source leaf. (B, D, F) Sink leaf. (Bars: A, C and E = 100 μm; B, D and F = 200 μm).

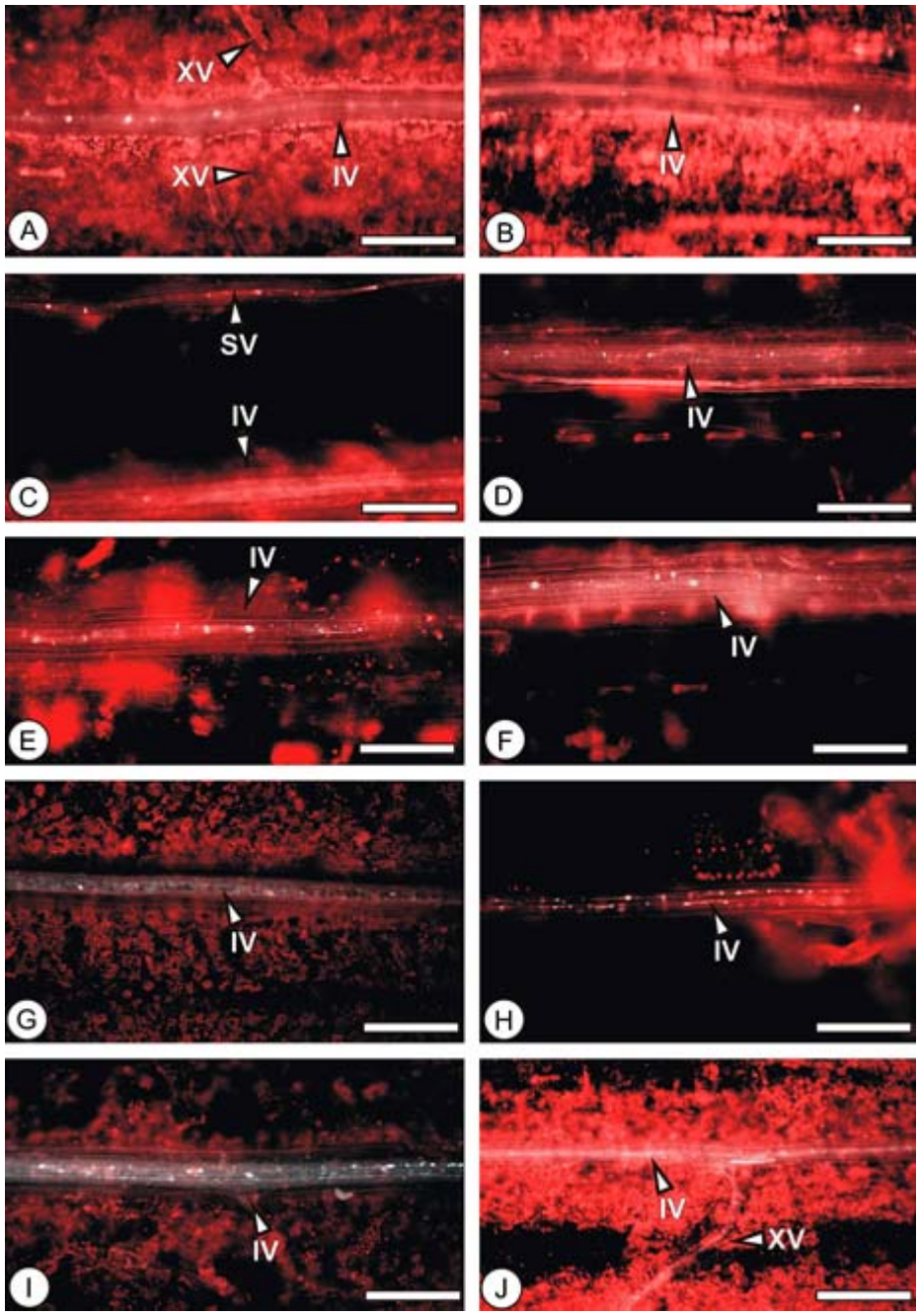

Fig. 2. Longitudinal sections of barley leaves after feeding by BCA, stained for callose distribution and viewed using a wide-field fluorescence microscope. (A–F) after 72 h of feeding, G-J after 14 days of feeding. (A, B, G) Part of leaf blade above (acropetal to) the aphid cage. (C, D, I, J) Section from leaf portion within the aphid cage. (E, F, H) Part of leaf blade below (basipetal to) the aphid cage. (A, C, E, G–J) Source leaf. (B, D, F) Sink leaf. (Bars: A, B, D–J = 100 μm; C = 200 μm).

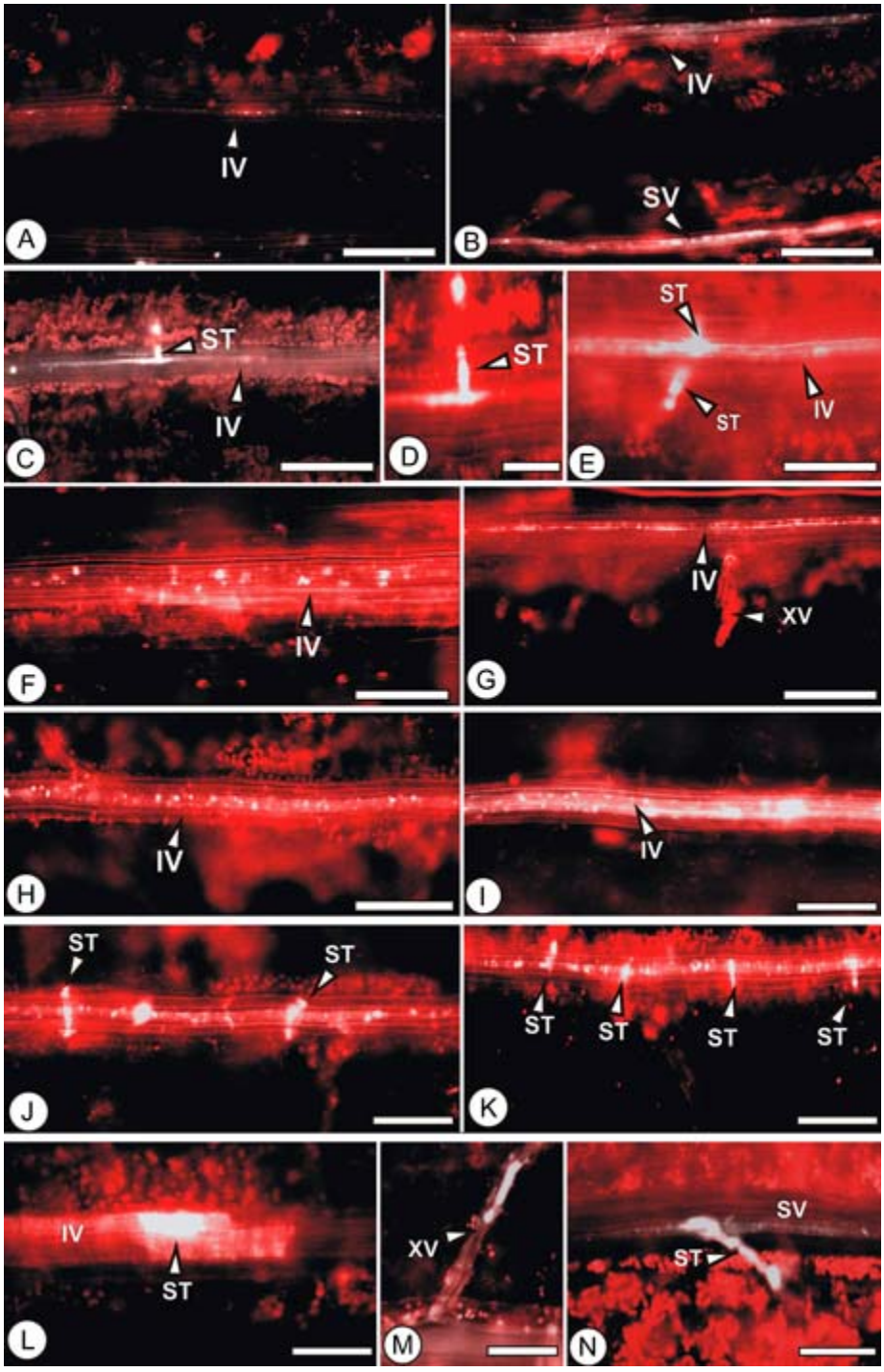

Fig. 3. Longitudinal sections of barley leaves after feeding by RWA, stained for callose distribution and viewed using a wide-field fluorescence microscope. (A–G) after 72 h of feeding. H-M after 14 days of feeding. (A, B, G, H) Part of leaf blade above (acropetal to) the aphid cage. (C, D) ST from C in high magnification (E, J–N) Section from leaf portion within the aphid cage. (F, I) Part of leaf blade below (basipetal to) the aphid cage. (A, C, D, F, H–N) Source leaf. (B, E, G) Sink leaf. Note the extensive deposition of callose in vascular parenchyma and phloem elements along longitudinal intermediate veins (IV, J-L) and cross veins (XV, M and N). Many ST are completely occluded with callose in these veins. (Bars: A, B = 200 μm; C, E–K = 100 μm; D, L–N = 50 μm).

Callose deposition in BCA-infested tissues

The accumulation of callose in leaf blades as a response to feeding by BCA is illustrated in Fig. 2. Sections from leaves taken after short-term feeding (72 h) did not differ from control tissue: callose-associated fluorescence in SV, IV and XV was limited to that associated with SPs and PPUs, and there was no callose deposition in longitudinal veins (Fig. 2. A–F). There was also little deposition of callose observed after 7 days of BCA feeding (data not shown), but after 14 days of continuous feeding there were limited callose depositions (Fig. 2G–J). Little or no callose appeared along the longitudinal vein upstream of the aphid feeding site (Fig. 2G). However, downstream of the aphid site (Fig. 2H) and at the area of the feeding site (Fig. 2I, J), limited callose was characteristically found in longitudinal veins. It is important to note that leaves, which started out as sinks at the beginning of the experiment described here, would have matured into strong sources of assimilate during the 14 days duration of the experiments.

Callose distribution in RWA-infested tissues

In leaf tissue that had been infested by RWA, callose formation appeared within 24 h (data not shown) becoming more evident after 72 h (Fig. 3A–G). Fig. 3A shows limited callose deposition in longitudinal veins acropetal to the aphid feeding zone in a source leaf. In contrast, deposition upstream of the aphid feeding zone in a sink leaf was extensive (Fig. 3B). In the aphid feeding zones (Fig. 3C–E) callose deposition occurred in longitudinal veins, in both source (Fig. 3C) and sink (Fig. 3E) leaves. Aphid stylet tracks (ST) indicated strong callose formation (Fig. 3C, D). Callose was widespread below the feeding sites in source leaves (Fig. 3F), (compare Fig. 3F to Fig. 3A, which is an upstream source). Callose was also formed below the feeding sites in sink leaves (Fig. 3G), but generally less intense in comparison with the upstream sink (Fig. 3B). Callose deposition increased progressively between 7 and 14 days of RWA feeding, but after 14 days (Fig. 3H–M) no further increase was observed (not shown). Comparison of feeding sites above (Fig. 3H) or below (Fig. 3I) aphid feeding zones, respectively, illustrate that callose was always more extensively basipetal in source leaves.

Regulation of callose synthases and β-1,3-glucanases at RNA level

Changes in steady state levels of transcripts from callose synthase and β-1,3-glucanase genes were examined using RT-PCR and real-time RT-PCR. Primers were designed for all eight putative callose synthase sequences identified in database searches, of which four are present on the Barley1 GeneChip (Table 1). Of the eight barley sequences, all but one (Hv.4615, which is represented as contig4949 on Barley1) was found by RT-PCR to be expressed in the tissue (data not shown). There was no clear regulation of any of the sequences detectable. To be sure of this result, the seven putative callose synthase-coding sequences that were expressed were analyzed by real-time RT-PCR, but none of them showed any change in transcript accumulation upon aphid infestation. Fig. 4 shows real-time results for two representative examples, *HvGSL1* and Hv.22049. They are selected because of the expressed sequences, *HvGSL1* represents the only full-length *GSL* from barley and Hv.22049 had the highest similarity to *AtGSL6*, which was induced by silverleaf whitefly nymphs and the cabbage aphid (*Brevicoryne brassicae*) in *Arabidopsis* (Kempema et al. 2007, Kusnierczyk et al. 2008). For the study of β-1,3-glucanase transcriptional regulation, primer pairs were designed for 12 out of 16 unigenes identified in a database search (four were too similar in sequence to other glucanases for the design of specific primers). Of the 16 unigenes identified, 6 are annotated on the Barley1 GeneChip; the other 10 are highly similar to Barley1 contigs (Table 1). Of the 12 sequences that were analyzed, only 5 (Hv.8964, Hv.18110, Hv.19837, Hv.24036 and Hv.26605) were expressed, while no RT-PCR products

were detected for the other seven sequences (data not shown). Real-time (RT)-PCR analysis revealed that all five were upregulated by RWA, three of them (Hv.8964, Hv.24036 and Hv.26605) as early as 1 day after infestation (Fig. 5). The effect of BCA infestation was much less evident, with upregulation being significant only at 2 and 3 days for Hv.18110 and at 2 days for Hv.24036. For the other seven β-1,3-glucanase sequences, no transcripts could be detected by RT-PCR, and real-time analysis gave no indication of regulation either (data not shown).

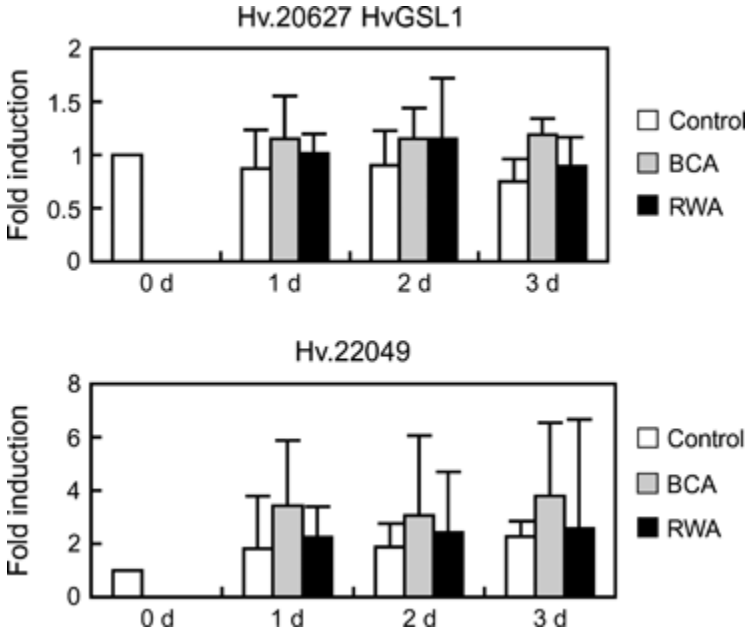

Fig. 4. Transcript accumulation of two of the putative callose synthase sequences during aphid infestation. Real-time (RT)-PCR with primers for callose synthase sequences *HvGSL1* and Hv.22049. RNA was extracted after 0, 1, 2 and 3 days of aphid infestation as indicated. Transcript accumulation was normalized with actin and fold induction is calculated relative to day 0. Columns represent means of three biological replicates and standard deviations are indicated by bars.

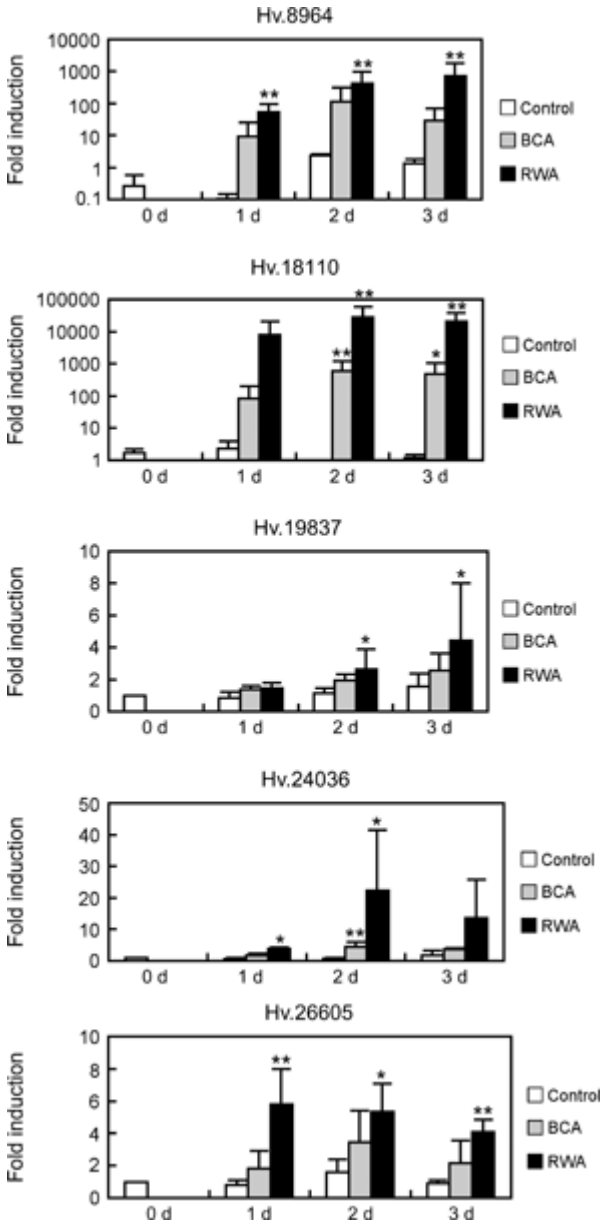

Fig. 5. Difference in β-1,3-glucanase transcript accumulation depending on aphid species and time of infestation. Real-time (RT)-PCR for the five β-1,3-glucanases that were transcribed in barley leaves. RNA was extracted after 0, 1, 2 and 3 days of aphid infestation as indicated. Transcript accumulation was normalized with actin and fold induction is calculated relative to day 0. The unigene numbers correspond to contigs on Barley1 GeneChip as follows: Hv.8964 to contig11289, Hv.18110 to contig1637_s (isozyme GII), Hv.19837 to contig10477, Hv.24036 to contig1636 (isozyme GIII) and Hv.26605 to contig1639. Columns represent means of three biological replicates, with standard deviations indicated by bars. Asterisks indicate statistically significant regulation as compared with control at the same time point (determined by *t*-test; $*P < 0.05$; $**P < 0.01$).

Discussion

The current investigation confirms the earlier findings that the pattern of callose deposition in barley as caused by the two aphid species, BCA and RWA, differs (Saheed et al. 2007). At moderate infestation,

BCA did not cause callose deposition within 7 days of infestation, whereas callose was seen already after 24 h in RWA-infested tissue. However, after 14 days of infestation, BCA caused limited callose deposition. This was also observed within shorter infestation times with a higher aphid load of BCA (not shown). This indicates that both aphid species are able to induce callose, but that the callose-inducing signal from BCA is weaker as compared with RWA or that the plant defense against BCA is more efficient. In RWA-infested tissue, ST were always associated with extensive callose, suggesting that components of the RWA saliva trigger callose formation.

Our data demonstrate that SP pores, PPU and related plasmodesmata are blocked by callose deposition in RWA-infested plants, which eventually would result in a marked decrease and possibly cessation of transport of assimilates via the phloem. This may be the reason for the symptoms of yellow, white or purple streaks and leaf rolling that are a consequence of RWA infestation (Walters et al. 1980). The detection of callose depositions within 24 h is in line with earlier findings that reduction in transport of assimilates becomes apparent when RWA feeds in wheat leaves (Botha and Matsiliza 2004) and within the first 24 h of infestation in barley (S. S. A., B. C. E. J.).

Transport of assimilates in mature leaves is known to follow classical source to sink pattern (see review by Turgeon 1989), with basipetal (lamina tip to base) movement in source leaves and acropetal (lamina base to tip) movement in sink leaves. Transition leaves can transport in either direction, but toward the stronger sink region. Our data suggest that callose-inducing signals are transported from the aphid feeding site in the sieve elements and that the direction of transport is influenced by source–sink relationships. For example, in RWA-infested source leaves, extensive callose deposition was observed below the area of feeding compared with the area above the feeding site. The reverse was true for sink leaves where more callose deposition occurred above the feeding sites than below them. Limited acropetal transport could have accounted for the callose deposition in areas above the feeding site in source leaves and limited basipetal transport in the area below feeding sites in sink leaves.

We examined whether the callose accumulation was because of increased accumulation of *GSL* gene transcripts in barley leaf blades. Our results did not support transcriptional regulation, as the seven genes that were expressed in leaf blades did not show any consistent changes in transcript accumulation upon aphid infestation. Based on barley EST libraries, Li et al. (2003) concluded that in barley at least six *GSL* genes could be expected. This number corresponds to the eight sequences that we were able to identify. However, in *Arabidopsis* 12 and in rice 10 putative callose synthase genes were annotated (Verma and Hong 2001, Yamaguchi et al. 2006). We therefore cannot exclude the possibility that there are more than eight GSL sequences in barley and that callose biosynthesis in barley leaf blades resultant from aphid attack might be caused by increased transcriptional accumulation of another as yet unidentified *GSL* gene. In *Arabidopsis*, *GSL6* is the only *GSL* gene that was induced by phloem-feeding silverleaf whitefly nymphs, in tissue where callose deposition was found at feeding sites and in vascular tissue (Kempema et al. 2007). The same gene was found to be induced in *Arabidopsis* by the cabbage aphid, where callose depositions were also detected at the stylet insertion sites (Kusnierczyk et al. 2008). Another *Arabidopsis GSL* gene, *GSL5,* has been functionally characterized and shown to be involved in wound callose and papillary callose formation (Jacobs et al. 2003). The *Arabidopsis GSL* genes have been positioned in a phylogenetic tree with callose synthase genes in rice, where 10 *OsGSL*s were identified (Yamaguchi et al. 2006). Of the eight barley sequences studied here, Hv.4615 (contig4949) was most similar to *AtGSL6* [84.9% identity over 589 base pairs (bp)], but it was not expressed. The other two sequences with similarity to *AtGSL6* were Hv.22049 (80.3 % identity over 279 bp) and Hv.17389 (71.6 % identity over 101 bp). However, these similarities are based on partial overlaps, as no full-length cDNA sequence for the three barley unigenes are available and may thus not reflect the phylogenetic relationship correctly. The only full-length *GSL* from barley, *HvGSL1*, has highest similarity among the *Arabidopsis GSL* sequences to *AtGSL10*. None of the eight barley sequences studied here seems to be orthologous to *GSL5* from *Arabidopsis*.

The findings that none of the eight putative barley *GSL* sequences studied here was regulated transcriptionally upon aphid attack suggest that callose deposition is regulated at the protein level rather than at the transcriptional level. Other evidence for regulation at protein level is for instance that wound

callose often appears within 5–10 min of injury stimulation (Nakashima et al. 2003) and that callose synthesis can be activated by changes in the intracellular distribution of a glucoside activator (Ohana et al. 1993). Strong evidence exists that callose synthase can occur in membrane-associated complexes with several other proteins (Verma and Hong 2001). The release, or binding of separate units from such a complex could rapidly switch callose synthesis on and off, with no need for de novo synthesis of glucan-β-1,3-synthase. Furthermore, in vitro studies have shown callose synthesis to be activated by calcium and by proteases (Kauss 1985, Nakashima et al. 2003). It is possible that penetration of aphid stylets in some cases causes leakage of extracellular calcium into sieve elements and thereby an increase of internal calcium levels (Will and van Bel 2006). Analysis of the protein content of saliva from the aphid *Megoura viciae* revealed the presence of several Ca^{2+}-binding proteins (Will et al. 2007). It could be that differences in the Ca^{2+}-binding capacity of the saliva from BCA and RWA could lead to differences in callose deposition through activation of callose synthases. In conclusion, none of the eight putative barley *GSL* sequences identified here was regulated transcriptionally by infestation with two aphid species that differ in their ability to induce callose deposition. Thus the role of regulation of the activity of *GSL*s at protein level and the involvement of aphid salivary proteins in this process need to be investigated to reveal their involvement in callose deposition upon aphid attack.

Callose is degraded by β-1,3-glucanases. In database searches at NCBI we identified 16 putative β-1,3-glucanase genes, 12 of which were analyzed further. Five were expressed in leaves and induced by RWA. Two of them are almost identical to earlier studied barley β-1,3-glucanases: Hv.18110 (contig1637_s; AJ271367; isoenzyme GII) and Hv.24036 (contig1636; X67099; isoenzyme GIII), both of which have a potential N-terminal signal peptide and basic pI; 9.8 for Hv.18110 (isoenzyme GII) and 10.3 for Hv.24036 (isoenzyme GIII) (Hrmova and Fincher 1993). Isoenzyme GII is expressed in the aleurone (Wang et al. 1992), but was induced in leaf tissue by barley powdery mildew (Xu et al. 1992) and the pathogen *Bipolaris sorokiniana* (Jutidamrongphan et al. 1991). Isoenzyme GIII is expressed in young developing leaves (Wang et al. 1992). It was not induced by *Blumeria graminis* (Xu et al. 1992), but more recently, it has been shown that activation of the promoter is salicylic acid-dependent and that the gene is induced by salicylic acid (Li et al. 2005). Both isoenzymes GII and GIII were also induced in barley leaves infected with the leaf scald fungus (*Rhynchosporium secalis*) (Roulin et al. 1997). The results suggest that the two basic β-1,3-glucanases that were initially shown at protein level to be induced by BCA in barley probably correspond to Hv.18110 and Hv.24036 (Forslund et al. 2000). With regard to β-1,3-glucanases, earlier shown to be upregulated in wheat by RWA (van der Westhuizen et al. 1998, 2002), there is no sequence information available and we therefore cannot speculate whether they correspond to the barley genes identified here. We hypothesized that the difference in callose accumulation between RWA and BCA might be because of a stronger induction of β-1,3-glucanases leading to a more efficient breakdown of callose in BCA-infested tissue. However, as transcript accumulation was higher after infestation with RWA than BCA, there is no support for this idea.

We have found before that callose induced by RWA feeding in wheat persisted for up to 48 h after removal of aphid colonies (Botha and Matsiliza 2004). In a separate study we have found that in barley callose persists for up to 120 h (data not shown) after aphid removal. This suggests that aphid-induced callose, in contrast to wound callose that may disappear over the course of days (Currier and Webster 1964), is not degraded or removed either by the feeding aphids or the affected plant.

In conclusion, the results presented here indicate that callose deposition is aphid species-specific in the barley cultivar studied, with RWA causing a much stronger response than BCA. The differences in callose induction between RWA and BCA are most probably caused by a stronger synthesis of callose in RWA-infested leaves. Further studies will be needed to find the mechanism responsible for this process.

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