Lack of the plant signalling component SGT1b enhances disease resistance to *Fusarium culmorum* in Arabidopsis buds and flowers

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Summary

• *Fusarium culmorum* causes ear blight disease on cereal crops resulting in considerable losses to grain yield, quality and safety. This fungus can also infect Arabidopsis floral tissues. In this study, the Arabidopsis floral infection model was used to assess the impact of five defence mutants on disease.

• *Fusarium culmorum* was spray inoculated onto the floral tissues of the mutants *eds1*, *lms1*, *rar1*, *sgt1a* and *sgt1b* involved in basal and resistance gene-mediated defence to pathogens. Floral disease development was assessed quantitatively.

• Only the *sgt1b* mutant exhibited a significantly different interaction phenotype compared with wild-type plants. The buds and flowers were more resistant to infection and developed milder symptoms, but had wild-type levels of deoxynivalenol (DON) mycotoxin. Microscopic studies indicated that to cause disease, *F. culmorum* requires plant cells in the invaded tissues to be competent to activate both a cell death response and a sustained oxidative burst. The *sgt1a* mutant exhibited a weak trend towards greater disease resistance in the new silique tissues.

• This study highlights that the SGT1-mediated signalling cascade(s), which had previously only been demonstrated to be required for Arabidopsis resistance against biotrophic pathogens, is causally involved in *F. culmorum* disease symptom development.

Introduction

The ascomycete fungi *Fusarium culmorum* and *Fusarium graminearum* (teleomorph *Gibberella zeae*) cause ear blight disease, which is a global problem on wheat, maize, barley and rye, reducing grain yield, quality and safety. This disease is also referred to as head blight or head scab (Parry *et al.*, 1995; Goswami & Kistler, 2004). *Fusarium* species synthesize various mycotoxins *in planta*, including the B-type sesquiterpenoid epoxide trichothecenes, deoxynivalenol (DON), 3-acetyl DON, 15-acetyl DON and nivalenol, which are of particular concern to human and animal health (Hohn *et al.*, 1998). In wheat, resistance to *Fusarium* is species nonspecific and no race structure within a single *Fusarium* species has been shown to exist (Bai & Shaner, 2004). Several types of host resistance have been defined, the main ones experimentally verified are Type I to primary infection and Type II to subsequent colonization after infection (Mesterházy, 1995). Currently, the most useable forms of resistance are polygenically inherited and several major quantitative trait loci (QTL) have been defined. The 3BS QTL contributes to the Type II resistance and detoxification of the DON mycotoxin (Waldron *et al.*, 1999; Lemmens *et al.*, 2005). This QTL has recently been Mendelized and is now referred to as the *Fhb1* resistance locus (Cuthbert *et al.*, 2006). Although the genetic basis of resistance has been established, the molecular basis of resistance to *Fusarium* ear blight in cereals remains poorly understood.

In plant defence, many functional plant resistance (*R*)-genes have been identified and cloned as well as components of the downstream defence signalling network underlying R-protein, basal and nonhost resistance and those of importance to the induction of systemic responses (Hammond-Kosack & Parker, 2003; Jones & Dangl, 2006; Hammond-Kosack & Kanyuka, 2007). Key defence signalling components include RAR1 (required for *Mla12* resistance) and SGT1 (suppressor of G2 allele of *skp1*). These proteins are involved in triggering various

responses including production of reactive oxygen species (ROS), localized programmed plant cell death (the hypersensitive response (HR)) and the accumulation of salicylic acid (SA) and antimicrobial pathogenesis-related (PR) proteins (Hammond-Kosack & Parker, 2003). RAR1 and SGT1 are required for the function of many R-proteins in both monocotyledonous and dicotyledonous plants (Azevedo *et al.*, 2002; Liu *et al.*, 2002; Muskett *et al.*, 2002; Tor *et al.*, 2002; Tornero *et al.*, 2002; Shirasu & Schulze-Lefert, 2003). They have also been implicated in basal and nonhost resistance (Peart *et al.*, 2002; Ham *et al.*, 2007; Noel *et al.*, 2007). In Arabidopsis there are two *SGT1* gene homologues, coding for the SGT1a and SGT1b proteins which exhibit 87% homology (Austin *et al.*, 2002). SGT1b has been shown to have a role in defence (Azevedo *et al.*, 2006). Heat shock protein 90 (HSP90) is able to interact with SGT1 and RAR1 in barley and tobacco as well as SGT1a, SGT1b and RAR1 in Arabidopsis to regulate R-protein stability (Hubert *et al.*, 2003; Takahashi *et al.*, 2003; Liu *et al.*, 2004). Upstream of the SGT1/RAR1/HSP90 complex is the EDS1 (enhanced disease susceptibility 1) protein which has some homology to lipases. In Arabidopsis, EDS1 is required for the function of several R-proteins (Aarts *et al.*, 1998), basal resistance to virulent isolates of several pathogens (Parker *et al.*, 1996; Aarts *et al.*, 1998; Xiao *et al.*, 2005) and nonhost resistance to two biotrophic pathogens of *Brassica oleracea* (Parker *et al.*, 1996).

Plant pathogens are generally classified as biotrophs, which derive nutrients from living host tissues, necrotrophs, which derive nutrients from dead or dying cells, and hemibiotrophs, which are initially in a biotrophic interaction that subsequently becomes partially or completely necrotrophic (Agrios, 1997). Host defence against biotrophic pathogens generally requires R-proteins which rapidly trigger programmed cell death (PCD) and activate the defence responses associated with the SA and the NPR1 (nonexpresser of pathogenesis protein 1) signalling pathway. Activation of the latter leads to the expression of numerous defence-related genes, for example *PR-1*. By contrast, necrotrophic pathogens benefit from host cell death, and so are not limited by cell death and SA-dependent defences. For effective defence against necrotrophs, functional jasmonic acid (JA) and ethylene (ET) signalling pathways are required and result in the expression of a different suite of defence genes, for example *PDF1.2* (Thomma *et al.*, 1998; Hammond-Kosack & Parker, 2003; Glazebrook, 2005).

The floral tissues of Arabidopsis are susceptible to infection by *F. culmorum* and *F. graminearum* (Urban *et al.*, 2002; Cuzick *et al.*, 2008a,b). Following spore germination, a mass of superficially colonizing hyphae develop, and then the mycelium engulfs the entire floral tissue. Subsequently, tissue penetration and colonization occurs which causes the formation of a grey/brown necrotic area initially on petals and sepals and eventually constriction of pedicel and upper stem tissues. Deoxynivalenol mycotoxin production occurs in the infected floral tissues. Parallel microscopic studies revealed that the *Fusarium* hyphae initially advance through living host tissue and cell death was only evident well behind the hyphal front. Floral spray inoculation of 236 Arabidopsis ecotypes failed to identify single genotypes exhibiting either enhanced resistance or susceptibility to either *Fusarium* species (Urban *et al.*, 2002). More recent studies have revealed that full disease can occur in the absence of fungal DON mycotoxin production (Cuzick *et al.*, 2008b). Using the floral inoculation protocol devised by Urban *et al.*, (2002), it was revealed that floral tissues of the *esa1* mutant were significantly more susceptible to *F. culmorum* than the corresponding wild-type ecotype Col-0 (Van Hemelrijck *et al.*, 2006). Previously, the *esa1* mutant was demonstrated to exhibit enhanced susceptibility to *Alternaria brassicicola* and other necrotrophic fungal pathogens (Tierens *et al.*, 2002). The *ESA1* gene has not yet been isolated. By contrast, over-expression of the Arabidopsis *NPR1* gene in wheat led to a reduction of *F. graminearum* disease levels in the floral spikelets (Makandar *et al.*, 2006). In a recent study exploring mutations in the SA, JA and ET defence signalling pathways, we reported that the *npr1* mutant but not the *sid2* mutant led to an increase in *F. culmorum* floral disease compared with the Col-0 wild-type plants (Cuzick *et al.*, 2008a). This increased disease susceptibility also resulted in higher levels of DON production. However, the effects of mutations in the other two defence signalling pathways were found to be either absent (JA/ET combined), absent/minimal (ET) or inconclusive (JA). In the Arabidopsis root–*Fusarium oxysporum* pathosystem, SA has been shown to be required to activate both systemic acquired resistance as well as local root resistance to this fungus. Also the SA, ET and JA signalling pathways have been demonstrated to interact in a positive way to activate resistance to this necrotrophic vascular invading pathogen. While abscisic acid (ABA) signalling is thought to function in plant resistance to *F. oxysporum*, its precise role is currently unclear (for review see Berrocal-Lobo & Molina, 2008).

To further understand the defence signalling pathways involved in *Fusarium* floral disease of Arabidopsis, single gene mutants of key defence signalling genes typically required for R-protein and basal resistance to biotrophs were assessed for their effect on the interaction outcome because the advancing *F. culmorum* hyphae grow through living plant tissue. Also, little is known about the role of defence signalling proteins in floral tissues, because they have generally been identified within pathosystems involving leaves, roots or the stem base. In this study, Arabidopsis defence mutants in the Landsberg *erecta* (L*er*-0) and Wassilewskija (Ws-2) genetic backgrounds were investigated. The gene mutants investigated were *eds1*, *rar1*, *sgt1a* and *sgt1b*, which are known to be critical for defence against biotrophic pathogens. The infection biology of *F. culmorum* in floral tissue is similar to the initial leaf infection phase by the hemibiotrophic fungus *Leptosphaeria maculans* which infects *Brassica* crops (Hammond & Lewis, 1986). Therefore, the floral defence of the Arabidopsis *lms1* mutant, which causes enhanced *L. maculans* susceptibility in leaves,

was also assessed (Bohman *et al.*, 2004). This study has revealed that the removal of the function of the plant defence-signalling component SGT1b, results in enhanced floral disease resistance to *F. culmorum*, although DON mycotoxin levels remain similar to wild-type. The *eds1*, *lms1* and *rar1* mutants tested had no discernable effect on the interaction outcome.

Materials and Methods

Arabidopsis germplasm, *Fusarium culmorum*, growth and maintenance

Arabidopsis seed stocks Ler-*eds1-2*, Ler-*sgt1b-1* and Ler-*rar1-10* were a gift from Jane Parker (Max Planck Institute for Plant Breeding, Germany), Ws-*sgt1a-1* seed was provided by Ken Shirasu (Riken, Japan) and the L*er*-*lms1-1* seed was obtained from Christina Dixelius (SLU, Sweden). Ws-2 and Ler-0 were obtained from the Nottingham Arabidopsis Stock Centre, UK. Plants were grown as described previously (Cuzick *et al.*, 2008b).

The *F. culmorum* strain 98/11, was propagated and prepared for plant inoculations as described previously (Cuzick *et al.*, 2008a).

Fusarium–Arabidopsis floral inoculation and scoring

Floral spray inoculation of plants was done as described previously (Cuzick *et al.*, 2008a,b). Each plant was sprayed with approx. 500 000 conidia (0.5 ml of a 1×10^6 conidia ml^{-1} suspension in water). The numerical *Fusarium*–Arabidopsis disease individual floral component (FAD-I value) scoring system was used to assess macroscopic disease symptoms at 8 d post inoculation (dpi). Apically wounded silique inoculations were done as described previously (Cuzick *et al.*, 2008b), by placing a 2 μl droplet of inoculum consisting of approx. 2000 conidia for strain 98/11 on the wounded tip of each green silique (*c.* 10 mm length).

Trichothecene mycotoxin analysis

The commercially available Ridascreen Fast DON enzyme-linked immunosorbent assay (ELISA) kit (R-Biopharm AG, Darmstadt, Germany) was used to quantify DON mycotoxin as described previously (Cuzick *et al.*, 2008a). In each experiment, floral tissue from six plants was pooled and each experiment was done in triplicate.

Microscopy

Light, UV and scanning electron microscopy were done as described previously (Cuzick *et al.*, 2008a,b).

Histochemical stains

Floral tissues were stained with lactophenol–trypan blue to identify regions of cell death (Koch & Slusarenko, 1990). Samples were harvested into the stain and boiled for 1 min. The chlorophyll was then cleared using 2.5 g ml^{-1} chloral hydrate solution, and finally the tissues were mounted in 70% (v : v) glycerol for light microscopy.

Staining for hydrogen peroxide was done by immersing tissue in an aqueous solution of 3,3′-diaminobenzidine (DAB) (1 mg ml^{-1} at pH 3.8) for 24 h (Thordal-Christensen *et al.*, 1997). Staining for superoxide was done by immersing tissue in a nitroblue tetrazolium (NBT) solution (1 mg ml^{-1} dissolved in 10 mM potassium phosphate buffer at pH 7.8 with 1 mM sodium azide) for 1 h (Jabs *et al.*, 1996). Stained tissues were subsequently cleared of chlorophyll using 3 : 1 (v : v) ethanol–dichloromethane, 0.15% (w : v) trichloroacetic acid and stored in 70% (v : v) glycerol before examination by light microscopy. Water-only sprayed or *F. culmorum* conidia inoculated floral tissues were stained at 2, 4 and 7 dpi. Noninoculated floral and leaf tissues were stained after 5 wk of plant growth.

Statistical analyses of *Fusarium*–Arabidopsis data

A total of 14 independent experiments, consisting of randomized block designs, were done to compare *F. culmorum* disease severities between each Arabidopsis mutant and the corresponding wild-type genotype (either L*er*-0 or Ws-2). Each genotype was tested in at least three independent experiments. Statistical analysis was done separately for each genetic background (L*er*-0 or Ws-2) using GENSTAT 8.0 (Payne *et al.*, 2005) as described previously (Cuzick *et al.*, 2008a).

Results

Relative transcript abundance in floral tissue for genes implicated in basal and R-protein mediated resistance

In most studies, Arabidopsis defence gene activation has been studied in root, stem base or leaf tissue, as part of the local or systemic response to pathogen attack. To determine whether the same defence signalling genes were also expressed in floral tissues the Gene Atlas tool from GENEVESTIGATOR (Zimmermann *et al.*, 2004) was queried for tissue specific expression of *EDS1*, *HSP90.1*, *HSP90.2*, *RAR1*, *SGT1a* and *SGT1b* (see the Supporting Information, Fig. S1 and Text S1). The *LMS1* gene has not yet been isolated. This GENEVESTIGATOR analyses revealed that all the defence signalling genes investigated within this study were expressed in some Arabidopsis floral tissues.

Inoculation of five different defence mutants revealed that *sgt1b* buds and flowers were more resistant to *F. culmorum* infection

Arabidopsis defence signalling mutants previously implicated in basal and R-protein mediated resistance were spray inoculated with *F. culmorum* strain 98/11. Floral disease levels

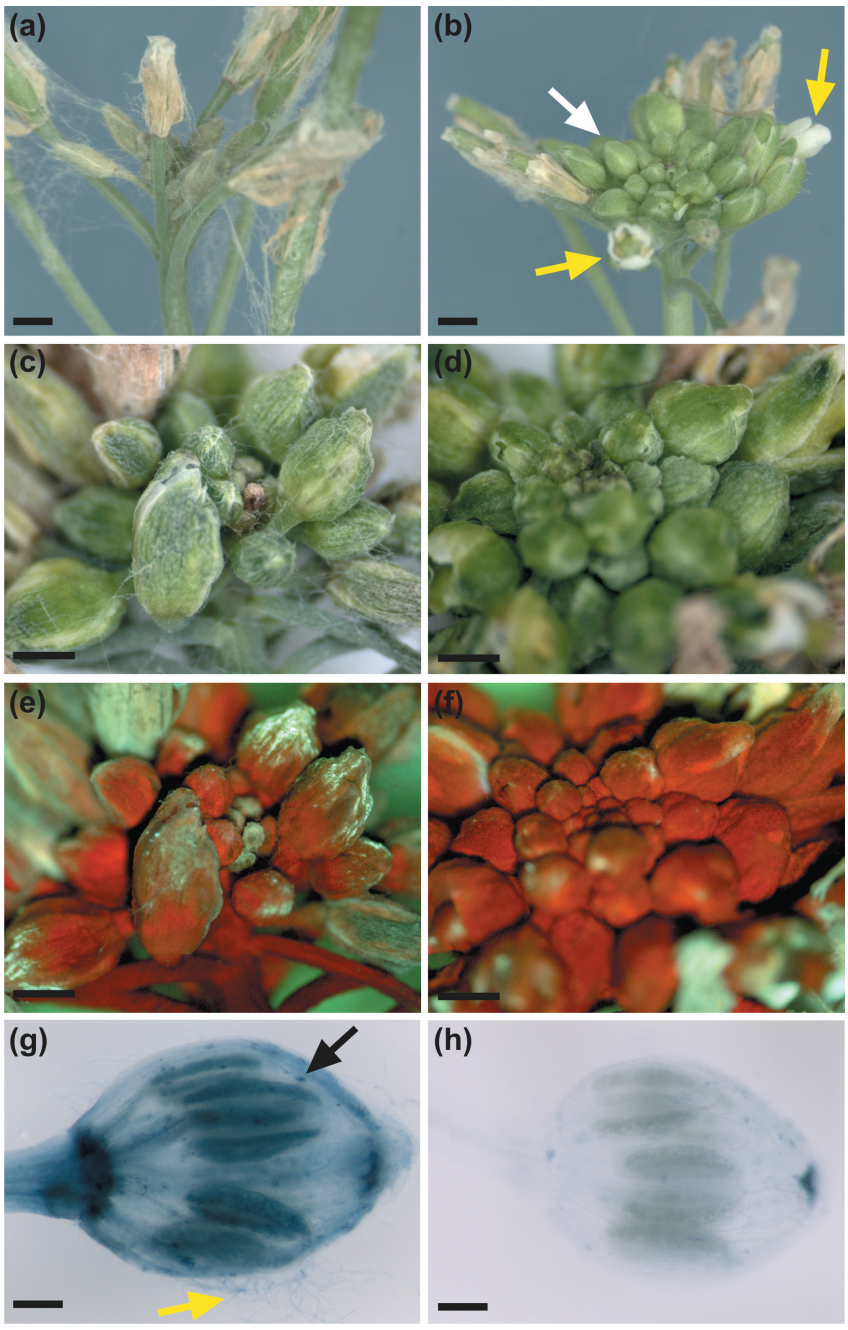

Fig. 1 The *sgt1b* flowers exhibit less disease caused by *Fusarium culmorum* than wild-type L*er*-0. (a,b) Wild-type (a) and *sgt1b* mutant (b) Arabidopsis floral tissues spray-inoculated with *F. culmorum* conidia and photographed at 7 d post inoculation (dpi). Wild-type floral tissues were covered with aerial mycelium; most buds were drying out and had failed to open. Lower levels of aerial mycelium were visible on *sgt1b* floral tissues; buds generally remained green (white arrow) and flowers continued to open (yellow arrows). Bar, 1 mm. (c–f) Close-up of wild-type (c,e) and *sgt1b* mutant (d,f) flower buds viewed under light (c,d) or UV light (e,f) at 7 dpi. Under UV light the chlorophyll present in the healthy green tissues autofluoresced red, whereas blue-green autofluorescence was visible from the colonized tissues. More blue-green autofluorescent regions were present on the heavily diseased wild-type L*er*-0 plants (e) than the *sgt1b* mutant plants (f). Bar, 0.5 mm. (g,h) Trypan blue stained representative buds taken from either (c) or (d). Patches of dead cells (black arrow) and fungal hyphae (yellow arrow) were visible on the heavily colonised wild-type buds (g). Bar, 0.2 mm.

were assessed to determine whether disease levels were altered between the single gene mutant *eds1*, *lms1*, *rar1*, *sgt1b* and the wild-type L*er*-0 or *sgt1a* and the wild-type Ws-2. The assessments for disease phenotypes were divided into two floral subcomponents: (1) unopened and opened flowers and (2) new siliques that were fully open flowers at inoculation. Although the entire plant received inoculum, all the genotypes tested in the present study again only permitted disease development in the floral tissue (Urban *et al.*, 2002). No obvious differences were seen in plant heights, general stature or floral morphology between any of the mutants and their corresponding wild-types before inoculation (data not shown). The infection phenotypes of the four mutations in the L*er*-0 background are reported first and the *sgt1a* mutation which is in the Ws-2 background is reported last.

Both the flowers and the new siliques of the *eds1*, *lms1* and *rar1* L*er*-0 background mutants all had wild-type levels of disease. However, the flowers from the *sgt1b* mutant plants had significantly less disease than the L*er*-0 wild-type flowers whereas the *sgt1b* siliques had an equivalent level of disease (Table 1, Fig. 1). In the infected floral tissues the DON levels were indistinguishable between the L*er*-0 and the *sgt1b*

Table 1 *Fusarium culmorum* disease formation on the floral tissue of various defence signalling mutants and the corresponding wild-type (wt) ecotype

	Tissue type						Experimental design	
	Flowers			New siliques			Replication	
Arabidopsis genotype	Mean[a]	SEM[b]	*P*-value[c]	Mean[a]	SEM	*P*-value	Plants[d]	Experiments[e]
L*er*-0 (wt)[f]	3.04	0.28	na[g]	1.70	0.12	na	135	11
eds1	3.55	0.34	ns[h]	1.84	0.23	ns	51	4
lms1	2.87	0.39	ns	1.30	0.27	ns	36	3
rar1	3.41	0.30	ns	1.41	0.17	ns	75	7
sgt1b	2.07	0.31	< 0.0001	1.28	0.17	ns	76	7
Ws-2 (wt)[f]	0.28	0.13	na	1.89	0.20	na	60	3
sgt1a	0.27	0.13	ns	1.30	0.21	0.05	59	3

[a]Estimated mean disease values determined using mixed model analysis for flowers and new siliques.
[b]The standard error of the mean.
[c]Represents the disease comparison between the Arabidopsis mutant genotype and the wild-type (wt). A *P*-value < 0.05 is considered to be statistically significant.
[d]Total number of plants inoculated per Arabidopsis genotype.
[e]Number of experiments in which the Arabidopsis mutant genotype was tested against the corresponding wild-type background.
[f]Degrees of freedom for L*er*-0 are 116 and for Ws-2 are 34.
[g]Not applicable.
[h]Not significant.

Table 2 Deoxynivalenol (DON) mycotoxin analysis of wild-type L*er*-0 (wt) or *sgt1b* mutant Arabidopsis floral tissue spray inoculated with either *Fusarium culmorum* strain 98/11 or water

	DON production (p.p.m.)[a]	
Treatment	wt	*sgt1b*
F. culmorum inoculated[b]	2.9 ± 1.9[c]	3.0 ± 1.6
Water only	< 0.2[d]	< 0.2

[a]DON levels quantified by competitive enzyme-linked immunosorbent assay (ELISA) and each p.p.m. value is based on plant dry weight.
[b]Conidial spray inoculation, the associated disease scores are given in Table 1. Combined floral and upper stem tissues were harvested at 8 d post-inoculation.
Six plants were pooled, freeze dried, ground and analysed for the presence of DON.
[c]Mean DON value ± standard deviation obtained from three experiments.
[d]DON value below the detection limit of 0.2 p.p.m.

mutant genotypes (Table 2). In addition, using a previously published root DON sensitivity bioassay (Masuda *et al.*, 2007), no differences were found between *sgt1b* and wild-type seedlings (see Fig. S2, Text S1).

Aerial mycelium was frequently visible on the flowers of both L*er*-0 and *sgt1b*. With L*er*-0 this was associated with the drying of all the floral subcomponents, and by 8 dpi most of the floral tissue appeared grey and sunken (Fig. 1a,c). Within 2–3 d constriction of the L*er*-0 main stem was observed. By contrast, the inoculated *sgt1b* floral tissue contained fully open flowers, with healthy petals and anthers as well as numerous unopened healthy green buds at different developmental stages. Using the previously devised floral nomenclature (Smyth *et al.*, 1990) we considered younger buds to be at stage 9, medium buds at stage 10 and older buds to be at stage 11–12. Only towards the periphery of the *sgt1b* floral apex were a few grey and dried-out flowers with light brown petals visible (Fig. 1b,d). When viewed under UV light, healthy chlorophyll-containing tissues autofluoresce red. In the diseased wild-type buds this red autofluorescence was reduced and replaced by blue-green autofluorescence (Fig. 1e). More blue-green autofluorescence was present on the heavily diseased wild-type L*er*-0 plants than the *sgt1b* mutant plants (Fig. 1e,f). Buds were stained with trypan blue to visualize nonviable cells (Koch & Slusarenko, 1990). The heavily diseased L*er*-0 buds stained an intense blue, with patches of cell death visible on the sepals and an intensely stained region at the base of the bud adjacent to the pedicel (Fig. 1g). The extensive surface hyphae and the pollen within the anthers also stained blue. By contrast the lightly diseased *sgt1b* mutant buds exhibited very low levels of trypan blue staining except at the apical tip of the sepals adjacent to the tip of the nonprotruding stigma (Fig. 1h). This latter staining pattern was also found in the wild-type (Fig. 1g).

Scanning electron microscopy imaging of wild-type L*er*-0 and *sgt1b* mutant buds inoculated with only water revealed that the outer surface of the sepals had long turgid epidermal cells interspersed with smaller epidermal cells and stomata (Fig. 2a,b). By contrast, analysis of the floral tissues at 7 dpi

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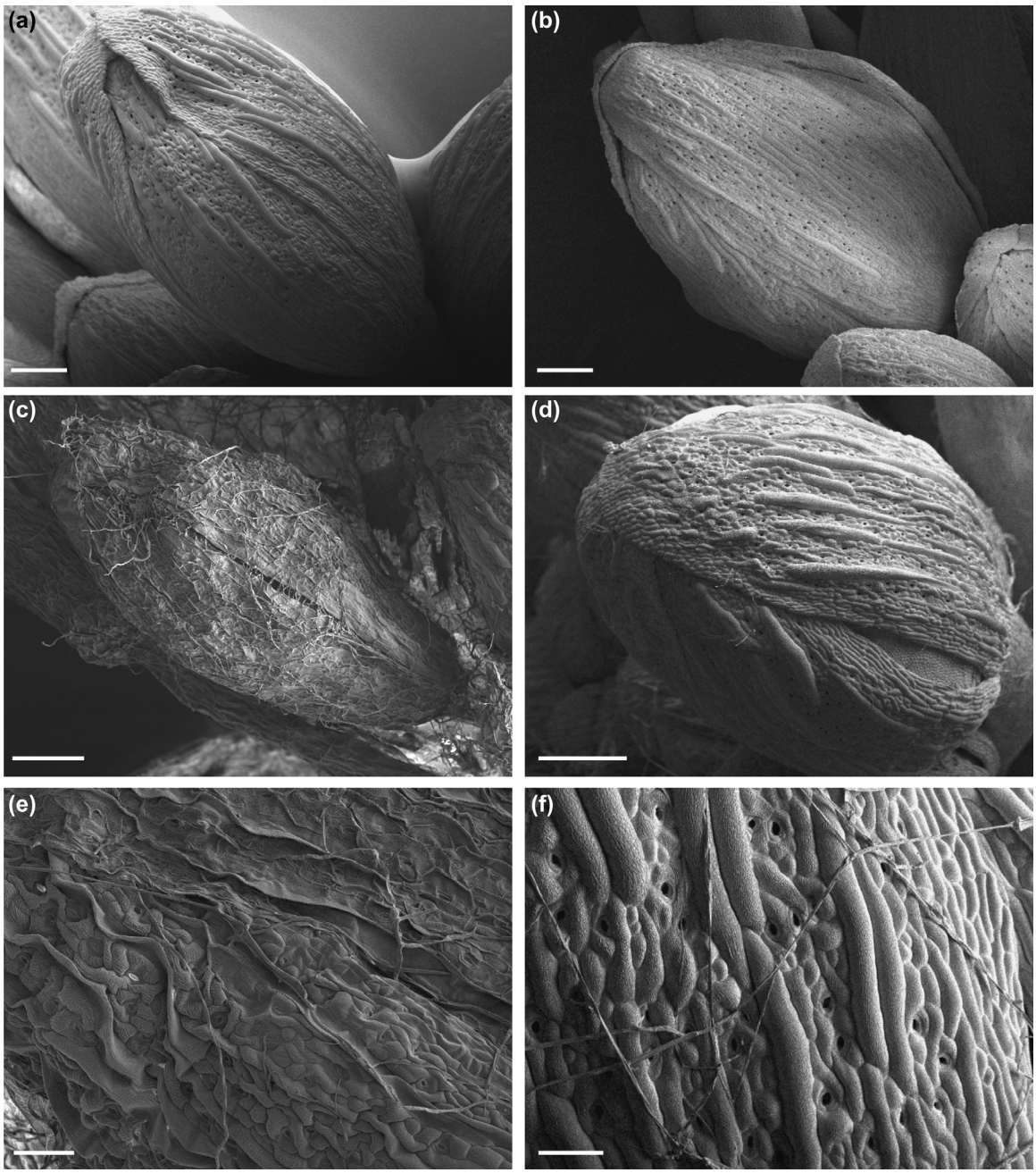

Fig. 2 Scanning electron micrographs of mock and *Fusarium culmorum* spray-inoculated wild-type and *sgt1b* mutant Arabidopsis floral tissue. (a,b) Buds examined from mock-inoculated wild-type (a) and *sgt1b* mutant (b) Arabidopsis plants. The outer surface of the sepals of both genotypes contain long epidermal cells, stomata and a fringe of smaller cells. Bar, 200 μm. (c,d) Representative images of buds examined from wild-type (c) and *sgt1b* mutant (d) Arabidopsis plants 7 d after inoculation with *F. culmorum* conidia. The older wild-type buds were engulfed in mycelium and the epidermal cell layer on the sepals had collapsed. By contrast the epidermal cells on the abaxial and adaxial sepals of the *sgt1b* mutant buds were still turgid even while in contact with the relatively lower levels of mycelium. Bar, 200 μm. (e,f) Close-up images from (c and d, respectively). The sepals of the inoculated wild-type buds revealed extensive epidermal cell collapse, particularly the large epidermal cells. While in the *sgt1b* mutant, hyphae were present in contact with the surface of the large epidermal cells, but minimal or no host cell collapse was visible at this time. No conidia formation was visible on either genotype. Bar, 50 μm.

revealed that the older buds of the wild-type plants were covered with *F. culmorum* mycelium and extensive cell collapse was visible (Fig. 2c). The younger wild-type buds had less mycelium and the long epidermal cells had collapsed. No conidia formation was seen in the mycelium covering the buds of either genotype (Fig. 2e and data not shown). The older buds of the *sgt1b* mutant had less mycelium and most cells remained turgid, with the occasional partially collapsing

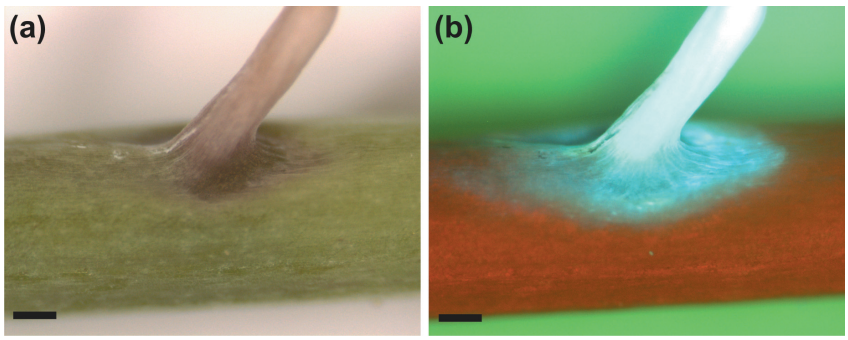

Fig. 3 Disease progression into wild-type stem tissue 13 d post-inoculation of *Fusarium culmorum* conidia onto the cut surfaces of apically wounded Arabidopsis siliques. Viewed under light (a) or UV light (b). Similar results were evident with the *sgt1b* mutant (data not shown). The chemistry responsible for the blue-green autofluorescence (b) is not known. Bar, 0.2 mm.

long epidermal cell (Fig. 2d). The medium-aged and younger buds had a small quantity of mycelium present compared with the wild-type; however, the epidermal cells remained turgid even when directly underneath the surface hyphae (Fig. 2f).

Host cell death occurred in regions of pathogen colonization as seen in images in Figs 1 and 2. The SGT1 protein was recently shown to be required for the activation of a PCD mechanism in tobacco leaves by the necrotrophic pathogen *Botrytis cinerea*. This resulted in the release of nutrients required for *in planta B. cinerea* growth (El Oirdi & Bouarab, 2007). Also, direct injection of DON mycotoxin into the apoplastic spaces of healthy wheat leaves has recently been shown to induce cell death within 24 h accompanied by DNA laddering, which is a hallmark of PCD (Desmond *et al.*, 2008). To determine whether a similar PCD mechanism was responsible for the extensive cell death occurring in the *Fusarium*–Arabidopsis floral pathosystem, detected by the heightened trypan blue stain, DNA was extracted from a time-course of *F. culmorum*-infected wild-type L*er*-0 and *sgt1b* mutant plants. However, over the selected time-course (days 0, 2, 5 and 8) there was no evidence for the DNA laddering that is a hallmark of PCD in other host–pathogen interactions (data not shown). No cell death was seen in the floral tissues of healthy wild-type or *sgt1b* mutant plants, when viewed under UV light. Therefore, the *sgt1b* mutation alone does not result in spontaneous cell death in floral tissue (Fig. S3).

To explore in greater detail the differential response of the bud/flower and silique tissues of the *sgt1b* mutant to *F. culmorum* infection, single wounded green siliques (*c.* 10 mm long) were droplet-inoculated with conidia (Cuzick *et al.*, 2008b). *Fusarium culmorum* was able to colonize the entire silique resulting in dark-brown shrivelled seed engulfed with mycelium by 6 dpi and disease symptoms were indistinguishable from the wild-type *Ler*-0 (data not shown). The silique surface became brown and a blue-green autofluorescent compound was visible where the infected tissue was necrotic. The colour of the autofluorescence was similar to that previously detected on the spray inoculated wild-type buds (Fig. S4). By 10 dpi mycelial colonization from the pedicel base into the main stem tissue was evident in both *sgt1b* and wild-type L*er*-0 plants (Fig. 3). This additional data indicates that the effect of the *sgt1b* mutation on limiting *F. culmorum* disease development is restricted to the buds and flowers. Biochemical experiments have indicated that this autofluorescent compound is a novel phenolic which appears to be of host origin (J. L. Ward *et al.*, unpublished).

In the Ws-2 background, there were no significant differences in the disease levels occurring on *sgt1a* and the wild-type flowers. A trend towards less disease was observed in the *sgt1a* newly formed siliques, although this was not as obvious as the effect of the *sgt1b* mutation in the L*er*-0 flowers. Overall there was less disease in the Ws-2 wild-type flowers than the L*er*-0 wild-type flowers. This may be caused by differences in inflorescence morphology; L*er*-0 had shorter and more tightly arranged inflorescences than Ws-2. Compact floral architecture in L*er*-0 was previously reported to enhance *Fusarium* disease levels (Urban *et al.*, 2002).

The *sgt1b* mutation does not affect *F. culmorum*-induced accumulation of reactive oxygen species

The Arabidopsis *sgt1b* mutant has previously been shown to have a compromised oxidative burst upon pathogen infection of leaves (Austin *et al.*, 2002; Tor *et al.*, 2002). Therefore, the tissue specific gene expression of genes involved in ROS generation and scavenging was extracted from GENEVESTIGATOR. This analysis confirmed that several of the genes encoding ROS generating or scavenging capabilities are highly expressed in floral tissue (Fig. S5, Text S1).

The floral tissue of the 5-wk-old L*er*-0 and *sgt1b* Arabidopsis plants just before inoculation was consistently found to contain high levels of superoxide and hydrogen peroxide in specific regions. The floral subcomponents, which had high ROS levels included the stigma, sepals and the petals/sepals abscission zone. By contrast, leaves taken from 5-wk-old noninoculated L*er*-0 and *sgt1b* plants that had grown in soil under nonsterile conditions and were ready for floral spray inoculation had low ROS levels except for occasional patches of staining for both superoxide and hydrogen peroxide towards the leaf periphery (Fig. S6). These patches of ROS indicate that at the time of inoculation both genotypes had already responded similarly to the standard growth room conditions.

To determine whether an alteration in the reactive oxygen species levels occurred post-*F. culmorum* inoculation, tissues were stained at various time-points for ROS. Greater intensities of ROS staining were observed in inoculated floral tissues than in the water-only sprayed controls from 4 d onwards, particularly in the green sepals, suggesting that a host oxidative burst had occurred in response to the pathogen infection. However, there were no obvious differences between the levels of ROS staining in the wild-type L*er*-0 and the *sgt1b* mutant post-inoculation. When the initial inoculations were done using a 100 times lower initial spore concentration, although the overall intensity of ROS staining was considerably lower, no differences in ROS staining between the two genotypes were observed. It was intriguing to observe that although a differential response was seen between levels of blue-green autofluorescence in inoculated wild-type L*er*-0 and *sgt1b* mutant buds at 7 dpi (Fig. 1e,f), this was not reflected with either of the ROS stains.

Discussion

To gain further insights into the globally important *Fusarium*-wheat interaction, the *Fusarium*–Arabidopsis floral pathosystem (Urban *et al.*, 2002) was used to assess a suite of Arabidopsis defence signalling mutants involved in basal and R protein-mediated defence. The defensive role of these important gene mutations has not previously been explored in the floral tissue of any species. This study has identified that the buds and flowers of the *sgt1b* mutant were significantly more resistant to infection and disease formation. This is the first time that a *sgt1b* mutation has been reported to result in enhanced pathogen resistance in Arabidopsis. The siliques of the *sgt1a* mutant also revealed a trend towards increased disease resistance. The infected *sgt1b* buds exhibited reduced epidermal cell death, reduced blue-green autofluorescence and reduced dehydration compared with the wild-type buds, but the fusarium DON mycotoxin accumulated to similar concentrations in both infected *sgt1b* and wild-type L*er*-0 floral tissues. By comparison mutations in *eds1*, *rar1* and *lms1* did not alter the interaction outcome.

In Arabidopsis, the *SGT1a* and *SGT1b* genes are functionally redundant in the early stages of plant development, but the double mutant is lethal (Azevedo *et al.*, 2006). Although *SGT1b* has been documented to have a greater role in resistance than *SGT1a*, both were induced upon pathogen inoculation of leaves and were able to confer resistance once a certain protein level was attained (Azevedo *et al.*, 2006). Historically, *SGT1b* has been reported to be required for R-protein mediated defence in leaves of Arabidopsis to many pathogens including the oomycete *Hyaloperonospora parasitica* (Tor *et al.*, 2002) and the powdery mildew fungus (Xiao *et al.*, 2005). Recently *SGT1b* has also been implicated in basal resistance to *H. parasitica* in Arabidopsis (Noel *et al.*, 2007). The Arabidopsis *SGT1a* gene but not the *SGT1b* gene is required for club root resistance in roots mediated by the *RPB1* gene (resistance to *Plasmodiophora brassicae*) (J. Siemens, pers. comm.). By contrast, in this study, we report a role in disease susceptibility for *SGT1b* in bud and flower tissues, but not siliques tissues, and a trend for a similar role for *SGT1a* in silique tissues but not bud or flower tissues. This may be another example of tissue-specific roles of the two SGT1 proteins in Arabidopsis, although caution must be taken in interpretation of these results because the single gene mutations were only available in different ecotypes.

The *F. culmorum*-infected *sgt1b* floral tissue, although supporting less disease and less host cell death, had DON levels equivalent to those present in the heavily diseased L*er*-0 wild-type floral tissue. This would suggest that per unit biomass the fungus produced more DON in the *sgt1b* mutant interaction. We also conclude that normal DON accumulation can occur in the absence of widespread plant cell death and that host cell death *per se* is not the trigger for sustained DON mycotoxin accumulation. In contrast to this discovery, we recently demonstrated that the *npr1* and *eds11* mutations in the Col-0 background supported both greater levels of *F. culmorum* disease and DON accumulation than the Col-0 wild-type floral tissue. Although the different ecotype backgrounds may be responsible for these contrasting results, it is more likely that these specific mutations have a direct or indirect effect on altering host–pathogen signalling pathways involved in inducing and/or suppressing DON production and/or degradation. Interestingly, *F. graminearum* strains engineered by deletion of the *Tri5* gene to be non-DON producers retain the ability to cause full disease symptoms on Arabidopsis floral disease (Cuzick *et al.*, 2008b). The DON requirement for *F. culmorum* to cause disease on Arabidopsis is not known. In this study, it is unclear whether DON is being made in the surface aerial mycelium or in the penetrating hyphae in close contact with the plant tissue. The use of a *Fusarium* reporter strain harbouring the *TRI5* promoter fused to a suitable reporter protein to monitor the onset of mycotoxin production (Jansen *et al.*, 2005) may help to further explore these interesting observations.

Transient reduction in expression of the *EDS1* gene or the single *SGT1* gene in tobacco leaves by virus-induced gene silencing (VIGS), resulted in reduced necrosis and enhanced resistance to the necrotrophic pathogen *B. cinerea* (El Oirdi & Bouarab, 2007). The authors concluded that activation of these signalling genes triggered an HR-form of PCD that enhanced *B. cinerea* colonization, whereas in the VIGS plants the HR was not triggered and the plants exhibited significantly reduced levels of disease. To further support their model, El Oirdi & Bouarab (2007) generated stable transgenic tobacco plants expressing the baculovirus anti-apoptotic protein p35 to compromise the establishment of an HR. These transgenic plants were also more resistant to *B. cinerea* than wild-type plants. Although we report here that the *sgt1b* mutation causes increased resistance to a floral invading pathogen, to

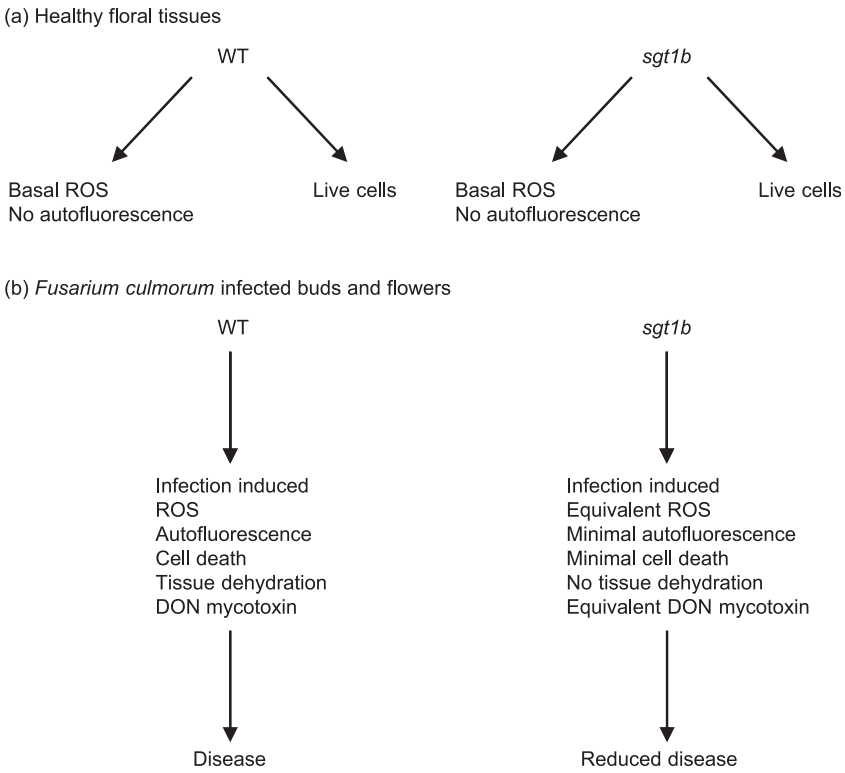

Fig. 4 Summary of the characteristic features of healthy wild-type (WT) and *sgt1b* mutant Arabidopsis floral tissue and the responses induced in both genotypes following *Fusarium culmorum* infection. (a) Healthy water-only sprayed wild-type and *sgt1b* mutant plants both produce moderate basal levels of reactive oxygen species (ROS) in specific floral regions, but do not exhibit spontaneous cell death. (b) *Fusarium culmorum* infections trigger the wild-type flowers to undergo an oxidative burst, host cell death, tissue dehydration and to accumulate blue-green autofluorescence and the DON mycotoxin. Collectively, this leads to a high level of disease. By contrast, in the *sgt1b* mutant, infection induces the oxidative burst but only minimal accumulation of autofluorescent material, minimal cell death and no tissue dehydration. Collectively, this culminates in reduced disease levels, but DON mycotoxin levels remain wild-type.

date we have not found evidence for a PCD mechanism in the *Fusarium*–Arabidopsis interaction. Unlike El Oirdi and Bouarab's study, lack of EDS1 function did not alter the *Fusarium* disease outcome. Therefore, it may be informative to test an Arabidopsis line expressing an anti-apoptotic protein and evaluate the outcome on both *Fusarium* disease and DON mycotoxin levels or evaluate specific cell types using a more sensitive method to detect apoptosis such as TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling) (Gavrieli *et al.*, 1992).

Although the plant defence signalling components RAR1 and SGT1b are able to interact and form a complex, the requirements of each for gene-for-gene mediated resistance in Arabidopsis to downy mildew conferred by *RPP* (resistance to *Peronospora parasitica*) genes, has been uncoupled. Therefore, the lack of differences in the floral disease outcome in the *rar1* mutant is highly informative in dissecting the functional requirements of the *F. culmorum* defence signalling pathway.

Figure 4 illustrates a comparison of the overall characteristics of both Arabidopsis wild-type L*er*-0 and *sgt1b* mutant floral tissue before and post *F. culmorum* infection. The major differences include minimal blue-green autofluorescence, minimal cell death and no tissue dehydration, which collectively contribute to reduced disease levels in the *sgt1b* mutant. Curiously, no obvious differences were observed with the ROS staining, possibly indicating that *F. culmorum* triggers different signalling networks from those conferred by R protein-mediated resistance. The latter often triggers both an oxidative burst and cell death via *SGT1*. In plants two ROS-generating mechanisms involving either plasma membrane NADPH oxidases or cell wall peroxidases have now been well characterized (Bolwell *et al.*, 2002, Torres *et al.*, 2002). Potentially pertinent to this study, is the observation that Arabidopsis leaves infiltrated with an elicitor from *F. oxysporum* produced high levels of ROS following the rapid activation of an apoplastic peroxidase (Bindschedler *et al.*, 2006). It is plausible that in the *sgt1b* mutant background, the host cells responding to the presence of *Fusarium* hyphae were induced to generate ROS by a different mechanism from that generating ROS in the wild-type *Ler*-0 plants. Alternatively, the *Fusarium* infections may have resulted in a reduction in the efficacy and/or the levels of alternative oxidase enzyme in the mitochondria and this led to elevated ROS levels arising from electron transport. In cultured tobacco cells, this source of elevated ROS has been demonstrated using an antisense approach (Maxwell *et al.*, 1999).

This report has not included gene expression studies of *F. culmorum* infected or mock-control treated floral tissues in wild-type or mutant plants because of the complexity of floral tissues. Manual dissection of specific floral region of interest, would have been extremely time-consuming, difficult and resulted in severe wounding. As new techniques such as laser capture microscopy (LCM) of plant tissues become available, this approach may be more feasible for sampling a selection of individual cells that can subsequently be used for gene expression studies (Ramsay *et al.*, 2006).

The *Fusarium*–Arabidopsis floral pathosystem has proved an invaluable tool to assess the impact of single gene mutations on this interaction. Similar assessments in the *Fusarium*–wheat pathosystem would be far more challenging, because the generation of stable transgenics in hexaploid wheat is still a time-consuming process. However, identification of candidate genes required for defence or susceptibility in the *Fusarium*–Arabidopsis floral pathosystem are ideal targets to disrupt in wheat and assess for altered disease outcomes. The *SGT1*, *RAR1* and *HSP90* genes have each been transiently silenced in wheat using VIGS and resulted in reduced resistance to a fungal rust (Scofield *et al.*, 2005). In the future, we plan to use VIGS to silence *SGT1* in hexaploid wheat floral tissue, with the predicted outcome of reduced levels of *Fusarium* ear blight disease while retaining DON mycotoxin production. Previously, transgenic wheat lines overexpressing the Arabidopsis *NPR1* gene were found to exhibit increased type II ear resistance to *F. graminearum* but DON levels were not reported (Makandar *et al.*, 2006). In the future a combination of VIGS and stable transformants should yield useful functional information on the signalling networks in wheat floral tissue and determine whether there are further similarities and differences between wheat and Arabidopsis.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Arabidopsis microarray analysis of the tissue-specific expression profiles of the selected defence signalling genes *EDS1*, *HSP90.1*, *HSP90.2*, *RAR1*, *SGT1a* and *SGT1b*.

Fig. S2 Root inhibition of wild-type and *sgt1b* mutant seedlings in the presence of DON mycotoxin.

Fig. S3 Healthy Arabidopsis wild-type L*er*-0 and *sgt1b* mutant floral tissues were found not to contain patches of spontaneous cell death when viewed under UV light.

Fig. S4 Photographs of Arabidopsis wild-type L*er*-0 and *sgt1b* mutant buds dissected from the floral apex 7 d after spray inoculation with water or *Fusarium culmorum* conidia.

Fig. S5 Arabidopsis microarray analysis of the tissue specific expression profiles of ten respiratory burst oxidase homologue (*RBOH*) genes, seven superoxide dismutases (SD), three catalases (CAT) and nine peroxidases (PX).

Fig. S6 Healthy and *Fusarium culmorum* infected wild-type L*er*-0 and *sgt1b* mutant Arabidopsis tissues stained for different reactive oxygen species.

Text S1 Relative transcript abundance of selected Arabidopsis genes in floral tissue using GENEVESTIGATOR and the DON sensitivity root bioassay.

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