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A novel function for a redox-related LEA protein (*SAG21*/AtLEA5) in root development and biotic stress responses

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ABSTRACT

SAG21/AtLEA5 belongs to the late embryogenesis-associated (LEA) protein family. Although it has been implicated in growth and redox responses, its precise roles remain obscure. To address this problem, we characterized root and shoot development and response to biotic stress in *SAG21/AtLEA5* over-expressor (OEX) and antisense (AS) lines. AS lines exhibited earlier flowering and senescence and reduced shoot biomass. Primary root length was reduced in AS lines, as was the number of laterals relative to the primary root. Root hair number was unchanged but root hair length was proportional to *SAG21/AtLEA5* expression level, with longer root hairs in OEX lines and shorter root hairs in AS, relative to wild type. Growth of the fungal nectroph, *Botrytis cinerea* and of a virulent bacterial pathogen (*Pseudomonas syringae* pv. *tomato*) was affected by *SAG21/AtLEA5* expression; however, growth of an avirulent *P. syringae* strain was unaffected. A SAG21/AtLEA5-YFP fusion was localized to mitochondria, raising the intriguing possibility that SAG21 interacts with proteins involved in mitochondrial ROS signalling, which in turn, impacts on root development and pathogen responses.

Key-words: *Arabidopsis*; disease; LEA proteins; mitochondria; redox signalling; ROS; root hair; senescence.

INTRODUCTION

Late embryogenesis abundant (LEA) proteins, as the name implies, were first identified as proteins abundant in the later stages of seed development (Dure, Greenway & Galau 1981; Grzelczak *et al.* 1982). Later they were also found to be expressed in a range of plant vegetative and reproductive tissues (Hundertmark & Hincha 2008), and related proteins were found in other eukaryotes and prokaryotes (Garay-Arroyo *et al.* 2000). Importantly, they are also induced by abiotic stresses, particularly by water deficit and cold (Bray 1997; Thomashow 1999). LEA proteins are characterized by being small, highly hydrophilic and of low complexity (Garay-Arroyo *et al.* 2000; Wise 2003), and are thought to function as molecular chaperones protecting against aggregation of proteins under water stress (Goyal, Walton & Tunnacliffe 2005).

In *Arabidopsis*, LEA proteins constitute a family of 51 members that can be subdivided into nine distinct groups with different constitutive and inducible expression patterns (Hundertmark & Hincha 2008). SAG21/AtLEA5 (At4g02380; hereafter referred to as *SAG21*) belongs to an anomalous LEA-3 group, specific to plants, whose members are predicted to be exclusively targeted to either plastids or mitochondria, and are more hydrophobic than average (Hundertmark & Hincha 2008). *SAG21* was first identified as a senescence-associated gene (SAG), expressed transiently at an early stage of leaf senescence just as leaves begin to yellow, and is also induced by darkness (Weaver *et al.* 1998). Light regulation was confirmed by Mowla *et al.* (2006) who further showed that *SAG21* expression was most abundant in roots and flowers and was up-regulated in leaves by dehydration and oxidants. Induction by dehydration is common to other LEA proteins in *Arabidopsis* (Hundertmark & Hincha 2008), but induction by oxidative stress has not been as widely reported, although some LEA proteins may have a role as antioxidants (Hara *et al.* 2003; Hara, Fujinaga & Kuboi 2004; Tunnacliffe & Wise 2007). *SAG21* is up-regulated by a range of oxidants (Mowla *et al.* 2006) including both H_2O_2 and superoxide ($O_2^{\bullet-}$)-generating agents such as menadione and Paraquat; however, oxidant induction was independent of *OXI1*, a gene implicated in reactive oxygen species (ROS) signalling in root hair growth and basal resistance to *Peronospora parasitica* (Rentel *et al.* 2004). ABA induces the expression of over half of the *Arabidopsis* LEA genes (Hundertmark & Hincha 2008), including *SAG21*, whereas induction by

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dehydration was dependent on ABA synthesis but independent of ABI1, a protein phosphatase which participates in ABA signalling (Mowla *et al.* 2006). Numerous reports, including data from publicly available microarrays (Zimmermann *et al.* 2004), indicate that *SAG21* expression is also up-regulated by a range of other stresses such as ozone (Miller, Arteca & Pell 1999), cold (Seki *et al.* 2001), low nitrate (Wang *et al.* 2000) and pathogen infection (*Colletotrichum higginsianum,* Liu *et al.* 2007), as well as the hormones ethylene (Weaver *et al.* 1998; De Paepe *et al.* 2004), jasmonate (Jung *et al.* 2007) and by sugar signalling (Xiao, Sheen & Jang 2000).

Expression of LEA proteins in transgenic plants can confer abiotic stress tolerance to drought, cold and freezing. For example, the expression of a barley LEA (*HVA1*) improved drought tolerance in rice and wheat (Xu *et al.* 1996; Sivamani *et al.* 2000); the expression of a citrus LEA (*CuCOR19*) conferred cold tolerance in tobacco (Hara *et al.* 2003); and freezing tolerance in strawberry leaves was increased by the expression of a wheat LEA (*WCOR410*) (Houde *et al.* 2004). However, these effects are not universal: for example, the expression of cold-induced spinach LEA proteins in tobacco did not improve cold tolerance (Kaye *et al.* 1998). In the case of *SAG21,* constitutive over-expression did not result in drought tolerance as might have been expected from its strong up-regulation during dehydration stress (Mowla *et al.* 2006). In fact, under drought conditions the over-expressing plants were less able to assimilate CO_2. Over-expression of *SAG21* did confer increased tolerance to H_2O_2 both in shoots and roots and, even under optimal conditions, resulted in increased biomass and primary root elongation compared with wild type (WT).

Thus, the role of *SAG21* remains enigmatic. Although its light repression and predominant expression in non-photosynthetic tissues (flowers and roots) preclude a direct antioxidant role in photosynthesis, its up-regulation in response to oxidants and the reduced sensitivity to H_2O_2 in over-expressing plants suggest a potential function in ROS-mediated signalling. However, its lack of dependence on *OXI1* indicates that the *SAG21* signalling pathway differs from that established for root hair growth and pathogen attack. A role in redox signalling is nevertheless possible in response to stresses such as drought, although its *ABI1*-independent expression suggests that the signalling pathway converging on *SAG21* is not exclusively via ABA-dependent ROS production.

Building on the work of Mowla *et al.* (2006), the present study was undertaken to determine the scope of physiological functions of SAG21 and to determine its intracellular localization. We have generated antisense (AS) lines with reduced *SAG21* expression and used these, together with over-expressor (OEX) lines to characterize shoot and root phenotypes in more detail. We also explored the subcellular localization of SAG21 using plants stably expressing a YFP fusion. We show here that SAG21 is located in mitochondria, and that its expression affects leaf senescence, root development and the ability of pathogens to proliferate

within leaves. Taken together, this allows us to suggest a new hypothesis regarding SAG21 function in redox signalling.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana (Col-0) were grown in a controlled environment growth chamber at 21 °C under long days [16 h light (300–400 μmol m^{-2} s^{-1}) and 8 h dark] except for plants used for pathogen inoculation. Seeds were surface sterilized, stratified for 24 h at 4 °C, and either sown onto soil or onto Petri dishes containing 1× Murashige and Skoog (MS) basal salts, 1% agar, pH 5.5–5.7 and sealed with micropore tape.

For analysis of root growth, OEX and AS lines were sown onto 1 × MS, 1% agar as described previously, with five seeds sown in a line at one end of the plate, and grown vertically for 20 d following germination.

For analysis of aerial growth, flowering time and senescence, seeds were either sown directly into pots containing 3:1 compost : soil mixture and grown to maturity as described previously, or seedlings were sown onto 1 × MS, 1% agar, grown for approximately 2–3 weeks and transferred to soil when they had four to six leaves. Soil was kept moist and care was taken not to subject the plants to any stress. Bolting and flowering time was noted, and after 10 weeks, the whole rosette was harvested. This experiment was repeated three times with independent batches of plants (data not shown).

Plants used for pathogen inoculation experiments were transferred from 1 × MS 1% agar to Levington Universal compost and placed in Polysec growth rooms (Polysec Cold Rooms Ltd, Worcester, Worcs, UK) fitted with six Sylvania Lynx Delux 840 fluorescent tubes (International Lamps Ltd., Essex, UK) per shelf, providing a light intensity of 160 μmol m^{-2} s^{-1} at shelf height, maintained at 24 °C with an 8 h photoperiod. In all experiments, at least three replicate plants for each line were used.

Genetic constructs and plant transformation

OEX lines were as described in Mowla *et al.* (2006). AS lines were generated by PCR amplification of the entire *SAG21* open reading frame using primers: 5′-CAAG<u>TCTAGA</u>CTTACTTCGAAAATGGC and 5′-CC<u>GGATCC</u> TCTCCTCTTAAAGACC containing *Xba*I and *BamH*I sites, respectively (underlined), and cloned into pBin19-35S (Höfgen & Willmitzer 1990) in the reverse orientation as described in Mowla *et al.* (2006). For assembly of the *SAG21* promoter-GUS construct, 1685bp of the *SAG21* upstream region was amplified by PCR using primers containing *Sal* I and *Xba* I sites, respectively (underlined): 5′- AATT<u>GTCGAC</u>TAATCTCCAAAA CATTGTG and 3′- ACTG<u>TCTAGA</u>TTTCGAAG TAAGTGGTTTC, and cloned into pGEM-T Easy (Promega, Southampton, UK). The insert was removed by restriction digestion and cloned into the pGPTV-KAN (Becker *et al.* 1992) upstream of the *uid* gene.

For the *SAG21 -YFP* fusion construct, the *SAG21* open reading frame was amplified with primers 5′-<u>CGGATCC</u> ATGGCTCGTTCTATCTCTAACG and 3′- TAATATA ATA<u>GCGGCC</u>GCCTGCTTGTTGTTCAAGAG including *Bam* HI and *Not* I restriction sites (underlined) and cloned into pGREEN0029 (Hellens *et al*. 2000) in frame with ATG-less YFP.

Constructs were transformed into *Agrobacterium tumefaciens* strain *GV3101* and *Arabidopsis* plants were transformed by the floral dip method (Clough & Bent 1998). Transformants were selected on $1 \times MS$, 1% agar plates containing 50 mg L^{-1} kanamycin. Primary transformants were tested by PCR followed by RT-PCR, GUS assays or YFP fluorescence depending on the line. Several lines were selected for each experiment, and tested for expression (data not shown), unless otherwise stated; representative data for a single homozygous line for each transgene are presented.

DNA and RNA extraction, cDNA synthesis and semi-quantitative RT-PCR

Genomic DNA was extracted from young seedlings for genotyping using the method of Edwards, Johnstone & Thompson (1991). RNA was extracted using TRI-reagent (Sigma), DNase treated with RQ1 DNase (Promega) and cDNA was synthesized using MLV-RT and oligo dT (Promega) essentially as described in Spadafora *et al*. (2011). Primers for genotyping and checking expression of the transgenic lines were: GUS1F: 5′-GAAACCCCAA CCCGTGAAATCA, GUS2R: 5′-AACCTTCACCCGG TTGCCAGA; CaMV1F: 5′-CGTAAGGGATGACGC ACA, *AtSAG21*F: 5′-ATCTTCCGACGTGGTTATGC; and *AtSAG21*R: 5′-CCGGTTTCGGGTCTGTAATA. For semi-quantitative RT-PCR, PCR cycle number was optimized for each specific primer pair and cDNA batch by testing a set of standard cDNA dilutions over a suitable range of cycles to ensure linearity between template and product concentration. 18S primers (PUV2 5′-TTCCATGCTAATGTATTCAGAG and PUV4 5′-ATG GTGGTGACGGGTGAC) were used for data normalization, relying on the high AT content of rRNA. Specific primers for *SAG21* were *AtSAG21*F and *AtSAG21*R, as detailed previously. This approach has been employed successfully in a range of experimental systems (e.g. Orchard *et al*. 2005; Price *et al*. 2008). All RT-PCR reactions were replicated at least three times and standards were included in each PCR run. Ethidium bromide fluorescence was quantified by using the Gene Genius Bioimaging System and Gene Tools software package (Syngene Ltd., Cambridge, UK).

Phenotypic analysis of root growth

Primary root length was measured and numbers of lateral roots and lateral root primordia were counted using a dissecting microscope (Nikon SMZ-2T, Tokyo, Japan). All root hairs were measured on five seedlings of each line using a BH-2 Olympus research microscope (Tokyo, Japan), with the 10× objective over 2.5 mm from the root tip. Images of the root hairs were captured using a FUJI 1× Digital Camera HC-300Z (Tokyo, Japan) and measured using Sigmascan (Jandel Scientific, San Rafel, CA, USA).

GUS expression analysis

For histochemical assays, whole seedlings, leaves and flowers were incubated at 37 °C for 2–6 h in: 0.5 M sodium phosphate pH 7, 200 mM potassium ferricyanide, 20 mg mL^{-1} chloroamphenicol, 0.01% Triton X-100, 5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc), following vacuum infiltration for 2–3 min. Tissues were decolorized in 96% ethanol. Fluorogenic assays were essentially as described in Jefferson (1987), measured using a BIO-TEK FL600 fluorometer (Potton, Bedfordshire, UK).

Abiotic stress and plant growth regulator treatments

Seedlings were sown onto $1 \times MS$ as described previously and grown vertically for 8 d. Petri dishes were then pre-treated for 12 h light (approximately 300–400 μmol m^{-2} s^{-1}) to ensure that *SAG21* expression was fully switched off prior to the treatment. Stress treatments were based on those described in Kilian *et al*. (2007). Drought treatment was applied by removing the seedlings onto filter paper and subjected to 30 min air flow in a laminar air flow cabinet (Microflow, Hampshire, UK). For cold treatment Petri dishes were placed onto crushed ice for 30 min. For salt and oxidant treatments, Petri dishes were flooded with 2 mL of 150 mM NaCl, or 10 mM H$_2$O$_2$, respectively, for 1 h prior to analysis. Untreated seedlings were used as controls.

Methyl jasmonate (Me-JA) (50 μM) or 100 μM salicylic acid (SA) was applied in aqueous solution to 10-day-old seedlings grown on $1 \times MS$, 1% agar, in a volume of 2 mL applied directly to the roots. Seedlings were pre-treated for 12 h in the light prior to application of the Me-JA or SA (again, to ensure that *SAG21* expression was fully switched off prior to the treatment) and maintained under continuous light throughout the treatment period. In all cases, a mock water treatment was used as a control. Samples were taken after 24 h for GUS staining. Controls were treated with distilled water. Gaseous ethylene (0.1 mg g^{-1}) was administered to seedlings grown and pretreated as previously by injection of the gas into the headspace of the Petri dish through a small hole which was resealed with Nescofilm® (Cottonwood, AZ, USA), and the Petri dishes were tightly sealed with Nescofilm® for the duration of the experiment. Air treatment was used as a control.

DAB staining for H$_2$O$_2$ detection in wounded leaf was conducted as described in Nakagami *et al*. (2006).

Biotic stress treatments

Botrytis cinerea (strain iMi 169558, International Mycological Institute, Kew, UK) was maintained on potato dextrose

agar (PDA; Oxoid Ltd., Cambridge, UK). *Botrytis cinerea* conidia were harvested from the surface of the plates in a carrier media with sterile PDB (Formedium, Hunstanton, UK) and diluted accordingly to 10^5 spores mL^{-1} as determined using a Neubauer haemocytometer (Fisher Scientific, Loughborough, UK). *B. cinerea* spores ($5\,\mu$L) were applied onto the adaxial surface of the leaf of 5-week-old *Arabidopsis* rosettes. A 50–80% relative humidity was obtained by keeping the plants under Stewart Micropropagators (H. Smith Plastics Ltd., Wickford, Essex, UK). Strains of *Pseudomonas syringae* pv. *tomato* DC3000 were cultured on Nutrient Agar (Sigma-Aldrich Ltd., Poole, Dorset, UK) at 28 °C. A single colony was inoculated into 10 mL Nutrient Broth (Sigma-Aldrich Ltd.) and incubated overnight at 28 °C. Subsequently, the bacteria were harvested by centrifugation ($650\,g$) and resuspended to a bacterial cell density of 10^6 mL as described in Mur *et al.* (2000). The bacterial suspension was injected into the abaxial side of *Arabidopsis* leaves using a 1 mL sterile needleless plastic syringe (BD Plastipak, Madrid, Spain).

Infected plants were assayed at time intervals of 12, 24 and 48 h. For *B. cinerea,* leaves were either stained for GUS as described above or using aniline blue as described in Routledge *et al.* (2004). Aniline blue stained leaves were observed using a fluorescence microscope (Olympus BX51 microscope (Olympus America Inc., Center Valley, PA, US) coupled with a Nikon Coolpix 990 camera (Nikon). Bacterial counting was performed as described in Mur *et al.* (2000). Single cores from three individual lesions were ground in 500 μL of 10 mM phosphate buffer (pH 7). Serial dilutions of the homogenous extract were plated onto nutrient agar (Sigma-Aldrich Ltd.) supplemented with 50 μg mL^{-1} rifampicin. Following incubation at 28 °C for 2 d, colonies were counted and the original population size was determined. Bacterial populations were expressed as colony forming units (cfu) per leaf.

Subcellular localization

Sequence analysis was performed using Mitoprot (Claros & Vincens 1996) and TargetP (Emanuelsson *et al.* 2007) software. YFP expression was recorded in 5-day-old seedlings by confocal laser scanning microscopy using either a Leica TCS SP2 AOBS spectral confocal microscope system (Leica Microsystems, Wetzlar, Germany) or a Zeiss LSM 510 META confocal microscope system (Carl Zeiss Ltd., Oberkochen, Germany). YFP was excited at 514 nm and detected in the 525- to 583 nm range. Chlorophyll autofluorescence was excited simultaneously with the 514 nm laser and emission was detected at wavelengths longer than 650 nm. Older seedlings were also examined by the same method with very similar results (data not shown). Mitotracker Deep Red FM (250 nM; Molecular Probes, Invitrogen, Paisley, UK) was used to stain mitochondria. Whole seedlings were immersed in the stain for 15 min prior to observing root epidermal cells by confocal microscopy. A 633 nm, He/Ne laser was used to excite fluorescence, which was detected using a 650 nm long pass filter.

RESULTS

### Effects of modulation of *SAG21* transcripts on plant growth and development

Previously, we reported that over-expression of *SAG21* resulted in increased vegetative growth (Mowla *et al.* 2006). Analysis of an *Arabidopsis* T-DNA insertion mutant of *SAG21* did not reveal any obvious phenotypes (Mowla *et al.* 2006); however, the insertion in this line is situated at the end of the coding sequence and *SAG21* transcript was present, suggesting that this does not represent a null allele (data not shown). Therefore to investigate further the role of *SAG21* in growth and development, we generated AS plants driven by the CaMV 35S promoter (Fig. 1a). Several independent lines were obtained, of which two were selected for further work. In these lines (AS-2 and AS-8), transcript levels were approximately fivefold reduced compared with WT (Supporting Information Fig. S1). Figure 1b shows a comparison of OEX, WT and AS lines after 10 weeks' growth on soil. There was a clear difference in above-ground biomass between the three genotypes
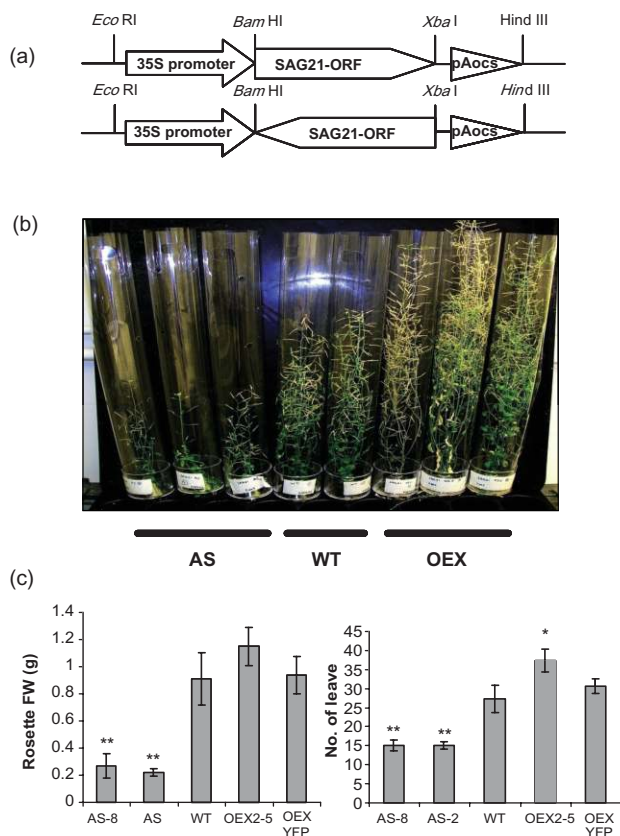
Figure 1. Aerial phenotype of *SAG21* transgenic lines. (a) Schematic representation of the constructs for phenotypic analyses: top, construct for over-expression (OEX); bottom, antisense (AS) construct. (b) Phenotypes of 10-week-old soil-grown plants (AS-2 and OEX2-5). (c) Whole rosette fresh weight and leaf number in two antisense and two over-expressor lines at 10 weeks ($\pm$SE, $n = 4$, $**P < 0.01$, $*P < 0.05$). Data are representative of three independent experiments.

(Fig. 1b,c), confirming our previous results for OEX plants (Mowla *et al.* 2006). Although all three genotypes had flowered, OEX lines produced taller plants with more flowering stalks. In contrast, the AS plants were of smaller stature, with fewer flowering stalks (Fig. 1b), significantly fewer leaves ($P < 0.01$) and significantly less rosette biomass ($P < 0.01$) (Fig. 1c). There was a significant difference in age at which the first flower appeared, and in bolting, occurring earlier in the AS lines and later in the OEX lines, relative to WT (Fig. 2a). As *SAG21* was originally identified as a senescence-associated gene (Weaver *et al.* 1998), we investigated whether senescence timing was affected in the transgenic lines in which *SAG21* is perturbed. A comparison of the six oldest leaves from 10-week-old plants revealed that the AS lines showed more visible symptoms of senescence relative to WT and OEX lines (Fig. 2b). Chlorophyll assays combining the oldest six leaves from three replicate plants revealed an increased chlorophyll content in the OEX line compared with WT ($P < 0.05$) and a further reduction in the AS line ($P < 0.05$) (Supporting Information Fig. S2).

In addition to the dramatic above-ground phenotypes, root growth and development were also markedly affected in *SAG21* OEX and AS lines (Fig. 3). At 20 d after germination, primary root length was similar in WT and OEX lines, but significantly shorter in AS lines (Fig. 3a,b). Strikingly, the number of lateral roots and lateral root
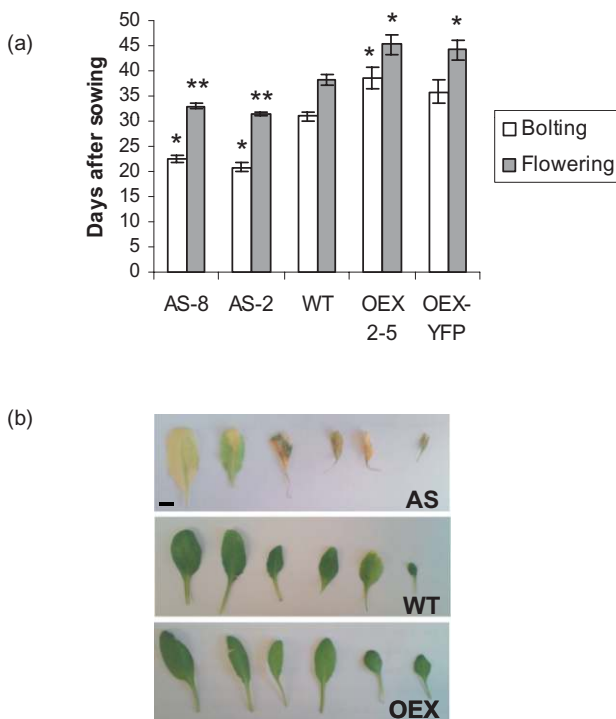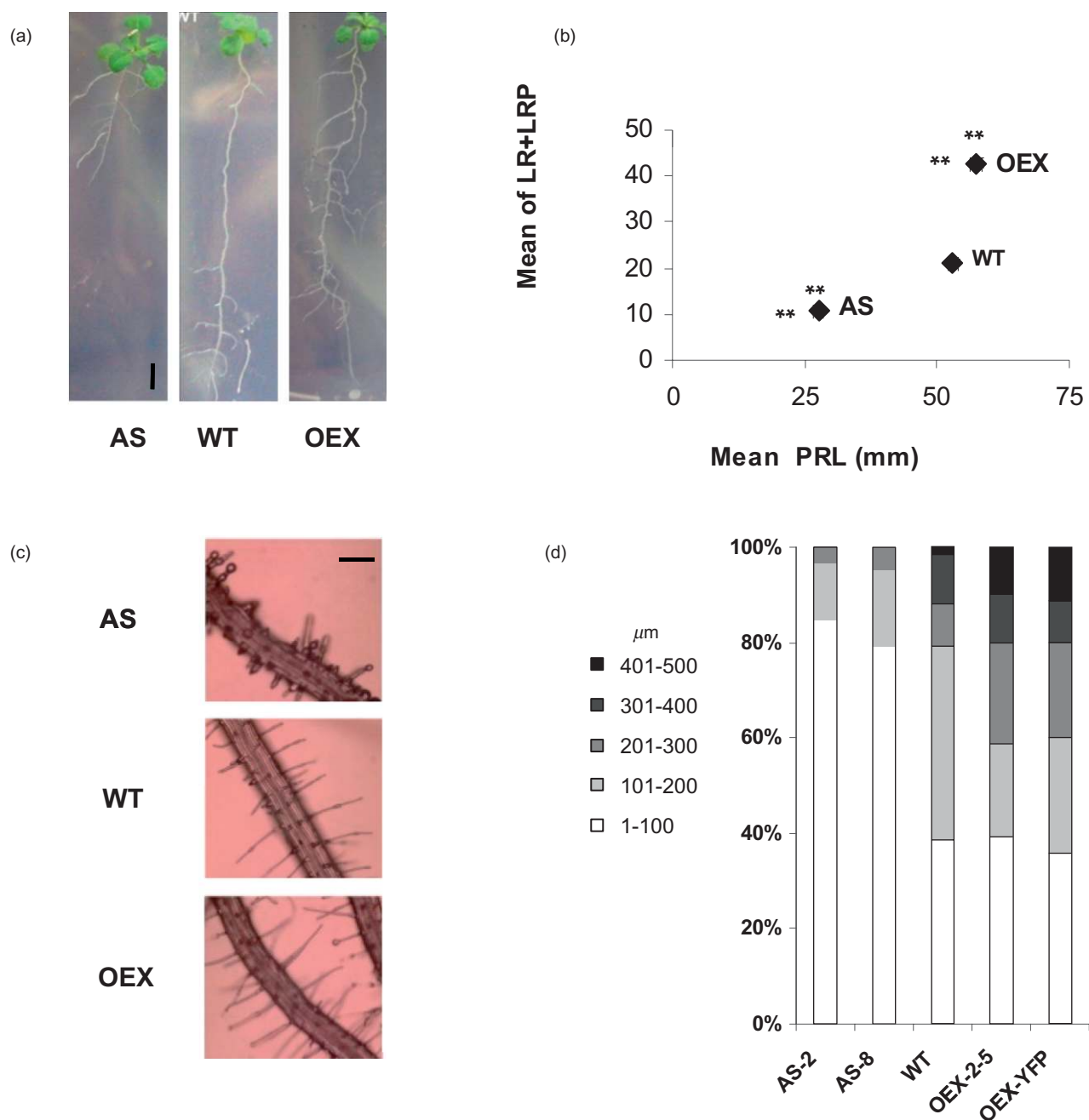
primordia correlated with *SAG21* expression, with increased abundance of laterals relative to primary root length in OEX and decreased abundance in AS (Fig. 3a,b). To establish whether cell elongation was affected, root hair morphology was examined, revealing a clear difference between the three genotypes (Fig. 3c). The proportion of longer root hairs was clearly increased in the OEX lines, whereas in the AS lines there were no hairs longer than 300 μm and the proportion of shorter hairs was vastly increased (Fig. 3d). However, the total number of root hairs was the same in the different genotypes (data not shown).

Tissue-specific and stress-responsive expression of *SAG21*

In order to investigate the spatial and temporal expression of *SAG21*, transgenic lines expressing a 1685 bp *SAG21* promoter-*GUS* construct (Fig. 4a) were produced, and homozygous lines were analysed for GUS expression. Expression was decreased in the light, relative to dark, as observed previously by Northern analysis (Mowla *et al.* 2006; Fig. 4b). In plants that had received an 8 h dark period, expression was observed in cotyledons and roots but was absent from rosettes (Fig. 4c), again in broad agreement with previous data (Mowla *et al.* 2006), although here expression in the root during the light period was less marked. In flowers, expression was restricted to the pollen (Fig. 4d), although very weak expression was also sometimes detected in petal veins. Expression was occasionally detected in the early stages of leaf senescence at the interface between the green and yellow regions of senescent leaves (Fig. 4e). In dark-treated seedlings, expression of *SAG21* was strong throughout the root tissues, although it was excluded from the primary and lateral root tips (Fig. 4f and Supporting Information Video S1). GUS expression was also clearly detected in root hairs (Fig. 4g).

GUS staining revealed strong up-regulation of *SAG21* expression in seedling roots pretreated in light to ensure that *SAG21* expression was fully switched off prior to the treatment, and then exposed to a range of abiotic stresses (Fig. 5). Stress treatments were performed as described in Kilian *et al.* (2007) to enable comparisons of results to the AtGenExpress stress array data. Expression was detected both in the maturation and elongation zones of the root, but excluded from the lateral root tips and primordia (Fig. 5a). As shown previously (Mowla *et al.* 2006), both drought and H_2O_2 up-regulated *SAG21* expression. In addition, we show here that both cold and salt also induce *SAG21* expression. Quantitative assays show that the strongest response was elicited by cold treatment followed by salt, with H_2O_2 and drought eliciting similar levels of up-regulation under the conditions tested (Fig. 5b,c). Similar experiments with young leaves did not result in up-regulation of the *SAG21* expression, although GUS activity in leaves was elicited by growth of the seedlings with restricted gas exchange (data not shown). Wounding also clearly induced strong expression in leaves along the

(a)

(b)

Figure 2. Flowering and senescence in *SAG21* transgenic lines. (a) Timing of bolting and flowering for each genotype (±SE, $n = 5$). Asterisks indicate significant difference to wild type (WT) at $P < 0.05$ (*) or $P < 0.01$ (**). (b) Appearance of the six oldest leaves of 10-week-old plants for each genotype (AS-2 and OEX2-5). Scale bar = 5 mm.

Figure 3. Root growth and development in *SAG21* transgenic lines. (a) Root architecture of 20-day-old seedlings. Scale bar = 5 mm. (b) Mean of [lateral root (LR) and lateral root primordia (LRP)] as a function of mean of primary root length (PRL) ($\pm$SE, $n = 30$, **$P < 0.01$) (c) Root hair zone of vertically-grown seedlings at 20 d after germination. Scale bar = 100 μm. (d) Distribution of root hair lengths 4 d after germination ($n = 5$ seedlings) over 2.5 mm from the root tip. Unless otherwise stated, lines used were AS-2 and OEX2-5.

cut edge and around severed vascular tissues (Fig. 5d). GUS staining corresponded to sites of H_2O_2 accumulation around the wound site.

The effect on *SAG21* expression of treatment with Me-JA, SA and ethylene was also tested. All three plant growth regulators strongly up-regulated *SAG21* expression in the root maturation zone (Fig. 5e), while mock treatments (water and air) did not elicit a response.

Relationship between *SAG21* and biotic stress tolerance

Challenge of *SAG21::GUS* plants with the necrotrophic fungal pathogen, *B. cinerea* elicited a strong up-regulation of expression at the site of necrotic lesions within 24 h after inoculation (Fig. 6a). To test whether perturbation of *SAG21* affected pathogen growth, the presence of fungal
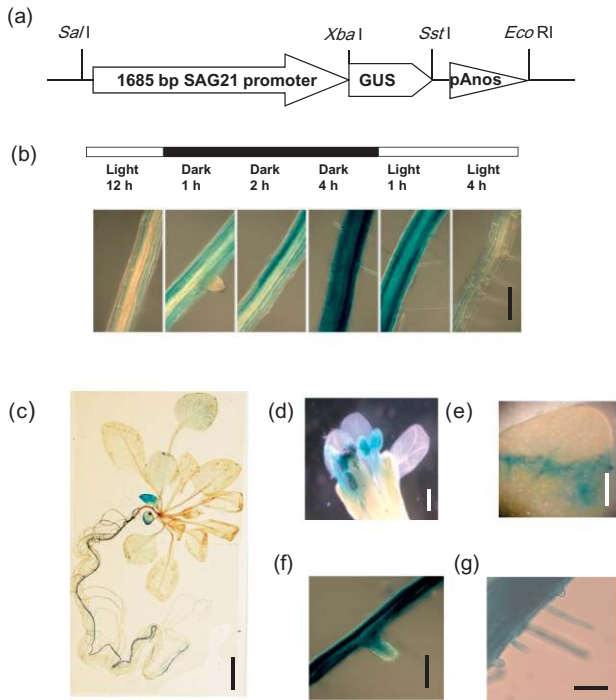
Figure 4. Spatial expression of *SAG21* promoter and response to light. (a) Schematic representation of the *SAG21* promoter-GUS construct; (b) response of *SAG21* promoter to light/dark regime in root (scale bar = 50 μm); (c–e) Tissue-specific expression of *SAG21* under normal development: (c) 3-week-old whole seedling (scale bar = 10 mm), (d) whole flower (scale bar = 1 mm), (e) interface between green and yellow regions of senescent leaf (scale bar = 5 mm), (e–g) 8-day-old roots: (f) primary root following dark treatment (scale bar = 200 μm), (g) root hair (scale bar = 100 μm).

hyphae was investigated using aniline blue staining 24 and 48 h after inoculation (Fig. 6b). While fungal germination and growth were observed in WT and the AS plants after 24 h, very little was observed in the OEX line. However, by 48 h, equivalent hyphal development was observed in WT and the transgenic lines (Fig. 6b). To test the effect of a bacterial pathogen, the same lines were also tested with virulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000; Fig. 6c), and avirulent *Pst* DC3000 *avrRpm1* (*Pst* DC3000 *avrRpm1*), which elicits a hypersensitive response (HR; Fig. 6d). In *Pst* DC3000-infected plants, bacterial numbers were significantly reduced in the *SAG21* OEX and increased in the AS line compared with WT (Fig. 6c). In contrast, there was no clear pattern of response of the three genotypes to the HR-eliciting avirulent line.

Subcellular localization of SAG21

As *in silico* analysis of targeting predicted the presence of either a mitochondrial or a chloroplast targeting signal (Mitoprot II: 0.9856, TargetP: 0.555 for mitochondria; Claros & Vincens 1996; Emanuelsson *et al*. 2007), the subcellular localization of SAG21 was investigated by

generating stable transgenic lines carrying a *SAG21*-YFP fusion driven by the 35S CaMV promoter (Fig. 7a). Confocal microscopy revealed that the YFP signal was associated with small subcellular compartments forming a punctate pattern suggesting mitochondrial localization (Fig. 7b). Time lapse imaging of these compartments in roots revealed that their appearance and behaviour is characteristic of mitochondria (Supporting Information Video S1). In cotyledons, autofluorescence from chloroplasts clearly shows that the YFP signal is not chloroplast associated, and therefore a plastid localization for SAG21 can be excluded
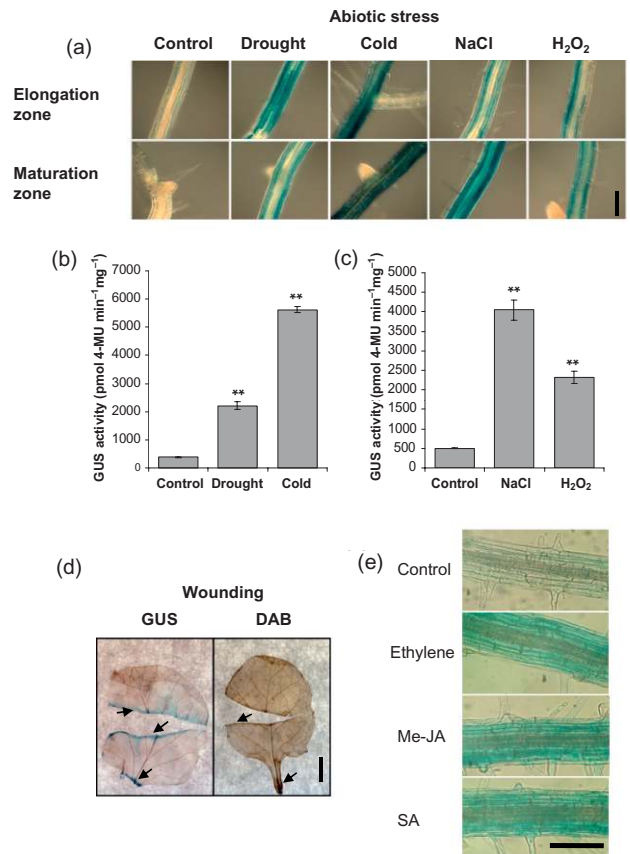
Figure 5. Response of *SAG21* promoter to abiotic stress and plant growth regulators. *SAG21* promoter-GUS lines were subjected to a range of stress treatments followed by histochemical GUS staining. (a) Seedlings (8 d after germination) were pretreated in 12 h light then subjected to drought (30 min under direct air flow), cold (30 min in crushed ice), 150 mM NaCl, or 10 mM H_2O_2. Untreated seedlings were used as control. Scale bar = 100 μm (b,c). Quantification of relative GUS activity in seedlings under optimal and stress conditions (±SE, $n = 10$). Significant differences from control **$P < 0.01$ are indicated. (d) Effect on wounding on *SAG21* promoter-GUS activity (left-hand panel) and on H_2O_2 levels, as indicated by DAB staining (right-hand panel). Black arrows indicate leaf wounding sites. Scale bar = 5 mm. (e) Seedlings (8 d after germination) were pretreated in 12 h light then subjected to distilled water (control), ethylene (0.1 mg g^{-1}), methyl jasmonate (Me-JA; 50 μM), salicylic acid (SA; 100 μM) followed by histochemical GUS staining (scale bar = 100 μm).
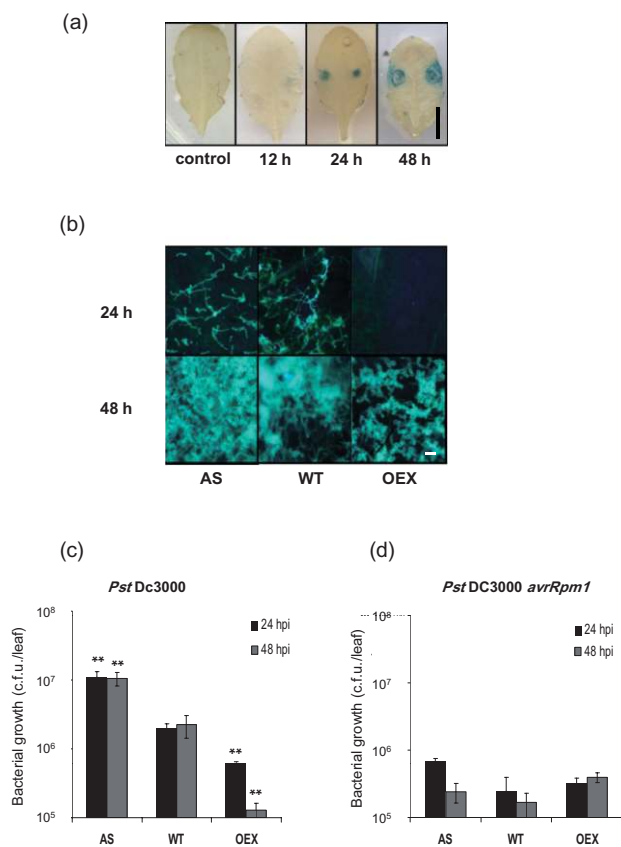
Figure 6. Response of *SAG21* expression to biotic stress and effects of *SAG21* expression on pathogen growth. (a) *SAG21* promoter-GUS activity at *Botrytis cinerea* inoculation sites. Infected 5-week-old plants were assayed at 12, 24 and 48 h after inoculation. Scale bar = 10 mm. (b) Aniline blue staining of *B. cinerea* hyphae in *SAG21* transgenic plants and wild type (WT) at 24 h and 48 h post-fungal inoculation. Scale bar = 50 μm. (c,d) Bacterial counts (see Methods for details) of *Pseudomonas syringae* pv. *tomato DC3000 (Pst DC3000)* and *Pst DC3000 avirulent Rpm1 (Pst DC3000 avrRpm1)* bacterial genotypes, as indicated. Data are means ± SE, *n* = 12. Significant differences from WT Col-0 ****P < 0.01 are indicated. AS, antisense; cfu, colony forming units; OEX, over-expression.

(Fig. 7b). The mitochondrial localization was confirmed with Mitotracker staining (Fig. 7c–e) of mature root epidermal cells.

DISCUSSION

LEA proteins are typically associated with protective functions, particularly in dehydrated tissues, where they are considered to act as chaperones, protecting other proteins from aggregation or desiccation (Tunnacliffe & Wise 2007). *SAG21* was isolated via its ability to complement an oxidant-sensitive yeast mutant and this initial study linked *SAG21* over-expression to an increase in growth (Mowla *et al.* 2006). The data presented in the current manuscript build on this observation and enable us to draw the following conclusions.

SAG21-YFP localizes to mitochondria

It was originally suggested that SAG21 might be a plastid protein (Mowla *et al.* 2006); however, confocal analysis of plants stably expressing a *SAG21*-YFP fusion protein was consistent with a mitochondrial location that fits well with predominant expression of *SAG21* in non-photosynthetic tissues (pollen and roots) and its light repression. A number of LEA proteins, both from plants and other organisms, are mitochondrially located and may be involved in protecting mitochondrial proteins from aggregation during stress (Grelet *et al.* 2005; Menze *et al.* 2009), although their precise roles are unknown.

Perturbation of *SAG21* expression affects above-ground biomass, flowering and timing of leaf senescence

Shoot biomass and leaf number were dramatically reduced in *SAG21* AS lines, and time to flowering was significantly increased in *SAG21* over-expressors, while it was decreased in the AS lines. Although plants deficient in antioxidant components often exhibit reduced growth and early flowering (e.g. Barth *et al.* 2004; Vandenabeele *et al.* 2004; Miller *et al.* 2007), in no case has over-expression of antioxidant enzymes produced the remarkable increase in biomass observed in *SAG21* OEX plants compared with WT (Mowla *et al.* 2006). However, the alteration of biomass and flowering time in the *SAG21* transgenic lines is consistent
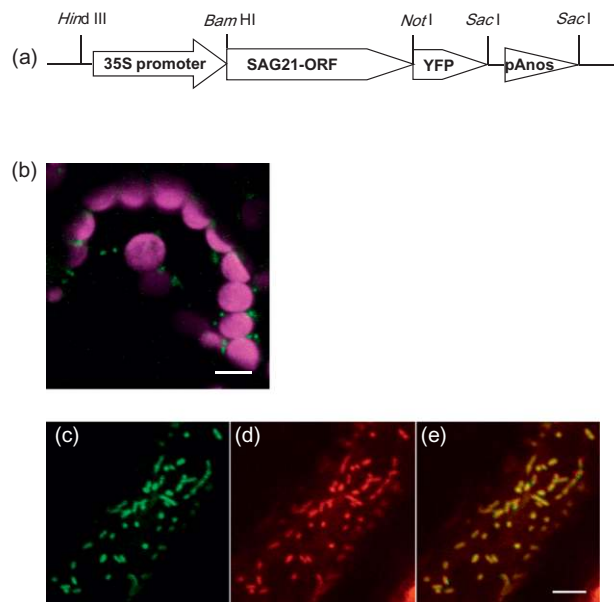
Figure 7. Subcellular localization of SAG21-YFP fusion protein. (a) Schematic representation of the construct for stable expression in plants. SAG21-YFP localization in (b) cotyledon mesophyll of 5-day-old seedlings. YFP is indicated by green signal; magenta signal indicates autofluorescence from chloroplasts. (c) SAG21-YFP localization in a mature epidermal root cell, (d) mitotracker staining, (e) overlay of (c) and (d) (scale bars = 5 μm). YFP; yellow fluorescent protein.

with a role for this protein in ROS responses. *SAG21* was originally identified as a senescence-associated gene and expression of a number of ROS-associated genes is up-regulated during senescence (Gepstein *et al.* 2003). Delayed senescence mutants *ore1*, *ore3* and *ore9* are also more tolerant of oxidative stress (Woo *et al.* 2004), as is the late-flowering mutant *gigantea* (Kurepa *et al.* 1998). Thus, there is also a relationship between lifespan and oxidative signalling. The mechanisms regulating the link between ROS, flowering and senescence are complex, but altering expression levels of senescence-linked transcription factors *WRKY53* and *WRKY70* affects both flowering time and senescence (Miao *et al.* 2004; Ülker, Mukhtar & Somssich 2007). A peak in H_2O_2 levels coinciding with bolting (Zimmermann *et al.* 2006) has been suggested to activate senescence-associated transcription factors such as *WRKY53*, coordinating developmentally induced leaf senescence. Thus, it is possible that *SAG21* is involved in signalling mechanisms leading to the control of lifespan and senescence. The 1685 bp promoter of *SAG21* used for the reporter gene studies contains four W-box elements within its upstream region, which are targets for *WRKY* transcription factor binding; however, further work will be required to establish whether it is indeed regulated by *WRKY* transcription factors.

SAG21 expression is restricted under optimal conditions but responds to abiotic stress and hormones

The strong expression of *SAG21* in pollen confirms reports from microarray data (Zimmermann *et al.* 2004) and the high expression noted in flowers from our previous work (Mowla *et al.* 2006). This fits well with the classical role of LEAs in dehydrated tissues: five other members of the LEA family in *Arabidopsis* are also expressed in pollen (At4g20450, At1g54410, At1g76180, At2g23120, At2g40170). Light had a strong negative effect on *SAG21* expression in both leaves and roots, consistent with previous Northern analysis (Mowla *et al.* 2006). Notably, we show here that *SAG21* is expressed throughout roots in darkness, with the exception of meristematic regions. Expression of *SAG21* in roots was clearly very responsive to a range of treatments, including abiotic stresses, ROS and plant growth regulators (ethylene, SA and MeJA), in agreement with microarray data (Zimmermann *et al.* 2004), and up-regulation in response to ROS in leaves (Mowla *et al.* 2006). Up-regulation of *SAG21* expression in leaves in response to stress treatments was found to be less consistent than in roots, perhaps reflecting greater sensitivity to growth conditions.

The promoter elements involved in *SAG21* expression remain to be characterized; however, as observed previously (Mowla *et al.* 2006) the 1685 bp promoter of *SAG21* used for the reporter gene studies contains four MYC and two MYB *cis* elements that may mediate responses to drought, salinity, ABA and cold. The involvement of MeJA in both senescence (He *et al.* 2001, 2002) and stress

responses (Balbi & Devoto 2008) is well established, and the four W-boxes may also mediate the SA and MeJA responses (Balbi & Devoto 2008) as JAs and SA are part of a complex network regulating responses to wounding, senescence and defence.

SAG21 is induced by biotic stress and infection patterns are altered in *SAG21* transgenic lines

As shown previously (Mowla *et al.* 2006), *SAG21* is not constitutively expressed in leaves; however, we show here that expression is induced within 24 h of inoculation with *B. cinerea*, a pathogen in which necrotic cell death is associated with increases in ROS production (Govrin & Levine 2000). *SAG21* expression is also responsive to wounding in leaves, specifically at the sites of ROS accumulation (Fig. 5d and Orozco-Cárdenas, Narvaez-Vasquez & Ryan 2001).

The potential link between *SAG21*, ROS and resistance to pathogens was investigated further using a bacterial and a fungal pathogen (Fig. 6c,d). The paucity of *B. cinerea* hyphal staining at 24 h after inoculation in the *SAG21* OEX line compared with WT suggests that the initial infection phase, where necrotic cell death has yet to be exhibited, was compromised and indicates increased plant resistance. Similar to *B. cinerea*, resistance of *SAG21* OEX plants to the bacterial pathogen, *Pst* DC3000, was increased and was also compromised in AS lines. Conversely, the response to the avirulent *Pst avrRpm1* was apparently unchanged by perturbation of *SAG21* expression. Taken together, these observations are consistent with SAG21 influencing plant defences that are not associated with HR-mediated programmed cell death. However, these findings are preliminary and could usefully be extended in future studies by examining the response of *SAG21* transgenics to a wider range of pathogens. Because basal resistance mechanisms are involved in responses to *B. cinerea* and *Pst* DC3000, our data suggest that *SAG21* expression specifically influences these pathways. Basal defences are elicited by pathogen-associated molecular patterns (PAMPs), which involve changes in cellular redox state (Gao *et al.* 2008). The data presented here suggest that the H_2O_2-responsive *SAG21* could be a component of this mechanism.

Perturbation of *SAG21* expression affects root growth, architecture and development

In common with the dramatic effect on above-ground organs of manipulating *SAG21* expression, the *SAG21* transgenic lines exhibited a marked root phenotype. Effects on primary root length noted previously (Mowla *et al.* 2006) were confirmed, although were less dramatic than previously noted, perhaps because of subtle differences in growth conditions. However, we show here that lateral root number and root hair elongation are significantly affected in *SAG21* AS and OEX plants.

It is now well established that redox regulation plays an important role in at least three distinct processes in root

development (Gapper & Dolan 2006): the cell cycle and maintenance of the root meristem (Vernoux *et al.* 2000), the transition from proliferation to differentiation in the root (Dunand, Crèvecoeur & Penel 2007; Tsukagoshi, Busch & Benfey 2010), and root hair formation (Foreman *et al.* 2003; Monshausen *et al.* 2007; Takeda *et al.* 2008). The cells in the quiescent centre of the root meristem are held in a highly oxidized state (Jiang & Feldman 2005; Jiang *et al.* 2006; De Tullio, Jiang & Feldman 2010). Moreover, glutathione is essential for root meristem development and in its absence, cells arrest in G1, as observed in the *Arabidopsis root meristemless* mutant (Vernoux *et al.* 2000). However, *SAG21* is not expressed in meristems, either in the primary or lateral roots, indicating that it does not have a role in the redox-mediated regulation of the cell cycle. This does not, however, preclude a role for *SAG21* in lateral root initiation, as demonstrated by the root phenotypes of AS and OEX lines. The initiation of the primary root is apparently not affected in *SAG21* transgenic lines, but the altered numbers of lateral roots relative to the primary root and the reduced length of the primary root suggest that *SAG21* plays a role in subsequent root development.

The data presented here strongly implicate *SAG21* in the control of root hair growth. The short root hair phenotype of AS plants is similar to that of the *rhd2* mutant, which lacks the plasma membrane-located respiratory burst oxidase, *AtrbohC* (Foreman *et al.* 2003; Dolan & Davies 2004; Carol & Dolan 2006; Gapper & Dolan 2006). Root hairs of *rhd2* mutants are 20% shorter than WT and the primary root is also shorter, although interestingly, *RHD2* expression is absent from root tips, as is *SAG21* (Gapper & Dolan 2006). Not only are root hairs shorter in *SAG21* AS lines, but the OEX lines have a higher proportion of longer root hairs (Fig. 3d). We conclude that *SAG21* plays a role in root hair elongation; moreover, its mitochondrial location suggests that this compartment has a controlling influence on this process. The pertinent question is: How can a mitochondrial protein participate in a pathway that leads to root hair expansion and does this involve *RHD2*, or is it a parallel pathway? Mitochondria are well established as sources of ROS (Rhoads *et al.* 2006; Noctor, De Paepe & Foyer 2007); and although we have not demonstrated a causal link with ROS and root hair growth in this study, *SAG21* expression is nevertheless linked to ROS (this study and Mowla *et al.* 2006). Both root hair elongation and the responses of plants to necrotrophic pathogens require the activation of different RBOH forms (Foreman *et al.* 2003; Miller *et al.* 2009). Thus, our data point to the possibility that *SAG21* plays a role in signalling pathways that involve RBOH. As a LEA protein, the most likely function of SAG21 is as an interacting protein that alters the function or stability of mitochondrial proteins involved in ROS production and/or signalling. This fascinating possibility awaits further investigation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Semi-quantitative RT-PCR of *SAG21* expression in transgenic lines. Wild type (WT), over-expressing line 2–5 and two independent antisense lines, AS-2 and AS-8, showing significantly different levels of transcript compared with WT ($\pm$SEM, $P < 0.01$).

Figure S2. Chlorophyll content in the oldest six rosette leaves. Over-expressing lines 2–5 and antisense line, AS-2 showing significantly different levels of chlorophyll compared with WT ($P < 0.05$).

Video S1. Timelapse imaging of *SAG21*-YFP in roots.

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