 Contribution of the drought tolerance-related Stress-responsive NAC1 transcription factor to resistance of barley to Ramularia leaf spot

GRAHAM R. D. MCGRANN1,*,†, ANDREW STEED1, CHRISTOPHER BURT1,‡, RACHEL GODDARD1, CLEA LACHAUX1, ANURADHA BANSAL1, MARGARET CORBITT1, KALINA GORNIAK2, PAUL NICHOLSON1 AND JAMES K. M. BROWN1,*

1Department of Crop Genetics, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, UK
2Crop Protection Team, Crop and Soil Systems Group, SRUC, West Mains Road, Edinburgh, EH9 3JG, UK

SUMMARY

NAC proteins are plant transcription factors that are involved in tolerance to abiotic and biotic stresses, as well as in many developmental processes. Stress-responsive NAC1 (SNAC1) transcription factor is involved in drought tolerance in barley and rice, but has not been shown previously to have a role in disease resistance. Transgenic over-expression of HvSNAC1 in barley cv. Golden Promise reduced the severity of Ramularia leaf spot (RLS), caused by the fungus Ramularia collo-cygni, but had no effect on disease symptoms caused by Fusarium culmorum, Oculimacula yallundae (eyespot), Blumeria graminis f. sp. hordei (powdery mildew) or Magnaporthe oryzae (blast). The HvSNAC1 transcript was weakly induced in the RLS-susceptible cv. Golden Promise during the latter stages of R. collo-cygni symptom development when infected leaves were senescing. Potential mechanisms controlling HvSNAC1-mediated resistance to RLS were investigated. Gene expression analysis revealed no difference in the constitutive levels of antioxidant transcripts in either of the over-expression lines compared with cv. Golden Promise, nor was any difference in stomatal conductance or sensitivity to reactive oxygen species-induced cell death observed. Over-expression of HvSNAC1 delayed dark-induced leaf senescence. It is proposed that mechanisms controlled by HvSNAC1 that are involved in tolerance to abiotic stress and that inhibit senescence also confer resistance to R. collo-cygni and suppress RLS symptoms. This provides further evidence for an association between abiotic stress and senescence in barley and the development of RLS.

Keywords: biotroph, endophyte, hemibiotroph, necrotroph, plant–pathogen interaction, senescence, transgenic resistance.

INTRODUCTION

Barley is the fourth most important cereal crop, grown in diverse environments worldwide (Newton et al., 2011). Barley yields are threatened by a wide range of biotic stresses, such as pests and diseases (Walters et al., 2012). As agricultural environments evolve through altered agronomic practices and climate change, crops such as barley are predicted to be at risk not only from current biotic stresses, but also from previously unrecognized pest and disease problems (Newton et al., 2011; West et al., 2012). Unfavourable environmental conditions caused by abiotic stresses, such as drought, flooding, extreme temperature, salinity and nutrient stress, can also cause yield losses that can be in excess of 50% (Bray et al., 2000). To maintain food production, crop protection needs to guard against losses caused not only by recognised diseases and abiotic stresses, but also by new and emerging threats.

Ramularia leaf spot (RLS), caused by the ascomycete fungus Ramularia collo-cygni, is a newly important disease of barley across Europe (Walters et al., 2008). The fungus is seed borne, but can also be spread by airborne spores that germinate on the leaf surface and penetrate through stomata (Havis et al., 2014; Stabentheiner et al., 2009). Ramularia collo-cygni lives as an asymptomatic endophyte during crop development, but can switch lifestyle late in the growing season to become a necrotrophic pathogen causing significant yield losses (Oxley and Havis, 2004; Walters et al., 2008). The expression of disease symptoms is associated with an overall decline in the host antioxidant system (Schützendübel et al., 2008) and progression of the disease results in an early onset of leaf senescence (Oxley et al., 2008). However, the precise conditions that contribute to the fungal transition from endophyte to pathogen are not fully understood. Exposure of barley plants to abiotic stresses, such as high light, results in enhanced RLS symptom development, implying that plant stress may be important to the development of this disease (Makepeace et al., 2008; Peraldi et al., 2014). If abiotic stress factors are required to elicit the fungal transition from endophyte to pathogen, crops with
enhanced tolerance to abiotic stress may also be more resistant to RLS.

Abiotic and biotic stress factors elicit somewhat different plant responses, but some components involved in resistance to different types of stress may be shared (Atkinson et al., 2013; Atkinson and Urwin, 2012; Narsai et al., 2013). The identification and characterisation of genes that control tolerance to multiple abiotic and biotic stress factors could help to reveal the mechanisms that enable plants to cope with stress, as well as being potential candidates for use in plant breeding programmes. Transcription factors act as molecular switches involved in the regulation of plant developmental programmes and responses to diverse stresses. One of the largest families of transcription factors is the plant-specific NAC [no apical meristem (NAM), Arabidopsis thaliana transcription activation factor (ATAF1/2) and cup-shaped cotyledon (CUC2)] transcription factor superfamily. NAC transcription factors are involved in the regulation of many developmental processes, including secondary cell wall biosynthesis, senescence and biotic and abiotic stress tolerance (Puranik et al., 2012). NAC genes can also promote and regulate reactive oxygen species (ROS) metabolism and homeostasis (Lee et al., 2012; Wu et al., 2012; You et al., 2013). Functional diversification within the NAC protein family predates the separation of the monocot and dicot lineages (Nuruzzaman et al., 2010) and many different NAC transcription factors have been characterised in cereals with various functions (Distelfeld et al., 2012; Uauy et al., 2006). Some cereal NAC transcription factors regulate responses to drought, cold and salinity stresses, whereas others act against pathogens (Chen et al., 2013; Jensen et al., 2007; Nakashima et al., 2007; Sun et al., 2013; Xia et al., 2010). However, many NAC genes respond to both abiotic and biotic stresses, and the regulation of multiple stresses appears to be a common function of NAC transcription factors, indicating a degree of functional redundancy within this gene family (Nuruzzaman et al., 2010).

NAC genes have been used in transgenic approaches to enhance abiotic stress tolerance in cereals. Stress-responsive NAC1 (SNAC1) was first characterised in transgenic rice (OsSNAC1), demonstrating a positive role for this transcription factor in drought and salt tolerance (Hu et al., 2006). OsSNAC1 regulates abiotic and oxidative stress tolerance by enhancing the expression of stress-responsive genes (Hu et al., 2006) and by targeting genes that control stomatal closure and ROS homeostasis (You et al., 2013). Over-expression of the orthologous barley gene HvSNAC1 also enhanced drought tolerance in barley plants (Al Abdallat et al., 2014).

In this article, we demonstrate that SNAC1 has a previously unknown positive function in plant–pathogen interactions. Over-expression of HvSNAC1 in barley reduces the severity of RLS symptoms as well as fungal colonisation by R. collo-cygni. This appears to be related to the delayed senescence of HvSNAC1 over-expression lines rather than alterations in antioxidant capacity or sensitivity to ROS. The specificity of the response to RLS and the link between HvSNAC1 over-expression and enhanced leaf longevity provide further insights into the involvement of senescence in the interaction between barley and RLS.

RESULTS

Disease development on HvSNAC1 over-expression lines

The role of HvSNAC1 in defence against fungal diseases was explored using two independent transgenic barley cv. Golden Promise (GP) lines, OE#3 and OE#11, which both exhibited consistently elevated HvSNAC1 transcript levels in seedling prophyll leaves (Fig. S1, see Supporting Information). Disease symptom expression was assessed on plants inoculated with R. collo-cygni, as well as the hemibiotrophic fungal pathogens Oculimacula yallundae, Magnaporthe oryzae and Fusarium culmorum, which differ in the duration of biotrophic growth before entering necrotrophic development, ranging from weeks to days, and the obligate biotroph Blumeria graminis f. sp. hordei. Typical development of R. collo-cygni was observed on cv. GP with the wild-type HvSNAC1 gene, a barley variety that shows a moderate degree of susceptibility to isolate Rcc09B4. RLS first became visible from 10 to 12 days post-inoculation (dpi). The number of spots increased over time with lesions coalescing as the leaf began to senesce (Fig. 1a). The development of R. collo-cygni symptoms was reduced in both transgenic lines compared with GP (P < 0.001; Fig. 1a,b). Significantly smaller amounts of R. collo-cygni DNA were recorded in both transgenic lines compared with GP (60%–75% lower than GP; Fig. 1c). No significant differences were observed in R. collo-cygni DNA levels in seeds of GP and the two transgenic lines that were used in the inoculation experiments. (P = 0.4; results not shown).

No significant differences were observed in the size of the lesions formed by F. culmorum (Fig. 2a; P = 0.2) or the number of lesions formed by M. oryzae (Fig. 2b; P = 0.3) on the leaves of either transgenic line compared with GP. Over-expression of HvSNAC1 also had no significant effect on disease symptom development of the stem base eyespot disease caused by O. yallundae (Fig. 2c; P = 0.3) or on colony formation by B. graminis f. sp. hordei (Fig. 2d; P = 0.8).

Expression analysis of HvSNAC1 transcript following R. collo-cygni inoculation

Time course analysis of HvSNAC1 transcript expression during R. collo-cygni infection in GP leaves indicated that HvSNAC1 was not differentially regulated during the early asymptomatic stages of the R. collo-cygni infection process, up to 10 dpi (Fig. 3). A small
increase in HvSNAC1 transcript level was observed at the later stages of infection when leaves exhibited severe symptoms and had begun to senesce (Fig. 1a and 3).

Effect of HvSNAC1 over-expression on leaf antioxidant transcript levels and sensitivity to ROS-induced cell death

The onset of RLS symptoms in the field has been associated with an overall decline in the host antioxidant system (Schützendübel et al., 2008). Transcript profiling of the major barley ROS scavengers, including catalases (CAT1 + CAT2), ascorbate peroxidases (APX1 + APX2), glutathione peroxidases (GPX1 + GPX2), glutathione reductase (GR1) and copper/zinc superoxide dismutase (CSD1), was used to assess the effect of HvSNAC1 over-expression on the antioxidant system of barley. Constitutive gene expression levels of all of the antioxidant transcripts tested were similar in both transgenic lines to those in GP (Fig. 4a). The role of HvSNAC1 over-expression on ROS-induced cell death was examined by testing the effect of different ROS donors on cell death lesion formation in lines OE#3 and OE#11 relative to GP. No significant differences in lesion size produced by the hydrogen peroxide donor alloxan ($P = 0.9$), the mitochondrial superoxide donor menadione ($P = 0.7$) and the chloroplastic superoxide donor methyl viologen ($P = 0.1$) were observed between either of the transgenic lines and GP (Fig. 4b).

Effect of HvSNAC1 over-expression on leaf senescence and stomatal conductance

In the field, RLS symptoms become visible during the later developmental stages of the host as the plant begins to flower and the leaves start to senesce (Schützendübel et al., 2008). It is not currently known whether leaf senescence is a cause or consequence of disease. Dark-induced senescence, a model system used to study leaf senescence in plants (Gan and Amasino, 1997), was used to test the effect of over-expression of HvSNAC1 on leaf senescence. All three lines showed a decline in relative chlorophyll content over time (Fig. 5a, $P < 0.001$). There was no significant difference between the relative chlorophyll contents of GP and the two transgenic lines at day 0 or after 2 days of dark treatment. From day 4 onwards, there was a significantly slower decline in relative chlorophyll content in both over-expression lines OE#3 and OE#11 compared with GP ($P < 0.001$). This slower decline in relative chlorophyll content was also observed at day 6 ($P < 0.001$) and day 8 ($P < 0.01$) in both transgenic lines, indicating that over-expression of HvSNAC1 delays dark-induced leaf senescence (Fig. 5a).
Given the role of HvSNAC1 in drought tolerance and that R. collo-cygni infects leaves through the stomata, the effect of the over-expression of HvSNAC1 on stomatal closure was examined. No significant differences in stomatal conductance between the transgenic lines OE#3 and OE#11 and GP were observed (Fig. 5b, \( P = 0.7 \)).

**DISCUSSION**

As a recently emerging problem of barley, the factors that result in the switching of the endophytic fungus R. collo-cygni to a necrotrophic pathogen, resulting in disease, are not fully understood. The development of RLS symptoms typically occurs late in the growing season and appears to be associated with the action of environmental stresses on the host (Makepeace et al., 2008; Oxley and Havis, 2004; Peraldi et al., 2014; Schützendübel et al., 2008). NAC transcription factors act as central modulators of stress responses that function as transcriptional activators to regulate the expression of stress-related genes, including those involved in defence, detoxification and redox homeostasis (Hu et al., 2006; Nakashima et al., 2007; Sun et al., 2013). SNAC1 transcription factors enhance the drought tolerance of cereals.
Overexpression of the barley SNAC1 gene confers a small increase in resistance to RLS, but has no effect on the interaction between barley and the fungal pathogens *B. graminis f. sp. hordei*, *F. culmorum*, *M. oryzae* and *O. yallundae*. This implies that there is a specific interaction between the endophytic parasite *R. collo-cygni* and SNAC1.

**Fig. 4** Effect of HvSNAC1 over-expression on barley redox system. (a) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of the constitutive transcript levels of the major reactive oxygen species scavengers in HvSNAC1 over-expression lines. Transcript levels are presented relative to wild-type Golden Promise leaves. APX1, ascorbate peroxidase 1; APX2, ascorbate peroxidase 2; CAT1, catalase 1; CAT2, catalase 2; CSD1, copper/zinc superoxide dismutase 1; GPX1, glutathione peroxidase 1; GPX2, glutathione peroxidase 2; GR1, glutathione reductase 1. Error bars indicate SE (b) Lesion development caused by the reactive oxygen species donors alloxan, menadione and methyl viologen in transgenic HvSNAC1 over-expression barley lines. Error bars indicate ±1SE.

**Fig. 5** Effect of HvSNAC1 over-expression (OE) on dark-induced senescence (a) and stomatal closure (b) compared with Golden Promise (GP). Error bars indicate ±1SE. ***P < 0.001 and **P < 0.01 for comparison of means of OE lines with GP.
Symbiotic interactions between fungal endophytes and plants are frequently benign or beneficial to the host. However, when host conditions become unfavourable for an endophytic lifestyle, these fungi can become pathogenic (Rodriguez and Redman, 2008). The development of RLS symptoms typically occurs following conditions that are expected to alter host ROS status (Makepeace et al., 2008; Peraldi et al., 2014; Schützendübel et al., 2008). Increased cellular ROS concentrations, if not controlled, can result in cell death, which promotes the development of necrotrophic pathogens (Heller and Tudzynski, 2011). NAC genes regulate ROS levels in plants under stressful conditions and the rice orthologue of HvSNAC1, OsSNAC1, regulates ROS homeostasis through interactions with genes, such as OsSRO1 (You et al., 2013). Over-expression of HvSNAC1 did not influence directly the regulation of ROS scavenger transcript expression or sensitivity to ROS-induced cell death (Fig. 4). This implies that the tolerance of transgenic plants over-expressing HvSNAC1 to RLS and restriction of the necrotrophic development of the fungus do not result from elevated resistance to ROS-related damage.

*Ramularia collo-cygni* is transmitted by infected seed (Havis et al., 2014) and spore-borne foliar infections that occur through stomatal openings (Stabentheiner et al., 2009). Seed-borne inoculum contributes to the initial infection, whereas spore-borne infections later in the season may play a role in secondary infections and fungal dissemination (Havis et al., 2014). The relative contribution of infection via both of these modes of transmission towards final disease levels is not fully understood. Stomata form points of entry for the fungus and, following spore or mycelium inoculation, *R. collo-cygni* grows intercellularly with conidiophores emerging from stomata (Stabentheiner et al., 2009; Thirugnanasambandam et al., 2011). The rice orthologue of HvSNAC1, OsSNAC1, can regulate stomatal closure (Hu et al., 2006) through its action on OsSRO1 (You et al., 2013), but we found no effect of HvSNAC1 over-expression in barley on constitutive levels of stomatal closure (Fig. 5b), corroborating the work of Al Abdallat et al. (2014). These data suggest that the reduced susceptibility of the HvSNAC1 over-expression lines to *R. collo-cygni* is unlikely to be a result of fewer infection events and reduced fungal penetration resulting from more tightly closed stomata. The effect of HvSNAC1 over-expression specifically on seed-borne RLS epidemiology remains to be determined.

Disease development occurs late in the growing season and has been associated with crop senescence (Oxley et al., 2008; Schützendübel et al., 2008). Over-expression of HvSNAC1 delayed dark-induced leaf senescence in both transgenic lines (Fig. 5a). HvSNAC1 transcript levels showed a weak induction in GP during the later stages of RLS symptom development (Fig. 3). However, HvSNAC1 transcripts are induced in senescing flag leaves (Christiansen et al., 2011), and so it is unclear whether the increased expression of this gene is a response to stress caused by fungal colonisation or is a consequence of premature leaf senescence associated with the later stages of disease development (Fig. 1a; Oxley et al., 2008; Walters et al., 2008). NAC transcription factors can positively or negatively affect senescence (Distelfeld et al., 2012; Lee et al., 2012; Uauy et al., 2006; Wu et al., 2012; Zhou et al., 2013). TTNAM-B1 and OsSNAP, which are related to the SNAC subgroup of cereal NAC genes (Nuruzzaman et al., 2010), promote leaf senescence in wheat and rice (Uauy et al., 2006; Zhou et al., 2013), respectively, whereas HvSNAC1 delays the process in barley (Fig. 5a). The results reported here imply that the over-expression of a gene which suppresses senescence in barley also suppresses the growth of *R. collo-cygni* and the development of RLS symptoms. NAC transcription factors regulate senescence through different processes, including manipulation of ROS and hormone pathways. Positive regulation of senescence by OsSNAP appears to be associated with jasmonate biosynthesis and signalling pathways (Zhou et al., 2013), but the relationship between SNAC1 and plant hormones is unknown. Whether the effect of SNAC1 on leaf senescence operates through previously characterised or novel hormone signalling pathways remains to be revealed.

The longevity of green leaf area has been genetically linked to drought tolerance in cereals (Foulkes et al., 2007). NAC gene mutants that delay senescence in the model plant *Arabidopsis thaliana* also show enhanced tolerance to abiotic stresses, implying an association between senescence and stress (Lee et al., 2012; Wu et al., 2012). In the field, there is some evidence that leaf senescence promotes RLS (Schützendübel et al., 2008), and barley varieties that are highly susceptible to RLS tend to senesce early as a result of the disease (Oxley et al., 2008). Although HvSNAC1 over-expression lines, which have reduced susceptibility to RLS (Fig. 1), have a small effect on reducing the rate of leaf senescence (Fig. 5), further experiments are required to elucidate the interactions of the signalling pathways regulated by HvSNAC1 with *R. collo-cygni*, stress and senescence.

Deciphering the signalling networks controlled by HvSNAC1 will further our understanding of the host processes that contribute to the expression of RLS. HvSNAC1 over-expression affected the development of RLS, but had no significant effect on four other diseases (Fig. 2), despite the fact that the HvSNAC1 transcript was regulated upwards and downwards by infection with *Fusarium* and *B. graminis*, respectively (Al Abdallat et al., 2014). Therefore, it appears that HvSNAC1 has a specific effect on RLS and is not a major factor contributing to the interaction between barley and other diseases (Fig. 2). The association between symptom development of this late season disease, plant stress and senescence implies that the delayed senescence phenotype of the HvSNAC1 over-expression lines is involved in the reduced growth of *R. collo-cygni* and expression of RLS symptoms. As a newly important pathogen, little is known about the host genetic components involved in the interaction with *R. collo-cygni*. Recent evidence that mutant *mlo* alleles, which confer resistance to the biotrophic...
powdery mildew fungus, increase susceptibility to RLS (McGrann et al., 2014) further implies that senescence is involved in the aetiopathology of RLS. The wild-type MLO gene responds to both biotic and abiotic stresses (Baker et al., 2000) and mutant mlo alleles accelerate the rate of leaf senescence once the process has begun (Pippanelli et al., 2002). Whether the mlo-enhanced susceptibility to RLS is specifically linked to senescence or to one or more of the other pleiotropic effects caused by the mlo mutation requires further experimentation. Interpretation of the link between RLS, senescence and stress responses may provide insights into why RLS has recently emerged as an important disease of barley.

EXPERIMENTAL PROCEDURES

Plant material

Two transgenic lines, OE#3 and OE#11, from independent transformation events over-expressing HvSNAC1 (HvSNAC003; Christiansen et al., 2011) were used in this study (Al Abdallat et al., 2014). Seeds were sown in 8 × 8 × 10-cm³ pots in Levington F2 compost medium (Scotts Professional, Ipswich, UK), and grown under a 16 h/8 h day/night photoperiod at day/night temperatures of 18/12 °C provided by 220 μmol/m²/s fluorescent lighting in a controlled environment room (Sanyo Gallenkamp PLC, Loughborough, UK).

Pathogen inoculations

Ramularia collo-cygni isolate Rcc0984 was used to inoculate barley plants following the protocol of Makepeace et al. (2008) with the modifications of Peraldi et al. (2014). RLS development was scored as the percentage leaf area covered with disease symptoms three to five times over 21 dpi and the area under the disease progress curve (AUDPC) was calculated. Three independent replicate experiments, each containing an individual 8 × 8 × 10-cm³ pot of 10 seeds of each line, were inoculated with R. collo-cygni. Oculimacula yallundae isolate P149 was inoculated at the stem base of 21-day-old plants using the method of Chapman et al. (2008). A single experiment was inoculated in a randomised block design, consisting of five blocks, with each block containing five plants of each line. The penetration of stem bases by O. yallundae was assessed 8 weeks post-inoculation using the scale of Scott (1971). Magnaporthe oryzae isolate BR32 was inoculated at a concentration of 10⁶ spores/mL in two independent experiments, as described previously (Tufan et al., 2009). In each experiment, 10 seeds of each line were sown in individual 8 × 8 × 10-cm³ pots and disease was assessed at 6 dpi as the number of blast lesions present on the second seedling leaf of each plant. The development of F. culmorum isolate Fu42 was assessed on detached leaves inoculated with two 5-μL droplets of 10⁶ conidia/mL amended with 75 μM deoxynivalenol (Chen et al., 2009). Photographs of disease lesion development were taken 48 h post-inoculation and the lesion area was measured using ImageJ software (Abramoff et al., 2004). Two independent replicate experiments, each containing a minimum of six replicate leaves of each line, were inoculated. Blumeria graminis f. sp. hordei isolate CC148 was used to inoculate leaf segments from 14-day-old prophyll leaves following the method of Boyd et al. (1994). Disease development was assessed at 14 dpi as the number of colonies observed per square centimetre of leaf area in four independent replicate experiments, each consisting of a minimum of eight replicate leaves of each line.

The response of HvSNAC1 over-expression lines to each pathogen was analysed separately using a general linear model (GLM) in GenStat v.15 (Payne et al., 2009). Each GLM assessed the variation attributable to experimental replicate, block and line, and calculated the predicted mean disease scores on each line. Predicted means were used to compare the disease development of each pathogen on the two transgenic lines and GP with t-tests using the residual standard error of the respective model.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) gene expression assays

Constitutive levels of HvSNAC1 and ROS scavenger gene transcripts were assessed in each of the two transgenic lines compared with GP using qRT-PCR. RNA was extracted from 14-day-old prophyll leaves, processed and converted to cDNA (Colebrook et al., 2012). Transcripts were amplified with gene-specific primers (Table S1, see Supporting Information; Shagimardanova et al., 2010) and the Sybr Green JumpStart™ Taq Ready mix system (Sigma-Aldrich, St. Louis, MO, USA), as described previously (Colebrook et al., 2012). cDNA samples were normalised with geNorm (Vandesompele et al., 2002) using five reference genes (elongation factor 1α, glyceraldehyde 3-phosphate dehydrogenase, cyclophilin, ubiquitin, α-tubulin; Burton et al., 2004; McGrann et al., 2009; Rostoks et al., 2003). Data were collected from three independent replicate experiments, each consisting of at least two independent samples of each line.

Expression of the HvSNAC1 transcript during the host response to R. collo-cygni infection was assessed in GP. Seedlings were inoculated as described above with either Rcc0984 or potato dextrose broth containing no fungus as the control. Two samples, each consisting of two pooled prophyll leaves from Rcc0984 and control inoculated plants, were collected at 5, 10, 15, 18 and 21 dpi. Samples were processed as above and the expression of the HvSNAC1 transcript was compared between Rcc0984 and control inoculated samples at each time point. Data were obtained from three independent replicate experiments.

qPCR quantification of R. collo-cygni DNA levels in planta

In order to estimate the amount of R. collo-cygni within host leaves, fungal DNA levels were measured by qPCR (Taylor et al., 2010). DNA was extracted from five leaf samples of each line collected at 21 dpi using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Fungal DNA levels in three independent replicate experiments were quantified by qPCR using a CFX96 thermocycler (Bio-Rad, Hercules, CA, USA). Differences in log₁₀ transformed fungal DNA levels between the two transgenic lines and GP were analysed using a GLM in GenStat v.15 with experimental replicate and line as factors. Ramularia collo-cygni DNA levels in seeds were assessed using the methods of Havis et al. (2014).

ROS-induced cell death assays

ROS-induced cell death assays were performed on barley leaves following the method of Saville (2011). Prophyll leaves from 14-day-old plants of
each transgenic line and GP were detached and suspended across agar (1% agar w/v supplemented with 100 mg/L benzimidazole; Sigma-Aldrich) bridges in clear plastic boxes. The sensitivity of each line to ROS-induced cell death was tested by adding 2 μL of solution, supplemented with 0.5% w/v Tween20, of the following ROS donors to the centre of each leaf: 200 mM alloxan (Sigma-Aldrich), 100 mM menadione (Sigma-Aldrich) and 25 μM methyl viologen (Sigma-Aldrich). Inoculated leaves were stored under constant light (15–20 μmol m⁻² s⁻¹) for 96 h at room temperature. After incubation, each box was photographed and the lesion size was measured using ImageJ. ROS-induced lesion formation was measured from three independent replicate experiments, each consisting of a minimum of 24 replicate leaves of each line for each ROS donor. General linear modelling was used to estimate the effects of replicate experiment and line. Each ROS donor was analysed separately.

Stomatal conductance measurements

The effect of over-expression of HvSNAC1 on stomatal conductance was measured using an AP4 cycling perometer (Delta-T Devices Ltd, Cambridge, UK) based on the methods described by Prats et al. (2006). Leaf water conductance measurements were taken from the prophyll leaf of 5–10 14-day-old seedlings of each line. The experiment was performed four times. Data were analysed using a GLM in GenStat v.15 with experiment and line as factors.

Dark-induced senescence assay

Dark-induced senescence in barley leaves was measured following the method outlined by Peraldi (2012). Prophyll leaves from 14-day-old seedlings were excised and an indirect measurement of leaf chlorophyll content was taken using a SPAD 502 Plus Chlorophyll Meter (Konica Minolta, Warrington, UK). Three measurements were taken across each leaf to cover the distal, middle and basal portions, and averaged. After the first measurement (day 0), leaves were placed on damp tissue paper in 10-cm² plastic Petri dishes, covered with aluminium foil and kept in the dark at room temperature to induce senescence. Further measurements were taken on each leaf after 2, 4, 6 and 8 days of dark treatment. Three replicate experiments were performed with measurements taken from six leaves of each line in each experiment. Linear mixed modelling of repeated measurements was used to evaluate differences in SPAD readings between each line at the different time points employing the uniform correlation/split plot in time covariance matrix. Fixed factors included day, experiment, line and the interactions between day and line, and experiment and day. The random factor was the leaf-by-day interaction term. Significant differences between lines at different days were subsequently assessed using a t-test.

ACKNOWLEDGEMENTS

We thank Ayed Al-Abdallat (University of Jordan) and Wendy Harwood John Innes Centre (IJIC) for seeds of the HvSNAC1 transgenic lines, and Mike Grimme (ADAS) for the loan of the AP4 cycling perometer. We also thank Didier Tharreau (CIRAD, Montpellier) for providing the Magnaporthe oryzae isolate BR32, which was used under Defra Plant Health Licence 51098/198798/1 (3/2013), and Neil Havis (SRUC) for useful discussions on the biology of Ramularia colo-cygni. This research was supported by the Biotechnology and Biological Sciences Research Council, RESAS, AHDB-HGCA and a consortium of 11 companies (BASF, Bayer, KWS, Lantmannen, Limagrain, LS, NIAB-TAG, Saaten-Union, Secobra, Sejeto, Syngeta) through Sustainable Arabic LINK, by grant BB/G024006/1 from BBSRC and by the John Innes Foundation. SRUC receives grant-in-aid from the Scottish Government. CL was funded by a student bursary from the British Society for Plant Pathology.

REFERENCES

Abraham, M.D., Magalhaes, P.J. and Ram, S.J. (2004) Image processing with ImageJ. Biophotonics Int. 11, 36–42.
Al Abdallat, A.M., Ayad, I.Y., Abu Elenein, J.M., Al Ajoumi, Z. and Harwood, W.A. (2014) Overexpression of the transcription factor HvSNAC1 improves drought tolerance in barley (Hordeum vulgare L.). Mol. Breed. 33, 401–414.
Atkinson, N.J. and Urwin, P.E. (2012) The interaction of plant biotic and abiotic stresses: from genes to the field. J. Exp. Bot. 63, 3523–3543.
Atkinson, N.J., Lilley, C.J. and Urwin, P.E. (2013) Identification of genes involved in the response of Arabidopsis to simultaneous biotic and abiotic stresses. Plant Physiol. 162, 2028–2041.
Baker, S.J., Newton, A.C. and Gurr, S.J. (2000) Cellular characteristics of temporary partial breakdown of mlo-resistance in barley to powdery mildew. Physiol. Mol. Plant Pathol. 56, 1–11.
Boyd, L.A., Smith, P.H., Green, R.M. and Brown, J.K.M. (1994) The relationship between the expression of defense-related genes and mildew development in barley. Plant–Microbe Interact. 7, 401–410.
Bray, E.A., Bailey-Serres, J. and Weretilnyk, E. (2000) Response to abiotic stresses. In: Biochemistry and Molecular Biology of Plants (Gruissem, W., Buchanan, B. and Jones, R., eds), pp. 1158–1203. Rockville, MD: American Society of Plant Physiologists.
Burton, R.A., Shirley, N.J., King, B.J., Harvey, A.J. and Fincher, G.B. (2004) The CesA gene family of barley. Quantitative analysis of transcripts reveals two groups of co-expressed genes. Plant Physiol. 134, 224–236.
Chapman, N.H., Burt, C., Dong, H. and Nicholson, P. (2008) The development of PCR-based markers for the selection of eyespot resistance genes Pch1 and Pch2. Theor. Appl. Genet. 117, 425–433.
Chen, X., Steed, A., Travella, S., Keller, B. and Nicholson, P. (2009) Fusarium graminearum exploits ethylene signalling to colonize dicotyledonous and monocotyledonous plants. New Phytol. 182, 975–983.
Chen, Y.-J., Pereira, V., Christiansen, M.W., Holme, I.B., Gregersen, P.L., Grant, M.R., Collinge, D.B. and Lyngkjaer, M.F. (2013) The barley HvNAC6 transcription factor affects ABA accumulation and promotes basal resistance against powdery mildew. Plant Mol. Biol. 83, 577–590.
Christiansen, M.W., Holm, P.B. and Gregersen, P.L. (2011) Characterization of barley (Hordeum vulgare L) NAC transcription factors suggests conserved functions compared to both monocots and dicots. BMC Res. Notes, 4, 302.
Colebrooke, E.H., Creissen, G., McGann, G.R.D., Dreas, R., Lamb, C. and Boyd, L.A. (2012) Broad-spectrum acquired resistance in barley induced by the Pseudomonas system pathosystem shares transcriptional components with Arabidopsis systemic acquired resistance. Mol. Plant–Microbe Interact. 25, 658–667.
Distelfeld, A., Pearce, S.P., Avni, R., Scherer, B., Uauy, C., Piston, F., Slade, A., Zhao, R. and Dubcovsky, J. (2012) Divergent functions of orthologous NAC transcription factors in wheat and rice. Plant Mol. Biol. 78, 515–524.
Foulkes, M.J., Sylvester-Bradley, R., Weightman, R. and Snape, J.W. (2007) Identifying physiological traits associated with improved drought resistance in winter wheat. Field Crops Res. 103, 11–24.
Gan, S.S. and Amasino, R.M. (1997) Making sense of senescence—molecular genetic regulation and manipulation of leaf senescence. Plant Physiol. 113, 313–319.
Havis, N.D., Nyman, N. and Osley, S.J.P. (2014) Evidence for seed transmission and asymmetric growth of Ramularia colo-cygni in barley (Hordeum vulgare). Plant Pathol. 63, 929–936. doi: 10.1111/ppa.12162.
Heller, J. and Tuzdynski, P. (2011) Reactive oxygen species in phytopathogenic fungi: signaling, development, and disease. Annu. Rev. Phytopathol. 49, 369–390.
Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q. and Xiong, L. (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc. Natl. Acad. Sci. USA, 103, 12 987–12 992.
Jensen, M.K., Rung, J.H., Gregersen, P.L., Gjetting, T., Fuglsang, A.T., Hansen, M., Joehnk, N., Lyngkjaer, M.F. and Collinge, D.B. (2007) The HvNAC6 transcription...
factor: a positive regulator of penetration resistance in barley and Arabidopsis. Plant Mol. Biol. 65, 137–150.

Lee, S., See, P.J., Lee, H.-J. and Park, C.-M. (2012) A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in Arabidopsis. Plant J. 70, 831–844.

Makepeace, J.C., Havis, N.D., Burke, J.J., Oxley, S.J.P. and Brown, J.K.M. (2008) A method of inoculating barley seedlings with *Ramularia collo-cygni*. Plant Pathol. 57, 991–999.

McGrann, G.R.D., Townsend, B.J., Antoniv, J.F., Asher, M.J.C. and Mutasa-Goettens, E.S. (2009) Barley elicits a similar early basal defence response during host and non-host interactions with *Polymyxa* root parasites. Eur. J. Plant Pathol. 123, 5–15.

McGrann, G.R.D., Stavrinides, A., Russell, J., Corbitt, M.M., Booth, A., Charrtain, L., Thomas, W.T.B. and Brown, J.K.M. (2014) A trade-off between nlo resistance to powdery mildew and increased susceptibility of barley to a newly important disease, *Ramularia* leaf spot. *J. Exp. Bot.* 65, 1025–1037.

Nakashima, K., Tran, L.-S.P., Van Nguyen, D., Fujita, M., Maruyama, K., Todaka, D., Ito, Y., Hayashi, N., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2007) Functional analysis of a NAC-type transcription factor OsNACs involved in abiotic and biotic stress-responsive gene expression in rice. *Plant J.* 51, 617–630.

Narsai, R., Wang, C., Chen, J., Wu, J., Shou, H. and Whelan, J. (2013) Antagonistic, overlapping and distinct responses to biotic stress in rice (*Oryza sativa*) and interactions with abiotic stress. *BMC Genomics* 14, 93.

Newton, A.C., Flavell, A.J., George, T.S., Leat, P., Mullhallan, B., Ramsay, L., Revoredo-Giha, C., Russell, J., Steffenson, B.I., Swanton, J.S., Thomas, W.T.B., Waugh, R., White, P.J. and Bingham, J.I. (2011) Crops that feed the world 4. Barley: a resilient crop? Strengths and weaknesses in the context of food security. *Food Secur.* 3, 141–178.

Nuruzzaman, M., Manimekalai, R., Sharoni, A.M., Satoh, K., Kondoh, H., Ooka, H. and Kikuchi, S. (2010) Genome-wide analysis of NAC transcription factor family in *Brachypodium distachyon* exhibiting compatible interactions with *Polymyxa* root parasites. *J. Exp. Bot.* 61, 991–999.

Polymyxa grisea during host and non-host interactions with *Magnaporthe grisea*. *Plant Mol. Biol.* 81, 41–56.

Shagimardanova, E.I., Geuse, O.A., Sychev, V.N., Levinskikh, M.A., Sharipova, M.R., Il’inskaya, O.N., Bingham, G. and Sugimoto, M. (2010) Expression of stress response genes in barley *Hordeum vulgare* in a spaceflight environment. *Mol. Biol.* 44, 734–740.

Stabenheimer, E., Minihofe, T. and Huss, H. (2009) Infection of barley by *Ramularia collo-cygni*: scanning electron microscopic investigations. *Mycopathologia* 168, 135–143.

Sun, L., Zhang, H., Li, D., Huang, L., Hong, Y., Ding, X.S., Nelson, R.S., Zhou, X. and Song, F. (2013) Functions of rice NAC transcriptional factors, ONAC122 and ONAC131, in defense responses against *Magnaporthe grisea*. *Plant Mol. Biol.* 81, 41–56.

Taylor, J.M.G., Paterson, L.J. and Havis, N.D. (2010) A quantitative real-time PCR assay for the detection of *Ramularia collo-cygni* from barley (*Hordeum vulgare*). *Lett. Appl. Microbiol.* 50, 493–499.

Thriugnanasambandam, A., Wright, K.M., Havis, N., Whisson, S.C. and Newton, A.C. (2011) *Agrobacterium*-mediated transformation of the barley pathogen *Ramularia collo-cygni* with fluorescent marker tags and live tissue imaging of infection development. *Plant Pathol.* 60, 929–937.

Tufan, H.A., McGrann, G.R.D., Magusin, A., Morel, J.B., Michele, L. and Boyd, L.A. (2009) Wheat blast: histopathology and transcriptome reprogramming in response to adapted and nonadapted *Magnaporthe* isolates. *New Phytol.* 184, 473–484.

Uauy, C., Distelfeld, A., Fahima, T., Bleich, A. and Dubcovsky, J. (2006) A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. *Science*, 314, 1302–1305.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, research0034.0031–research0034.0011.

Walters, D.R., Havis, N.D. and Oxley, S.J.P. (2008) *Ramularia collo-cygni*: the biology of an emerging pathogen of barley. *FEMS Microbiol. Lett.* 279, 1–7.

Walters, D.R., Avrova, A., Bingham, I.J. and Fitt, B.D.L. (2014) Comparative biology of different plant pathogens to estimate effects of climate change on crop diseases in Europe. *J. Eur. Plant Pathol.* 133, 315–331.

Wu, A., Allu, A.D., Garapati, P., Siddiqui, H., Dortay, H., Zanor, M.-L., Asensi-Fabado, M.A., Munne-Bosch, S., Antonio, C., Tohge, T., Fernie, A.R., Kaufmann, K., Xue, G.-P., Mueller-Roever, B. and Balazadeh, S. (2012) *JUNGBRUNNER1*, a reactive oxygen species-responsive NAC transcription factor, regulates longevity in Arabidopsis. *Plant Cell*, 24, 482–506.

Xia, N., Zhang, G., Sun, Y.F., Zhu, L., Xu, L.S., Chen, X.M., Liu, B., Yu, Y.T., Wang, X.J., Huang, L.L. and Kang, Z.S. (2010) TaNAC8, a novel NAC transcription factor gene in wheat, responds to stripe rust pathogen infection and abiotic stresses. *Physiol. Mol. Plant Pathol.* 74, 394–402.

You, J., Zong, W., Li, X., Ning, J., Hu, H., Li, X., Xiao, J. and Xiong, L. (2013) The *SNAC1*-targeted gene *OsSRO1c* modulates stomatal closure and oxidative stress tolerance by regulating hydrogen peroxide in rice. *J. Exp. Bot.* 64, 569–583.

Zhou, Y., Huang, W., Liu, L., Chen, T., Zhou, F. and Lin, Y. (2013) Identification and functional characterization of a rice NAC gene involved in the regulation of leaf senescence. *BMC Plant Biol.* 13, 132.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) confirmation of constitutive increase in Hv5SNAC1 transcript levels in transgenic barley over-expression (OE) lines. Error bars indicate ± 1SE. ***P < 0.001 and **P < 0.01 for comparison of means of OE lines with Golden Promise (GP).

Table S1 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) primers used in this study.