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The role of a cytosolic superoxide dismutase in barley-pathogen interactions

Damien J Lightfoot\textsuperscript{1,2}, Graham RD McGrann\textsuperscript{3,4}, Amanda J Able\textsuperscript{1,*}

\textsuperscript{1} School of Agriculture, Food and Wine, Waite Research Institute, PMB 1, Glen Osmond SA 5064 Australia
\textsuperscript{2} Biological and Environmental Sciences & Engineering Division, King Abdullah University of Science and Technology, Thuwal Saudi Arabia (current address)
\textsuperscript{3} Department of Crop Genetics, John Innes Centre, Norwich NR4 7UH United Kingdom
\textsuperscript{4} Crop Protection Team, Crop and Soil Systems Group, SRUC, Edinburgh EH9 3JG United Kingdom (current address)

*Corresponding author:
Professor Amanda J Able
School of Agriculture, Food and Wine
Waite Research Institute
PMB 1 Glen Osmond SA 5064
+61 8 8313 7245
amanda.able@adelaide.edu.au

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SUMMARY

Reactive oxygen species (ROS), including superoxide ($O_2^-$/$HO_2^-$) and hydrogen peroxide ($H_2O_2$), are differentially produced during resistance responses to biotrophic pathogens and during susceptible responses to necrotrophic and hemi-biotrophic pathogens. Superoxide dismutase (SOD) is responsible for catalysing the dismutation of $O_2^-$/$HO_2^-$ to $H_2O_2$ regulating the redox status of plant cells. Increased SOD activity has been previously correlated with resistance in barley to the hemi-biotrophic pathogen *Pyrenophora teres f. teres* (*Ptt*, the causal agent of net form of net blotch disease) but the role of individual isoforms of SOD has not been studied. A cytosolic CuZnSOD, *HvCSD1*, was isolated from barley and characterised as being expressed in tissue from different developmental stages. *HvCSD1* was upregulated during the interaction with *Ptt* and to a greater extent during the resistance response. Net blotch disease symptoms and fungal growth were not as pronounced in transgenic *HvCSD1* knockdown lines in a susceptible background (cv. Golden Promise), as compared to wild type plants, suggesting that cytosolic $O_2^-$/$HO_2^-$ contributes to the signalling required to induce a defence response to *Ptt*. There was no effect of *HvCSD1* knockdown on infection by the hemi-biotrophic rice blast pathogen *Magnaporthe oryzae* or the biotrophic powdery mildew pathogen, *Blumeria graminis f. sp. hordei* but *HvCSD1* also played a role in the regulation of lesion development by methyl viologen. Together these results suggest that *HvCSD1* could be important in maintaining cytosolic redox status and in the differential regulation of responses to pathogens with different lifestyles.
INTRODUCTION

Reactive oxygen species (ROS), including singlet oxygen ($^1\text{O}_2$), hydroxyl radical (OH), superoxide (O$_2^−$/HO$_2$), and hydrogen peroxide (H$_2$O$_2$); are toxic by-products of metabolism, potentially harmful to plant cell integrity (Dat et al., 2002; Mittler et al., 2004; Sutherland, 1991). However, ROS along with nitric oxide (NO) have been shown to be essential for signalling processes during metabolism and development as well as during responses to abiotic and biotic stress (Baxter et al., 2014; Groß et al., 2013; Lehmann et al., 2015). Upon recognition of a pathogen, a rapid oxidative burst can occur in the plant under attack and ROS production appears necessary for further plant defence reactions (Heller & Tudzynski, 2011; Lehmann et al., 2015; O’Brien et al., 2012) such as the hypersensitive response (HR) (Gadjev et al., 2008).

O$_2^−$/HO$_2$, H$_2$O$_2$ and NO have been shown as essential for the HR in various plant-pathogen interactions where the pathogen is known to be primarily biotrophic during its lifecycle (Able et al., 1998; Able et al., 2000; Delledonne et al., 2001; Levine et al., 1994). Tissue death resulting from ROS-induced HR impedes successful infection by biotrophs leading to host resistance. However, the HR might increase host susceptibility to necrotrophic pathogens providing dead tissue for nutritional purposes (Able, 2003; Barna et al., 2012). Indeed, ROS have been shown to be produced to a greater extent during susceptible plant responses to fungi with necrotrophic stages in their lifecycles, including in barley infected with *Rhynchosporium secalis* or *P. teres* (Able, 2003; Liu et al., 2015); wheat with *Zymoseptoria tritici* (syn. *Septoria tritici*, Shetty et al., 2003) and Arabidopsis with *Botrytis cinerea* (Govrin & Levine, 2000). Necrotrophic pathogens may therefore exploit ROS production by the plant or may even contribute to ROS production to
induce cell death, as has been suggested for *B. cinerea* (Govrin & Levine, 2000), *P. teres* (Able, 2003) and *L. maculans* (Li et al., 2008a; Li et al., 2008b). Removal of *in planta* H₂O₂ by infiltrating wheat leaves with the H₂O₂ scavenger catalase (CAT) during the necrotrophic stage of the infection by *Z. tritici* led to susceptibility due to enhanced growth of the pathogen but infiltration with H₂O₂ decreased growth of the pathogen (Shetty et al., 2007). These authors suggest that the fungus still grows *in planta* in spite of the ROS production and therefore may not actually need the ROS to be virulent. The observation that differences in the virulence of *P. teres* isolates was not correlated with their ability to produce ROS supports this suggestion (Able, 2003). In addition, infiltration of barley leaves with ROS scavengers did not affect the growth of *P. teres in planta*, but the extent of symptom development was partially reduced. Necrosis-inducing toxins produced by many of the pathogens discussed here also contribute to symptom development and possibly virulence (Ismail et al., 2014a; Liu et al., 2015; Sarpeleh et al., 2007; Sarpeleh et al., 2008). However, ROS are necessary for the regulation of various fungal processes associated with virulence including hyphal growth, fusion and branching; and; the differentiation of asexual spores, fungal cell walls, fruiting bodies, and appressorium (Dirschnabel et al., 2014; Georgiou et al., 2006; Scott & Eaton, 2008; Tudzynski et al., 2012). Given the complex associations between fungal growth rates, toxin production and virulence; the potential involvement of plant-produced ROS and their detoxification requires further study.

During biotic and abiotic stress, the levels of ROS will increase as a result of electron leakage from the electron transport chains in chloroplasts and mitochondria (Asada et al., 1974; Pastori & Foyer, 2002; Rhoads et al., 2006). Membrane-bound NADPH oxidases and cell wall peroxidases have also been shown to be the main
producers of ROS during plant-pathogen interactions (O’Brien et al., 2012). Elevated ROS levels induce the biosynthesis of non-enzymatic antioxidants, such as ascorbate, polyamines and glutathione (Blokhina et al., 2003; Conklin & Last, 1995), and increase the activity of the antioxidant enzymes superoxide dismutase (SOD), CAT and glutathione-S-transferase (GST) (Blokhina et al., 2003; Mittler et al., 2004); especially in cells that surround a HR (Levine et al., 1994). The main ROS produced, \( \mathrm{O}_2^-/\mathrm{HO}_2^- \), is usually converted to the less toxic \( \mathrm{H}_2\mathrm{O}_2 \) by SOD. \( \mathrm{H}_2\mathrm{O}_2 \) can be degraded more easily by antioxidants including CAT and ascorbate peroxidase (APX) (Groß et al., 2013). Given the demonstrated role of \( \mathrm{H}_2\mathrm{O}_2 \) in various plant-pathogen interactions (Able et al., 2000; Delledonne et al., 2001; Hückelhoven et al., 1999; Levine et al., 1994), changes to the levels of SOD may therefore also play a significant role during plant-pathogen interactions (Frederickson Matika & Loake, 2014), especially during the HR (Delledonne et al., 2001).

SODs, which are usually defined by their metal co-factors (Mn, Fe or CuZn), can be found at all sites of \( \mathrm{O}_2^-/\mathrm{HO}_2^- \) production (Alscher et al., 2002; Bowler et al., 1994; Miller, 2012). FeSODs are primarily located in the chloroplast for some plants (Van Camp et al., 1990) with three identified in Arabidopsis (Myouga et al., 2008). MnSODs are located in the mitochondria and peroxisomes in an independent manner (del Rio et al., 2003) while CuZnSODs are usually located in the chloroplast and the cytosol of plants (Alscher et al., 2002). The cytosolic form of CuZnSOD can also localise to the nucleus (Ogawa et al., 1996). Sequences for the cytosolic and chloroplastic forms of \( \text{CuZnSOD} \) are easily distinguishable due to differences in the numbers and positions of introns (Kliebenstein et al., 1998).

We have previously shown that total SOD activity increased significantly during resistant interactions between barley and \( \text{P. teres f. teres (Ptt)} \) when compared
to the susceptible response (Able, 2003). Furthermore, a suppressive subtractive hybridisation (SSH) screen identified a 181 bp fragment upregulated during the resistance response and with similarity to CuZnSOD (Bogacki et al., 2008). These results along with previous observations that $\text{O}_2^{-}/\text{HO}_2^-$ is produced during the susceptible response (Able, 2003) suggest that removal of $\text{O}_2^{-}/\text{HO}_2^-$ might be important during the resistance response to Ptt, a pathogen with a necrotrophic stage in its lifecycle (Lightfoot & Able, 2010). Here, we report the subsequent identification and characterisation of the upregulated CuZnSOD as HvCSD1. Using RNAi knock-down lines, we have also functionally analysed HvCSD1 for its potential role in ROS-induced lesion formation and during the interaction between barley and Ptt as well as another hemi-biotroph Magnaporthe oryzae and the obligate biotroph Blumeria graminis f. sp. hordei (Bgh).

RESULTS

Identification and characterisation of HvCSD1

A partial barley cDNA with high sequence homology to the rice CuZnSOD, OsSODCc1, was previously identified using SSH (Bogacki et al., 2008). Subsequently, a full length cDNA clone with 3’ and 5’ UTR (799 nt) was isolated (Fig. 1a). The deduced amino acid sequence for the open reading frame (152 amino acid residues) contains the conserved copper-binding (His-45, -47, -62, -119), zinc-binding (His-62, -70, -79, Asp-82) and conserved cysteine (56 and 145) residues common in CuZnSOD proteins. A lack of a signal peptide suggested that the protein is cytosolic while phylogenic analysis grouped the clone with cytoplasmic CuZnSODs such as AtCSD1 (Fig. 1b). Phylogenic analysis clearly separated cytoplasmic, peroxisomic and chloroplastic SODs. The exon structure of the genomic
DNA of the clone also suggested the protein would be cytosolic (Supplementary Table S1). The seven exons are of the same length as for other cytosolic CuZnSODs including *At CuZn Superoxide Dismutase 1*. Hence, we have named the gene characterised in this study as *HvCSD1* (Accession number KU179440). Predicted localisation to the cytoplasm (and nucleus) was confirmed using 35S::HvCSD1-GFP localisation (Fig. 1c).

Semi-quantitative RT-PCR revealed that *HvCSD1* gene expression levels are constitutive, regardless of tissue type (Fig. 2). *HvCSD1* gene expression is induced by infection with *Ptt*, in both susceptible (Sloop) and resistant (CI9214) cultivars (Fig. 3). The increase in *HvCSD1* expression was greater in the incompatible interaction between CI9214 and *Ptt* and occurred more quickly than in the compatible interaction between Sloop and *Ptt*. *HvCSD1* expression was greater from 18 hours post inoculation (hpi) in the resistant cultivar compared with the susceptible cultivar (Fig. 3), regardless of the lower expression of the internal control gene *HvGAPDH* in the infected resistant cultivar.

**Development of transgenic *HvCSD1* RNAi knockdown barley lines**

Based on the expression pattern of *HvCSD1* in response to *Ptt* in both resistant and susceptible interactions, transgenic RNAi-silencing knockdown lines for *HvCSD1* were generated to assess gene function during disease development. Two T2 homozygous transgenic lines, HvCSD1-RNAi1 and HvCSD1-RNAi2, were produced from two independent transformation events and contained a single copy of the gene (Supplementary Fig. S1). The extent of *HvCSD1* knockdown was confirmed using RNA expression analysis (Supplementary Fig. S2 a, b). Semi-quantitative RT-PCR analysis indicated that both HvCSD1-RNAi1 and HvCSD1-RNAi2 had lower levels
of *HvCSD1* transcript than the wild type cv. Golden Promise. Of the two lines, HvCSD1-RNAi1 appeared to have lower levels of the *HvCSD1* transcript (Supplementary Fig. S2a). Quantitative RT-PCR analysis of *HvCSD1* levels in HvCSD1-RNAi indicated that the target transcript levels were knocked-down to approximately 20% of the wild type level (Supplementary Fig. S2b). Assay of SOD activity also confirmed that the CuZnSOD activity was lower in both transgenic lines compared to wild type cv. Golden Promise (Supplementary Fig. S2c).

**Disease development on *HvCSD1* RNAi lines**

When *HvCSD1* was silenced, the development of disease symptoms during a compatible interaction with *Ptt* was significantly reduced (Fig. 4; *P*<0.05). The percentage of leaf area affected by necrosis and chlorosis was significantly reduced by 168 hpi in the RNAi lines (Fig. 4a, b; *P*<0.05), as was fungal development (Fig. 4c; *P*<0.05). At 120 hpi, fungal development was significantly lower for the resistant breeding line CI9214 and both RNAi lines (*P*<0.05). However, after 120hpi, fungal development proceeded further in the resistant breeding line CI9214 than in either of the knockdown lines (Fig. 4c; *P*<0.05) but did not reach the levels observed in the susceptible cv. Golden Promise. By 168 hpi, the leaves in the compatible interaction have collapsed (data not shown), the fungus is sporulating and its hyphae have spread throughout the entire tissue (a score of 9-10 on the fungal development scale of Lightfoot and Able, 2010). In comparison, the fungal hyphae have only just started to penetrate into the mesophyll in the *HvCSD1* knockdown lines and only some cell death is evident near the hyphae (a fungal development score of 6-7).

Irrespective of RNAi-silencing, *HvCSD1* gene expression was induced by *Ptt* in both transgenic lines (Fig. 4d). Increases in *HvCSD1* transcript levels were greatest
in the resistant breeding line CI9214. *HvCSD1* transcript levels were induced in the knockdown lines to a slightly greater extent than that in the susceptible cv. Golden Promise early in the interaction peaking at 24 hpi (Fig. 4*d*). CuZnSOD protein activity was also induced by *Ptt* in CI9214, Golden Promise and HvCSD1-RNAi1 (Fig. 4*e*). Increases in CuZnSOD activity were greatest by 24 hpi in the resistance response to *Ptt* (in CI9214).

The effect of silencing *HvCSD1* on the development of disease symptoms during compatible interactions with the hemi-biotrophic *M. oryzae* and the obligate biotroph *Bgh* was also examined. No significant differences were observed between the knockdown line HvCSD1-RNAi1 and the wild type cv. Golden Promise for the development of disease symptoms for either pathogen (Fig. 5*a*, *c*). There was no significant effect of *HvCSD1*-silencing on the number of lesions formed by the blast fungus *M. oryzae* (Fig. 5*b*; *P*=0.923) nor for the number of colonies formed by the powdery mildew fungus *Bgh* (Fig. 5*d*; *P*=0.417).

**Effect of *HvCSD1* transcript knockdown on sensitivity to ROS-induced cell death**

Whether silencing *HvCSD1* affected sensitivity to ROS-induced lesion formation was also examined. In general, the RNAi line HvCSD1-RNAi1 appeared to exhibit larger lesions caused by the H$_2$O$_2$ donor alloxan; the mitochondrial O$_2^−$/HO$_2^−$ donor, menadione; and the chloroplastic O$_2^−$/HO$_2^−$ donor, methyl viologen compared to the wild type cv. Golden Promise (Fig. 6*a*). However, only the observed increase in methyl viologen-induced lesion size was statistically significant between the knockdown line and wild type plants (Fig. 6*b*; *P*<0.05).

**DISCUSSION**
The role of ROS in resistance responses to biotrophic pathogens is well established (Heller & Tudzynski, 2011; Lehmann et al., 2015). ROS have also been identified during infection by fungi with necrotrophic stages in their lifecycle and could possibly contribute to cell death during susceptible responses such as observed for P. teres on barley (Able, 2003; Liu et al., 2015), and B. cinerea on Arabidopsis (Govrin & Levine, 2000). Even though the induction of ROS production has been correlated with susceptibility, ROS is not necessarily correlated with the ability of the pathogen to grow in planta (Able, 2003; Shetty et al., 2007). Various virulence-associated toxins and/or effectors, usually produced during the later stages of necrotrophic interactions, can also contribute to cell death, fungal growth, disease symptoms and the suppression of defence responses (Lo Presti et al., 2015). Ptt not only produces ROS at significant levels (Able, 2003; Liu et al., 2015) but also produces necrosis-inducing effectors (Ismail et al., 2014a; Liu et al., 2015; Sarpeleh et al., 2007; Sarpeleh et al., 2008) including a xylanase which appears necessary for the development of barley net blotch disease (Ismail et al., 2014b). Furthermore, its growth pattern suggests that Ptt does not become necrotrophic until after 48 h (Lightfoot & Able, 2010) with most effectors and toxins being produced in the greatest quantities after this time (Ismail et al., 2014b; Ismail and Able, unpublished data). Previous research in this interaction suggested that O$_2^-$/HO$_2^-$ removal might be important during resistance responses (Able, 2003). In this study, a 181 bp fragment previously identified using SSH as being upregulated during resistance to P. teres (Bogacki et al., 2008) was used to isolate HvCSD1, characterise its expression during the plant-pathogen interaction and predict its role through the use of knockdown lines. These lines were also used to establish that HvCSD1 appeared to play a limited or no role in the extent of disease development on barley as caused by M. oryzae and Bgh. HvCSD1 also appeared to
contribute to the regulation of ROS provided by the chloroplastic superoxide donor methyl viologen.

HvCSD1 was characterised as a 152 amino acid cytosolic CuZnSOD (Fig. 1), constitutively expressed in all barley tissue (Fig. 2). Although cytosolic CuZnSOD is usually constitutively expressed (Kliebenstein et al., 1998; Kwon & An, 2006), it can still be responsive to a range of stresses and treatments (Alsher et al., 2002; Kwon & An, 2006). HvCSD1 gene expression was increased in both susceptible and resistance responses of barley to Ptt, especially in the later necrotrophic stages, but to a greater extent in the resistance response (Fig. 3). CuZnSOD activity was also greater during the resistance response (Fig. 4). Similar observations for SOD activity have been made during various interactions with pathogens regarded as necrotrophic or hemi-biotrophic (Ding et al., 2011; Jindřichová et al., 2011; Taheri et al., 2014; Wang et al., 2015). These observations suggest that an increase in SOD is necessary to maintain redox status in response to pathogen attack. The observation that removal of $O_2^-/HO_2.$ with exogenous ROS scavengers partially reduced cell death and symptom development in the susceptible response to Ptt (Able, 2003), also supports an important role for inability to maintain redox status in the outcome of this interaction. We therefore expected that the knockdown in HvCSD1 in the net blotch susceptible cv. Golden Promise would facilitate symptom development during Ptt infection. However, disease severity was less in the knockdown lines but still greater than that observed in the resistance response (Fig. 4). Despite knockdown of HvCSD1 transcript level to as little as ~20% of wild type expression in the RNAi lines, HvCSD1 gene expression was induced in the knockdown lines during the interaction to a slightly greater extent than that in the susceptible cultivar early in the interaction. The apparent correlation between HvCSD1 expression levels and disease severity,
suggests that HvCSD1 could contribute to the lack of cell death and symptom development observed in the resistance responses of barley to *Ptt*. However, although CuZnSOD activity was induced in the RNAi lines, this induction was unexpectedly less than in the susceptible responses. Given the complexity of ROS metabolism (Mittler *et al.*, 2004) and its overlap with other signalling pathways during plant-pathogen interactions (O’Brien *et al.*, 2012), further investigation of the impact of HvCSD1 knockdown on other elements of ROS metabolism and signalling is therefore necessary.

*Ptt* development was also slower in the *HvCSD1* knockdown lines than in the susceptible cultivar suggesting that increased levels of O$_2^·$/HO$_2^·$ might impact the growth of the fungus. In the susceptible cultivar, the leaves have collapsed by 168 hpi, and the fungus is sporulating and has spread throughout the entire tissue. Most fungal growth and cell death occurred after 96 hpi. However, in the knockdown lines, the fungal growth after 96 h is much slower and by 168 hpi some cell death is evident near the hyphae, which has only just started to spread into the mesophyll. The pathogen may therefore be more sensitive to plant-produced O$_2^·$/HO$_2^·$ during the stages that lead to sporulation. Observations in other pathosystems that fungal antioxidant gene expression increases when plant-produced ROS levels are likely to be at their highest (Keon *et al.*, 2007), such as during necrotrophic stages of the interaction and when reproductive structures are forming (Keon *et al.*, 2005; Keon *et al.*, 2007); as well as the observation that virulence is lessened if the pathogen does not have appropriate antioxidant mechanisms (Veluchamy *et al.*, 2012); support this assumption.

However, in some pathosystems, there appears to be no evidence of *in planta* oxidative stress for fungi and fungal growth continues, regardless of the levels of
plant-generated ROS, especially H$_2$O$_2$ (Samalova et al., 2014; Shetty et al., 2003; Shetty et al., 2007; Temme & Tudzynski, 2009). O$_2^-$/HO$_2$ and H$_2$O$_2$ levels were limited during the early stages of the susceptible response of barley to Ptt but increased during necrosis development and just before sporulation occurred (Able, 2003). Infiltration of barley leaves with ROS scavengers also had no effect on the growth of Ptt in planta suggesting that it can cope with increased levels of ROS. The slowing of fungal growth in the knockdown lines could therefore be an indirect effect of the change to redox status on other elements of the plant-pathogen interaction such as fungal development (Dirschnabel et al., 2014; Georgiou et al., 2006; Scott & Eaton, 2008; Tudzynski et al., 2012) allowing the induction of the defence response (Pieterse et al., 2009), especially during the early stages before the switch to necrotrophy. HvCSD1 expression is not affected in barley lesion mimic mutants compromised in their redox status (McGrann et al., 2015b) suggesting the pathway through which HvCSD1 maintains redox balance is currently unknown. Given that HvCSD1 expression and CuZnSOD activity was increased during the resistance response (Fig. 4), if overexpression of HvCSD1 decreased disease severity then the mechanism by which disease severity is lessened will probably be different to that in the HvCSD1 knockdown lines. Indeed, our results might therefore reflect the potentially different roles and balance of each individual ROS (O$_2^-$/HO$_2$ versus H$_2$O$_2$) during the different stages of growth for the fungus. Determining whether (and how) overexpression of HvCSD1 affects the barley-Ptt interaction could therefore provide further insight.

Whether ROS production is modulated by toxin production during the necrotrophic stage also needs to be considered. Toxins from necrotrophs such as Cochliobolus sativus, Botrytis cinerea and Fusarium spp. induce H$_2$O$_2$ accumulation
in susceptible hosts (Kumar et al., 2001; Zhang et al., 2015; Desmond et al., 2008). The timing and extent of H$_2$O$_2$ production, fungal growth and cell death are diverse among interactions between Cochliobolus sativus and genotypes of wheat with different resistance and susceptibility (Rodríguez-Decuadro et al., 2014). A similar situation exists for Ptt where isolates with different virulence have different growth habits on susceptible barley cultivars (Ismail et al., 2014a) but there is no clear correlation between virulence and ROS production (Able, 2003) or between virulence and the capability to produce toxins that induce necrosis in susceptible barley cultivars (Ismail et al., 2014a).

Because of these complex associations between fungal growth, toxin production, virulence and ROS production in the interaction between barley and the hemi-biotroph Ptt; we also investigated whether the knock down of HvCSD1 would affect the interaction of the susceptible cv. Golden Promise with the facultative pathogen Magnaporthe oryzae and the obligate barley powdery mildew pathogen Bgh. Knockdown of HvCSD1 had no effect on the development of disease symptoms caused by either of these pathogens (Fig. 5). ROS have been shown to increase substantially during the resistance response of barley to Bgh but H$_2$O$_2$, rather than O$_2^-$ /HO$_2^-$, is more likely to play a role in HR and papillae formation (Hückelhoven et al., 1999; Hückelhoven & Kogel, 1998). Knockdown of the respiratory burst oxidase HvRBOHF2 did not affect HR-related cell death but enhanced susceptibility to penetration by virulent Bgh in barley (Proels et al., 2010), suggesting plasma membrane-associated O$_2^-$/HO$_2^-$ could also contribute to penetration resistance even though H$_2$O$_2$ was still observed where papillae and cell wall appositions were formed. However, application of H$_2$O$_2$ to barley prior to infection by Bgh prevents disease (Hafez and Király, 2003). Resistance in rice to M. oryzae also seems to be specifically
associated with H$_2$O$_2$ and penetration resistance (Huang et al., 2011). Knockdown of cytosolic *HvCSD1* would potentially lead to an increase in O$_2^-$/HO$_2^-$, and not necessarily biologically significant changes in H$_2$O$_2$, and, as observed, was therefore unlikely to have an effect. Furthermore the observations that whole barley leaf SOD activity did not change after inoculation with virulent and avirulent *Bgh* (Vanacker et al., 1998) and that minimal induction of SOD activity was observed in compatible or incompatible interactions of rice with *M. oryzae* (Matsuyama, 1983) suggest that O$_2^-$ /HO$_2^-$ and/or *HvCSD1* are unlikely to play an important role in the susceptible response of barley to *Bgh* or *M. oryzae*.

*HvCSD1* knockdown also significantly increased lesion formation due to application of the chloroplastic O$_2^-$/HO$_2^-$ donor methyl viologen at 25 μM (Fig. 6). Over-expressed chloroplastic CuZnSOD has been shown to have a protective effect but only at low concentrations of methyl viologen (<2μM) (Gupta et al., 1993). Cytosolic CuZnSODs, such as *HvCSD1*, usually help the plant cell deal with general stresses and/or overflow from the mitochondrial and chloroplastic O$_2^-$/HO$_2^-$ generation (Alscher et al., 2002; Kwon & An, 2006). The role that HvCSD1 appears to play in limiting lesion formation in the presence of the high concentration of methyl viologen further highlights the role of this gene in maintaining redox status and indicates that ROS produced in excess in the chloroplast are usually propagated out into the cytosol to influence its redox state (Lee & Jo, 2004).

The cytosolic CuZnSOD *HvCSD1* contributes to the response of barley to the hemi-biotroph *Ptt* but does not appear to play a role in the susceptible response to the hemi-biotroph *M. oryzae* or the biotroph *Bgh*. These differences mostly reflect the complex interactions between the timing of plant redox status changes and the lifecycle stages for each pathogen. Where and which ROS is central to the induction
of resistance or linked to symptom development, particularly cell death, also appears to be important. The observation that removal of HvCSD1 did not affect susceptibility to *M. oryzae* or *Bgh* but increased resistance to *Ptt* and slowed growth of the pathogen therefore confirms the differential role that H$_2$O$_2$ might play in different plant-pathogen interactions and suggests O$_2^\cdot$/HO$_2^\cdot$ contributes to the signalling responsible for induction of resistance. Because the induction of resistance to *Ptt* in the knockdown lines was not complete, other antioxidants and/or enzymes not regulated by redox status could also contribute to the defence response and should therefore be examined in further research. However, the ability of a plant to respond appropriately in maintaining or utilising redox status to control cell death appears to be an important determinant in the differential regulation of responses to pathogens with different lifestyles.

**EXPERIMENTAL PROCEDURES**

**Plant material**

Barley (*Hordeum vulgare* L.) cultivar Golden Promise, the barley breeding line CI9214, transgenic barley lines (described in more detail later) and tobacco (*Nicotiana benthamiana*) plants were grown under a 16 h day/8 h night photoperiod at 18-21°C/12-15°C supplemented with 220-250 µmol m$^{-2}$ s$^{-1}$ fluorescent lighting in controlled environment growth rooms.

**Isolation and sequence analysis of HvCSD1**

A 181 bp fragment of HvCSD1 was previously identified in a SSH screen as being upregulated in resistant barley plants compared with susceptible plants inoculated
with *P. teres* (Bogacki et al., 2008). This fragment was part of the 3’ UTR and contained the start of a poly-A tail. The full length coding sequence of *HvCSD1* was identified by locating and assembling overlapping publicly available EST sequences that corresponded to *HvCSD1*. The putative full-length *HvCSD1* cDNA clone was isolated by primary and nested PCR using primer pair 1 (Forward: 5’-ATGGTGAAAGCTGAGCTGTGCTT-3’ and Reverse: 5’-TTAGCCCTGGAGCCGATGAT-3’) and primer pair 2 (Forward: 5’-ACCGGCAGCGAGGGTGTC-3’ and Reverse: 5’-CCCGCAAGCAACGCGCG-3’) respectively. cDNA was synthesised from RNA, extracted from the leaves of 10 day old CI9214 seedlings using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA), with the SMART™ PCR cDNA Synthesis Kit (BD Biosciences Clontech, Palo Alto, CA, USA) as per manufacturer’s instructions. 5’- and 3’-RACE (GeneRacer Kit, Invitrogen) was performed on CI9214 cDNA as per manufacturer’s instructions to obtain the full length *HvCSD1* mRNA. The two forward primers from primer pairs 1 and 2 were used for 3’-RACE and the two reverse primers were used for 5’-RACE. The full length mRNA was also confirmed in cv. Sloop (Supplementary Figure S3). The *HvCSD1* genomic region encompassing the coding region of the *HvCSD1* mRNA was amplified using primer pair 1 using Sloop genomic DNA, extracted as previously described (Lightfoot et al., 2008).

The full length coding region of *HvCSD1* was used in TBLASTN searches to identify highly similar CuZnSOD sequences for evolutionary analysis. Sequences were aligned using ClustalW with the IUB DNA weight matrix utilising a gap opening penalty of 10 and a gap extension penalty of 5. Nucleotide trees were constructed using the neighbour-joining method and the p-distance model in the Molecular Evolutionary Genetics Program (MEGA) program as per Khoo et al.
The accession numbers of the nucleotide sequences used were as follows: NM_001112234 (ZmSOD4), U34727 (ZmSOD9), NM_001056653 (OsCuZn1), NM_0011111865 (ZmSOD2), AK243377 (OsCuZn2), NM_100757 (AtCSD1), AI727694 (GhCSD1), AJ002604 (Pship-SOD), AF034832 (McSOD2), NM_121815 (AtCSD3), EU597270 (GhCSD3), FJ393058 (PhipI-SODC1), AJ278671 (PhipI-SOD1), AJ278670 (Phip-SOD2), AY566699 (CiCuZnSODII), EU597268 (GhCSD2a), NM_128379 (AtCSD2), NM_001069049 (OsSODcp), EU408345 (ZmEU408345), AK248474 (HvAK248474), U69536 (TaSOD1.1), U69632 (TaSOD1.2) and U51242 (EcSODC). Sequences were analysed using Vector NTI version 10 (Invitrogen) and Genedoc version 2.6.002 (http://www.nrbsc.org/gfx/genedoc). The intron and exon structure of genes corresponding to the transcripts utilised in the phylogenetic analysis was determined where genomic and mRNA sequence data was available. The full length CDS for Sloop and CI9214 and the nucleotide sequence for the HvCSD1 gene in Sloop have been submitted to the GenBank database under accession numbers KU179439, KU179438 and KU179440 respectively.

Subcellular localisation of HvCSD1

The full length coding region of HvCSD1 was amplified from CI9214 genomic DNA (using the primers 5’-CACCATGGTGAAGGCTGTAGCTGTGCTT-3’ and 5’-GCCCTGGAGCCCGATGAT-3’) and cloned into the pCR8 entry vector (Invitrogen). HvCSD1 was then transferred into the pMDC83 binary vector (Curtis & Grossniklaus, 2003) using the LR Clonase™ Plus Enzyme Mix (Invitrogen) according to the manufacturer’s instructions. The 35S::HvCSD1-GFP fusion construct was introduced into Agrobacterium tumefaciens (strain AGL1) following
the method of An et al. (1989) and N. benthamiana leaves were infiltrated as previously described (Selth et al., 2004). Plant tissue was sampled after 3 d and analysed for GFP expression with a SP5 spectral scanning confocal microscope (Leica Microsystems, Wetzlar, Germany), using an excitation wavelength of 488nm. Z-step image collection occurred from the top to bottom of leaves at 1 µm intervals and Z-stack images prepared using Confocal Assistant (version 4.02). pMDC83 without the HvCSD1 insert was used as a control for GFP localisation. Subcellular localisation occurred in three independent experiments.

Production of HvCSD1-RNAi knockdown lines in barley

A 172 bp region of HvCSD1 3´ UTR was amplified from cDNA from CI9214 with the 5´-CAC CAC AGA TCT TGG CAC TTG AAG G-3´ and 5´-GAC AGA ACT GAA CTG TTC CAG TCA CG -3´ primers and cloned into the pENTR/D-TOPO® vector (Invitrogen) according to manufacturer’s instructions. The specificity of knockdown to HvCSD1 was confirmed by BLAST analysis of the barley genome assembly (GCA_000326085.1) using EnsemblPlants (plants.ensembl.org); alignment with HvCSD1 and the two other CSDs identified in the barley genome (MLOC_17760 previously characterised as HvSOD1, a chloroplastic CSD; and; MLOC_38479, a peroxisomal CSD or HvCSD3); and; detection of RNA interference targets of loci identified in the BLAST analysis and the CSDs with siFi (version 3.2, Snowformatics). The 172 bp region was specific to HvCSD1 (Supplementary Fig. S4, Supplementary Table S2) and was predicted to be specific to HvCSD1 with 54 effective hits (of 152 total hits). The 172 bp cassette was transferred into the hairpin RNAi vector pSTARGATE (Wesley et al., 2001) using the LR Clonase™ Plus Enzyme Mix (Invitrogen) according to the manufacturer’s instructions. The resultant
vector was utilised in *Agrobacterium*-mediated transformation of barley cv. Golden Promise and putative transformants screened as per previously established methods (Lloyd et al., 2007). Putatively transformed plants were screened at the T1 generation for the presence of the Hygromycin resistance gene by PCR with the primers, 5´-CTTTGCCCTCGGACGAGTGCTGGGGC-3´ and 5´-TGAACCTACCGCGACGTCTGTCGA-3´, under the following conditions: 94°C for 5 mins, 25 cycles of 94°C for 30 secs, 60°C for 30 secs, 72°C for 90 secs and a final extension step of 72°C for 7 mins. At the T2 generation, lines positive for the Hygromycin resistance gene were screened for homozygotes and transgene insertion number by Southern analysis with 20 μg genomic DNA digested with EcoRV or HindIII (New England Biolabs, Beverly, MA, USA). The Southern blots were screened with a fragment of the Hygromycin resistance gene amplified using the primers listed above (Supplementary Fig. S1). HvCSD1-RNAi1 and HvCSD1-RNAi2 are lines created from independent transformation events and both contain one copy of the transgene.

**HvCSD1 gene expression**

Reverse Transcription-PCR (RT-PCR) was initially used to confirm the extent of knockdown in the transgenic lines. RT-PCR was also used to determine *HvCSD1* gene expression in a tissue series from cv. Golden Promise and during the barley-*Ptt* interaction. RNA for the analysis of *HvCSD1* abundance in the transgenic lines was prepared from second leaves of three week old plants using TRIZOL reagent as per the manufacturer’s instructions. For the analysis of *HvCSD1* abundance in different Golden Promise tissue types, RNA was prepared from the first leaves of three week old plants, from flag leaves and immature heads of 18 week old plants and from roots.
and coleoptiles of 7 d old seedlings grown in 24-well microplates (Iwaki Glass Co., Funahashi, Japan) at 22°C in the dark. For the interaction between barley and *Ptt*, RNA was prepared from the second leaves of inoculated and mock-inoculated control plants at multiple time points during the interaction. All RT-PCR experiments were performed on at least three biologically distinct samples.

RT-PCR was performed using the SuperScript One-Step RT-PCR kit from Invitrogen as per the manufacturer’s instructions with the primers 5´-ACCGCACTTCAACCCCGCTGGTCATGTG-3´ and 5´-GAGCCCGATGATCCCGCAAGCAACGC-3´. RNA (100 ng) was used as template in each reaction: 50°C for 30 mins, 94°C for 2 mins, 32 cycles of 94°C for 30 secs, 60°C for 30 secs, 72°C for 1 min and a final extension step of 72°C for 10 mins. To monitor RNA loading, a region of *H. vulgare* *Glyceraldehyde-3-phosphate dehydrogenase* (*HvGAPDH*), considered to be an ideal reference gene in barley (Jarosova & Kundu, 2010), was amplified from the same RNA samples under the same conditions using the primers 5´-GTGAGGCTGGTGCTGATTACG-3´ and 5´-TGGTGCAGCTAGCATTTGAGAC-3´. Knockdown of expression in the *HvCSD1*-RNAi1 line was further confirmed in 14 day old prophyll leaves using quantitative RT-PCR (qRT-PCR) with *HvCSD1* transcript levels assessed using gene specific primers (Forward: 5´-ACCTCGGAAATGTGACAGC-3´ and Reverse: 5´-ACCCTTGCCAAGATCATCAG-3´) as previously described (McGrann *et al.*, 2015a; Tufan *et al.*, 2009).

**SOD protein activity**

Total protein was prepared from at least three biologically distinct samples as described by Van Camp *et al.* (1994) and checked by separation on 12% denaturing
SDS-PAGE gels by using Coomassie staining (Wang et al., 2007). To identify bands of SOD activity, 100 µg of total protein was run on 12% non-denaturing polyacrylamide gels and subsequently stained for SOD activity as previously described (Beauchamp & Fridovich, 1971) except for the use of a reduced nitro blue tetrazolium concentration of 1 mM. Different SOD isoenzyme activities were determined by differential inhibition (Fridovich, 1975) by soaking the gels in 3 mM H₂O₂ or 3mM KCN for 20 minutes prior to gel illumination.
**Barley-Pyrenophora teres f. teres interaction**

Barley plants at Zadok’s growth stage 14 (Zadoks et al., 1974) were inoculated with 20,000 spores mL\(^{-1}\) (in 0.1% v/v Tween-20) of *Ptt* isolate NB50 (kindly provided by Hugh Wallwork, South Australian Research and Development Institute) using an atomiser (Preval, Yonkers, NY, USA) as previously described (Lightfoot & Able, 2010). Symptoms of *Ptt* infection on second leaves were scored for the percentage of leaf area affected by necrosis and chlorosis using a scale of 0-5 where 0 = 0%, 1 = 1 to 10%, 2 = 11 to 25%, 3 = 26 to 50%, 4 = 51 to 75% and 5 = 76 to 100%. Images were also taken of the leaves using a CanoScan 5600F scanner (Canon, Japan). To visualise and assess fungal growth and development, microscopic analysis assessed ten germinated conidia on each of five cleared leaves using a 0-10 numeric scale that rates the development of *Ptt* during the interaction *in planta* (Lightfoot & Able, 2010). Data from two independent inoculation experiments were analysed with GenStat 11 (Lawes Agricultural Trust, VSN International Ltd) using analysis of variance (ANOVA). The least significant difference (l.s.d.) at *P* = 0.05 was used to determine significant differences between means.

**Barley-Magnaporthe oryzae interaction**

Plants at growth stage 13 (Zadoks et al., 1974) were spray inoculated with 100,000 spores mL\(^{-1}\) of *Magnaporthe oryzae* isolate BR32 as previously described (Tufan et al., 2009). Disease development was assessed by the number of blast lesions visible on the second leaf of each plant at 6 dpi from two independent inoculation experiments (for a total of 30 leaves). Data was analysed in GenStat15 using general linear modelling that took into account variation due the different lines and experiments.
Barley-Blumeria graminis f. sp. hordei inoculation

Detached prophyll leaves from 14 d old plants were cut into approximately 2 cm long segments, placed into clear plastic boxes containing 0.5% water agar supplemented with 100 mg L\(^{-1}\) benzimidazole and inoculated with B. graminis f. sp. hordei isolate CC148 following the method of Boyd et al. (1994). Pathogen infection was assessed as the number of colonies observed cm\(^{-2}\) leaf area from three independent inoculation experiments each consisting of a minimum of eight replicate leaves of each line. Data was analysed in GenStat15 using general linear modelling that took into account variation due the different lines and experiments.

Reactive oxygen species (ROS) induction of cell death by pharmacological agents

The H\(_2\)O\(_2\) donor, alloxan; the mitochondrial O\(_2^-/\)HO\(_2\) donor, menadione; and the chloroplastic O\(_2^-/\)HO\(_2\) donor, methyl viologen were used to induce cell death in detached prophyll leaves as per McGrann et al. (2015a). ROS-induced lesion size was measured from photographs of each box taken 96 h after ROS donor treatment using ImageJ (Abràmoff et al., 2004). Data for each ROS donor was analysed in GenStat15 using general linear modelling that took into account variation due the different lines and experiments.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

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

Supplementary Figure S1. Southern analysis of T2 HvCSD1-RNAi lines.

Supplementary Figure S2. RNAi silencing of HvCSD1 in transgenic barley plants.

Supplementary Figure S3. Comparison of full length coding sequences for HvCSD1 (a) and their translation (b) for the barley breeding line CI9214 and cv. Sloop.

Supplementary Figure S4. Comparison of the 172 bp region of the 3’ UTR of HvCSD1 used in the HvCSD1-RNAi construct with the full length coding sequences and 3’ UTR of other CSD in barley.

Supplementary Table S1. Exon structure of genes used in the phylogenetic analysis (Fig. 1B).

Supplementary Table S2. BLAST analysis of the barley genome assembly (GCA_000326085.1, EnsemblPlants) with the 172bp region used in the HvCSD1-RNAi construct.

FIGURE LEGENDS

Fig. 1 Characterisation of HvCSD1. (a) cDNA sequence of HvCSD1 (Accession number: KU179438; CI9214) and its derived amino acid residues. Conserved copper-binding residues are underlined with a solid line, conserved zinc binding residues are underlined with a dashed line and conserved cysteines which are required for disulphide bond formation have a ^ underneath them are. (b) A comparison of HvCSD1 (boxed) with known and predicted nucleotide sequences for CuZnSODs in other species including cytoplasmic, peroxisomic and chloroplastic CSD genes from Arabidopsis thaliana (underlined). Branch lengths (bar) infer evolutionary distances.
at 0.05 substitutions per site. Accession numbers are listed in ‘Experimental Procedures’. The species is indicated as follows: At (*Arabidopsis thaliana*), *Citrullus lanatus* (Cl), Ec (*Escherichia coli*), Gh (*Gossypium hirsutum*), Hv (*Hordeum vulgare*), Mc (*Mesembryanthemum crystallinum*), Os (*Oryza sativa*), Pship (*Pinus sylvestris* high isoelectric point), Pt (*Populus trichocarpa* high isoelectric point), Ta (*Triticum aestivum*), Zm (*Zea mays*). (c) Transient expression of 35S::GFP and 35S::HvCSD1-GFP in epidermal cells of *Nicotiana benthamiana* leaves. Cells were analysed 3 d after infiltration with *Agrobacterium tumefaciens* containing the appropriate vector. Composite images (Z-step stacks collected using confocal microscopy) are representative of three independent experiments. Bar = 25μm.

**Fig. 2** Expression of HvCSD1 transcript in different tissues. Spatial expression of HvCSD1 was analysed by semi-quantitative reverse transcription PCR (sqRT-PCR) using gene specific primers and 30 amplification cycles. *Glyceraldehyde 3-phosphate dehydrogenase* (HvGAPDH) was used as an internal control. Images shown are representative of three independent experiments for three biological replicates.

**Fig. 3** Temporal expression of HvCSD1 during the interaction between barley and *Pyrenophora teres f. teres* (Ptt). Transcript levels of HvCSD1 were monitored in the susceptible barley cv. Sloop and the resistant barley breeding line CI9214 at 0, 2, 4, 6, 18, 24, 48 and 72 h post inoculation with conidia from Ptt (Infected) or sterile nanopure water (Control). Material derived from the leaves was analysed with sqRT-PCR using gene-specific primers and 30 amplification cycles. *Glyceraldehyde 3-phosphate dehydrogenase* (HvGAPDH) was used as an internal control. Images
shown are representative of three independent experiments for three biological replicates.

**Fig. 4** Effect of *HvCSD1* silencing on the interaction between barley and *Pyrenophora teres* f. *teres* (*Ptt*). (a) Net blotch disease symptoms on the second youngest leaf, at 168 hpi. Images are representative of two independent experiments with a total of eight leaves. (b) Disease severity was determined for the second youngest leaf of *Ptt*-inoculated barley plants using a scale where 0 = 0% coverage of the leaf with symptoms (chlorosis and necrosis), 1 = 1-10%, 2 = 11-25%, 3 = 26-50%, 4 = 51-75% and 5 = 76-100%. No symptoms were observed on mock-inoculated controls. Data shown are means ± S.E. for n=21 across two experiments. The least significant difference (l.s.d.) at P<0.05 is shown for comparisons among treatments at each time point. (c) Fungal development scores using a scale where 0 = Conidia are visible but have not germinated and 10 = Stromata appear mature with conidiophores (Lightfoot & Able, 2010). Ten germinated conidia were assessed on each of five leaves. Data shown are means ± S.E. for n=50 across two experiments. The least significant difference (l.s.d.) at P<0.05 is shown for comparisons among treatments at each time point. (d) Temporal expression of *HvCSD1* during the interaction between *Ptt* and barley. *HvCSD1* transcript levels were analysed with sqRT-PCR using gene-specific primers and 30 amplification cycles. *Glyceraldehyde 3-phosphate dehydrogenase (HvGAPDH)* was used as an internal control. Images shown are representative of two independent experiments for four biological replicates. (e) Activity staining for CuZnSOD following non-denaturing PAGE of 100 µg total protein. Images shown are representative of two independent experiments for four biological replicates.
Fig. 5 Effect of HvCSD1 silencing on the development of disease symptoms, caused by Magnaporthe oryzae (a, b) and Blumeria graminis f. sp. hordei (c, d), on barley leaves. Blast disease symptoms (a) and number of blast lesions per leaf (b) were determined on the second youngest leaf, at 144 hpi. Representative images and data shown (means ± S.E.) are for n=30 across two independent experiments. Powdery mildew disease symptoms (c) and number of mildew colonies cm$^{-2}$ (d) were determined on the detached prophyll leaf, at 14 d post inoculation. Representative images and data shown (means ± S.E.) are for n=64 across three independent experiments.

Fig. 6 Effect of HvCSD1 silencing on reactive oxygen species (ROS)-induced lesion formation. ROS-related symptoms (a) and mean lesion size (mm) (b) were determined on the detached prophyll leaf of the wild type barley cv. Golden Promise and HvCSD1-RNAi1 at 96 h after treatment with a ROS donor (200 mM alloxan, 100 mM menadione or 25 µM methyl viologen). Representative images and data shown (means ± S.E.) are for n=24 across three independent experiments. * indicates a significant difference (P<0.05) between the wild type and HvCSD1-RNAi1 within a treatment.
Fig. 2

![Image](image-url)

- *HvCSD1*
- *HvGAPDH*
Fig. 3
Fig. 4

(a) Images of leaf samples:
- CI9214
- HvCSD1-RNAi1
- HvCSD1-RNAi2
- Golden Promise

(b) Graph showing disease severity over time (0-180 hours post inoculation):
- Golden Promise
- CI9214
- HvCSD1-RNAi1
- HvCSD1-RNAi2

(c) Graph showing fungal development over time (0-180 hours post inoculation):
- Golden Promise
- CI9214
- HvCSD1-RNAi1
- HvCSD1-RNAi2

(d) Western blot images for different time points (0-24 hours post inoculation):
- CI9214
- Golden Promise
- HvCSD1-RNAi1
- HvCSD1-RNAi2
- HvGAPDH

(e) Western blot images for different time points (0-24 hours post inoculation):
- CI9214
- Golden Promise
- HvCSD1-RNAi1
Fig. 5

(a) Golden Promise vs. HvCSD1-RNAi1 leaf samples

(b) Comparison of blast lesion numbers per leaf:
- Golden Promise: 3.8
- HvCSD1-RNAi1: 3.9

(c) Golden Promise vs. HvCSD1-RNAi1 mildew colony samples

(d) Comparison of mildew colonies per cm²:
- Golden Promise: 4.2
- HvCSD1-RNAi1: 4.3
Fig. 6

(a) Golden Promise vs. HvCSD1-RNAi1

(b) Lesion size (mm²)
Supplementary Table S1. Exon structure of genes used in the phylogenetic analysis (Fig. 1B). Genes were grouped according to whether their protein is normally found in the cytoplasm, peroxisome or chloroplast.

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Table S2

Supplementary Table S2. BLAST analysis of the barley genome assembly (GCA_000326085.1, EnsemblPlants) with the 172bp region used in the HvCSD1-RNAi construct.

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Supplementary Figure S1. Southern analysis of T2 HvCSD1-RNAi lines. gDNA was isolated from the wildtype cultivar Golden Promise and the T2 HvCSD1-RNAi lines that were PCR-positive for the hygromycin resistance gene. DNA was either digested with HindIII or EcoRV before probing using a fragment from the hygromycin resistance gene. Images shown are from individual Southern blots. Arrows indicate the two lines chosen for use in this research (left, HvCSD1-RNAi1; right, HvCSD1-RNAi2).
Fig. S2

Supplementary Figure S2. RNAi silencing of HvCSD1 in transgenic barley plants. (a) Semi-quantitative reverse transcriptase PCR (sqRT-PCR) of two independent RNAi knockdown lines, HvCSD1-RNAi and HvCSD1-RNAi2, and the wild type (WT) cv. Golden Promise. HvGAPDH was used as an internal control. Images shown are representative of two independent experiments for four biological replicates. (b) HvCSD1 expression in the transgenic line HvCSD1-RNAi1 relative to the WT. HvCyclophilin, HvEF1a, HvGAPDH, HvTUBA, HvUbiquitin were used as reference genes. Data shown are means ± S.E. for n=15 across three independent experiments. Means were significantly different (P<0.05). (c) Activity staining for SOD following non-denaturing PAGE of 100 µg total protein; without inhibitors, or with H2O2 (which inhibits CuZnSOD and FeSOD) and KCN (which inhibits CuZnSOD). Based on the expected size of proteins and the effect of activity inhibition, i = MnSOD activity and ii = CuZnSOD activity. Images shown are representative of two independent experiments with four biological replicates.
Supplementary Figure S3. Comparison of full length coding sequences for *HvCSD1* (a) and their translation (b) for the barley breeding line CI9214 and cv. Sloop. The nucleotide sequences have been submitted to GenBank under the accessions KU179438 and KU179439 respectively.
Supplementary Figure S4. Comparison of the 172 bp region of the 3' UTR of \textit{HvCSD1} used in the \textit{HvCSD1}-RNAi construct with the full length coding sequences and 3' UTR of other CSD in barley. \textit{HvSOD1}, is a chloroplastic CSD (MLOC\_17760). MLOC\_38479, is a peroxisomal CSD (or likely \textit{HvCSD3}) with 2 splice variants (38479.1 and 38479.3).