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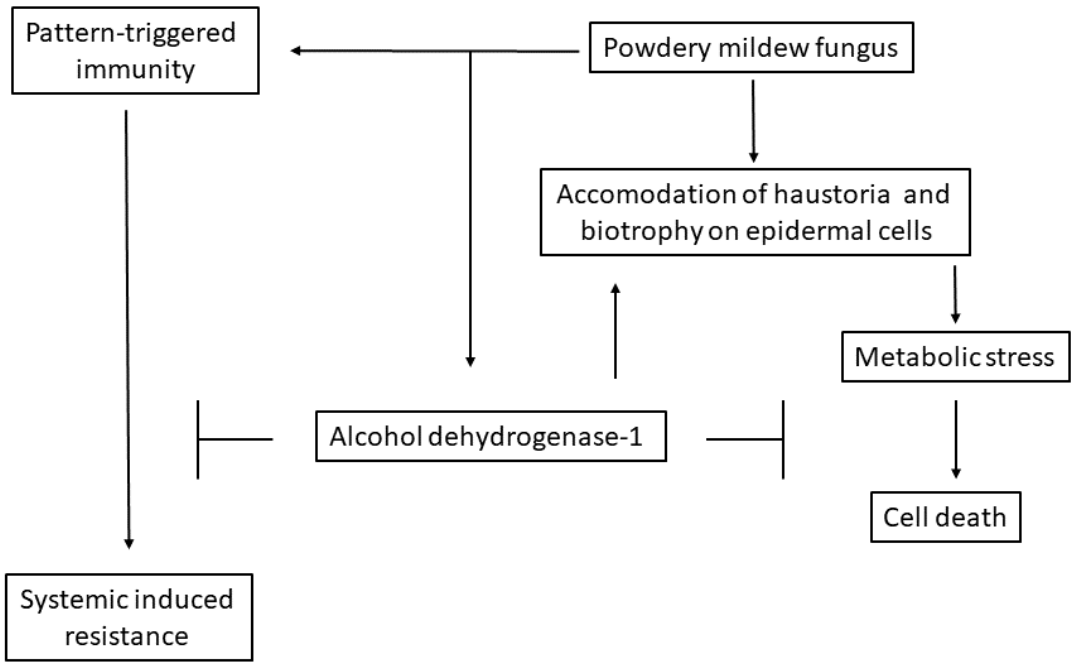

Barley ADH-1 modulates susceptibility to *Bgh* and is involved in chitin-induced systemic resistance

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Abbreviations
ADH, alcohol dehydrogenase; *Bgh*, *Blumeria graminis* f.sp. *hordei*; hai, hours after inoculation

SUMMARY

The plant primary energy metabolism is profoundly reorganized under biotic stress conditions and there is increasing evidence for a role of the fermentative pathway in biotic interactions. Previously we showed via transient gene silencing or overexpression a function of barley alcohol dehydrogenase 1 (HvADH-1) in the interaction of barley with the parasitic fungus *Blumeria graminis* f.sp. *hordei (Bgh).* Here we extend our studies on stable transgenic barley events over- or under-expressing *HvADH-1* to analyse ADH-1 functions at the level of whole plants. Knock-down (KD) of *HvADH-1* by dsRNA interference resulted in reduced and overexpression of *HvADH-1* in strongly increased *HvADH-1* enzyme activity in leaves of stable transgenic barley plants. The KD of *HvADH-1* coincided with a reduced susceptibility to *Bgh* of both excised leaves and leaves of intact plants. Overexpression (OE) of *HvADH-1* results in increased susceptibility to *Bgh* when excised leaves but not when whole seedlings were inoculated. When first leaves of 10-day-old barley plants were treated with a chitin elicitor, we observed a reduced enzyme activity of ADH-1/-1 homodimers at 48 h after treatment in the second, systemic leaf for empty vector controls and *HvADH-1* KD events, but not for the *HvADH-1* OE events. Reduced ADH-1 activity in the systemic leaf of empty vector controls and *HvADH-1* KD events coincided with chitin-induced resistance to *Bgh*. Taken together, stable *HvADH-1* (KD) or systemic down-regulation of ADH-1/-1 activity by chitin treatment modulated the pathogen response of barley to the biotrophic fungal parasite *Bgh* and resulted in less successful infections by *Bgh*.

INTORDUCTION

Plants are obligate aerobic organisms; the vast majority of ATP for cellular metabolism is provided by photosynthesis and oxidative phosphorylation. Under conditions of hypoxia/anoxia, plants mount adaptive mechanisms to compensate for the energy crisis. Thereby, two fermentative pathways, the ethanol- and the lactate fermentation play a critical role. Regarding ethanol fermentation, pyruvate decarboxylase and alcohol dehydrogenase (ADH) are induced as the central enzymes of the fermentative metabolism (Drew, 1997; Fukao and Bailey-Serres, 2004). By the sequential activity of those enzymes pyruvate is eventually converted to ethanol and the concomitant regeneration of NAD^+ is essential to maintain the energy metabolism via glycolysis and ATP production.

Due to a lack of an active transport system for molecular oxygen and a rather slow diffusion controlled transport through plant tissues, the oxygen pressure in tissues can vary widely. It may even happen that in the presence of sufficiently high external oxygen pressure the internal oxygen availability does not meet the demand. Hence, hypoxia or anoxia is a frequent metabolic status even during normal development, particularly in tissues with a high O_2 demand and/or restricted O_2 entry (Geigenberger, 2003). ADH has a well-established function in the protection against hypoxic stress after flooding (Kennedy et al., 1992; Bailey-Serres and Voesenek, 2008), during seed development (Hanson et al., 1984; Macnicol and Jacobsen, 2001) and in aerobic metabolism in pollen (Buchner et al., 1995).

There is mounting evidence for a critical role of the fermentative metabolism in plant-pathogen interactions. In *Arabidopsis thaliana* (Arabidopsis) glycolysis, respiration and fermentation are up-regulated at the site of powdery mildew attack and it is hypothesized that fermentation is favoured under cellular conditions associated with parasitic nutrient acquisition (Chandran et al., 2010; Wildermuth, 2010). This is in line with the observation of an increased carbohydrate sink mediated by an elevated invertase and hexose transporter expression at the site of pathogen attack (Fotopolous et al., 2003; Swarbrick et al., 2006; Proels and Roitsch, 2009). The barley ADH gene family consists of three members, *ADH-1*, *ADH-2* and *ADH-3* (Harberd and Edwards, 1983, Hanson and Brown, 1984, Trick et al., 1988). It was shown that HvADH-1 amounts to almost all of the ADH activity detected during aerobic growth (Good and Crosby, 1989). Following anaerobic induction the expression of *HvADH-1* and *HvADH-2* are induced. *HvADH-3* is also induced under low oxygen but at much lower extent

compared to *HvADH-1* and *HvADH-2* (Hanson and Brown, 1984; Good and Crosby, 1989). Previously, we showed that *HvADH-1* is the only constitutively expressed *ADH* gene in young barley leaves. HvADH-2 activity is induced after prolonged biotic stress and might be associated with senescence, whereas ADH-3 activity could not be detected in barley leaves under any tested condition (Proels et al., 2011). Moreover, we showed that ADH activity is induced in a compatible interaction of susceptible barley leaves with virulent *Blumeria graminis* f.sp. *hordei* (*Bgh*), a biotrophic ascomycete ectoparasite, that exclusively penetrates epidermal cells of barley. Genetic or pharmacological inhibition of barley ADH limited fungal development on barley. Transient overexpression of *HvADH-1* in epidermal cells resulted in increased susceptibility to invasion by fungal haustoria and a transient-induced knock-down of *HvADH-1* in a reduced susceptibility to *Bgh*, respectively (Pathuri et al., 2011). Interestingly, *Bgh* has lost its own fermentative pathway in the course of evolution to an obligate biotrophic pathogen (Spanu et al. 2009).

In this study the role of *HvADH-1* in the interaction of barley with *Bgh* is addressed by the use of stable transgenic barley. Knock-down of *HvADH-1* results in strongly reduced ADH activity and reduced susceptibility to *Bgh*. Overexpression of *HvADH-1* results in increased susceptibility to *Bgh* when leaf-explants were used. ADH-1 activity and susceptibility to *Bgh* could be systemically modulated by chitin treatment. This chitin-induced down-regulation of ADH-1 activity in the systemic leaf, associated with less susceptibility, does not manifest in *HvADH-1* overexpressing events.

RESULTS

For analysis of *HvADH-1* function in pathogenesis of powdery mildew at a whole-plant level, we designed an antisense-intron-sense vector for stable knock down (KD) of *HvADH-1* and a second construct for stable overexpression (OE) of *HvADH-1* in transgenic barley (*Hordeum vulgare* L., cultivar ‘Golden Promise’). For both constructs, we used the maize *Ubiquitin1* promoter *ZmUbi1*, which is ubiquitously active in barley (Hensel et al. 2008; Schultheiss et al. 2005). By the use of these constructs we produced stable transgenic barley by *Agrobacterium*-mediated gene transfer to totipotent cells of immature embryos. For phenotyping of transgenic events, we separated T1 or T2 generation material into either transgenic hygromycin-resistant and

PCR-positive individuals or hygromycin-sensitive azygous segregants. We pooled the azygous segregants from individual events and considered them as azygous controls in addition to the parent wild type control Golden Promise. For a first screening, we performed two independent inoculation experiments on leaf segments. For microscopic evaluation, we counted fungal penetration (haustorium formation in intact cells) and whole-cell DAB staining, which is indicative of H_2O_2 generation during pathogen-induced cell-death (Thordal-Christensen et al. 1997) (Figs. 1, Supplemental Figs. 1&2). The rest of the cells were non-penetrated cells without signs of cell death and usually contained cell wall appositions at the site of attempted penetration. We observed reduced susceptibility to fungal penetration in all five tested *HvADH-1* KD events and enhanced susceptibility in all four tested *HvADH-1* OE events (Supplemental Fig. 1 A/B). Following up, two events each for *HvADH-1* KD and OE were tested in two independent inoculation experiments for fungal penetrations on intact plants (Fig. 1, Supplemental Fig. 2). Inoculation of *HvADH-1* KD events 344-E10 and 344-E12 resulted in a similar rate of reduced susceptibility to fungal penetration as seen on excised leaf segments. Other than in excised leaves, *HvADH-1* OE events 346-E8 and 346-E13 did not show a consistent effect on susceptibility when *Bgh* was inoculated on whole seedlings (second leaves evaluated). The *HvADH-1* OE event 346-E13 showed a slight, yet not significant, increase in susceptibility in both experiments, whereas the *HvADH-1* OE event 346-E8 showed reduced susceptibility in one experiment (Figure 1) and enhanced susceptibility in the repetition (Supplemental Fig. 2). Therefore, OE of *HvADH-1* results in increased susceptibility to *Bgh* only when detached leaves were inoculated. Both *HvADH-1* KD events were characterized by a significant increase of *Bgh*-attacked cells showing whole-cell DAB staining, indicative of *Bgh*-induced cell-death (Fig. 1, Supplemental Fig. 2). Therefore, a critical amount of ADH-1 activity might be required for viability of cells in course of the interaction with *Bgh*.

The stable transgenic barley events (individuals expressing hygromycin-resistance) were checked for ADH-1 KD and OE efficiency by native protein-PAGE and in-gel ADH staining for ADH activity. The *HvADH-1* KD events 344-E10 and 344-E12 showed a significantly reduced ADH-1 activity reaching levels of about 53% and 39% compared to empty-vector controls, which were set to 100 per cent. The *HvADH-1* OE events 346-E8 and 346-E12 showed an enhanced ADH-1 activity of 283% and 327%

compared to empty-vector controls (Figure 2A). These effects were also observed for all events at the level of *HvADH-1* transcripts by semi-quantitative RT-PCR and further confirmed by RT-qPCR quantification. In the *HvADH-1* KD event 344-E12, *HvADH-1* transcript level was reduced to 0.34-fold compared to empty vector controls and in the overexpression event 346-E8 *HvADH-1,* expression was increased by 39-fold (Table 1).

To gain a deeper understanding of the function of barley ADHs in the interaction with *Bgh,* we analyzed epidermal cell layers for the presence of different ADH homo- and heterodimers by native-PAGE and in-gel ADH staining. First, we confirmed in *HvADH-1* KD and OE events a strongly reduced (KD event 344-E12) and increased (OE event 346-E8) ADH-1/-1 activity, respectively, in epidermal strips (Fig. 2B). As shown in Figure 3A, ADH-1/-1 is the major ADH isoform in leaves of young seedlings, with little contribution of ADH-2/-2 homo- and ADH-1/-2 heterodimers. ADH-1/-1 homodimers also appeared as the predominant isoform in epidermal strips, which located ADH-1 at the critical interface for interaction with *Bgh* (Fig. 3A). Subsequently, we tested the ADH activity after *Bgh*-inoculation in epidermal strips. Therefore, first leaves of 11-day-old barley plants were inoculated with *Bgh*-spores using a brush. Due to technical reasons it is possible to gain epidermal strips only from the abaxial (lower) side of the leaf. Using a brush enabled us to apply high amounts of spores to the lower side of the leaf on intact plants without much wounding or disturbing the leaves. As a control, we treated leaves with a separate brush that was not saturated with spores. Following spore treatment, the activity of HvADH-1/-1 was induced at 60 min when compared to the 0 min control treatments (set to 100 percent). The leaves also responded to the control brush-treatment with an induction of ADH-1/-1 activity within 20 minutes but this effect was statistically not significant. This early induced ADH-1/-1 induction leveled off and reached the non-treated control level after 60 min (Fig. 3B). The *Bgh*-induced ADH-1/-1 activity in epidermal strips reached a significant level at 120 min with about 150% of the brush-treated control. This supports a possible function of HvADH-1 in course of reorganization of the primary energy metabolism early in tissue directly interacting with *Bgh*.

In a further approach we tested whether ADH activity could be a target of systemic regulation in barley. Therefore, first leaves of 10-day-old barley plants were treated

with chitin as an elicitor of early innate immune responses in barley (Scheler et al. 2016). The second, non-treated leaf was harvested two days later and analyzed for ADH activity and susceptibility to *Bgh* (pustule formation) (Fig. 4A). Chitin treatment resulted in a reduced ADH-1/-1 activity in the systemic leaves of both the empty vector control and KD events. In front of the background of a constitutively reduced ADH-1/-1 activity in the KD event, we observed a further yet not statistically significant chitin-mediated systemic reduction in ADH-1/-1 activity. In the OE events this effect was least pronounced due to the very high overall ADH-1 activity in second leaves, which was not significantly changed by chitin treatment of first leaves (Fig 4B). Using further leaves from the same batch of plants we checked the influence of chitin-treatment on resistance of the systemic leaves to *Bgh* (pustule formation). Pustule formation was clearly reduced in the empty-vector control by 18% and *HvADH-1* KD events by 37% compared to water-treated controls. No such effect was observed in the *HvADH-1* OE event. These results associated the observed reduced ADH-1 activity in the systemic leaf of the empty-vector controls and *HvADH-1* KD events to reduced susceptibility to powdery mildew.

DISCUSSION

Previously we identified a function of *HvADH-1* in the interaction of barley with the parasitic fungus *Bgh* by the use of transient induced gene silencing and overexpression. Silencing of *HvADH-1* resulted in lower susceptibility, and overexpression in higher susceptibility to haustoria formation by *Bgh*. In agreement with the observation of an increased glycolytic/fermentative metabolic activity at the site of powdery mildew pathogen infection (Wildermuth, 2010), it was discussed that *HvADH-1* could function as susceptibility factor (Pathuri et al., 2011).

In this study we generated stable transgenic barley plants characterized by a KD or OE of *HvADH-1* to analyse ADH-1 functions at the whole-plant level. KD events showed a clearly reduced susceptibility to *Bgh*, both on intact plants and on excised leaves. The OE events, however, did not show a clear phenotype following inoculation of intact plants but after inoculation of excised leaves. We reported previously that basal transcripts level of *HvADH-1* decreases with leaf age (Proels et al., 2011). We therefore speculate that in the background of induced leaf senescence, as usually seen in excised leaves, OE of *HvADH-1* might support metabolic activity of host cells, which might support penetration by a biotrophic pathogen. This phenotype might not manifest

when intact plants are inoculated, because basal and pathogen-induced ADH activity in barley wild types suffice to support fungal success (Pathuri et al. 2011; Proels et al. 2011). Based on the fact that *HvADH-1* KD events are characterized by a strongly reduced fungal success in haustorium formation (Fig. 1, Supplemental Figs. 1&2) we assume that a critical amount of ADH-1 activity is required for a compatible interaction with *Bgh*. From this we conclude that *Bgh* profits from barley *HvADH-1*, which we thus consider a susceptibility factor in this interaction. Loss of disease susceptibility might be associated with specific trade-offs, because susceptibility factors usually fulfil important functions for the host plant that are not associated with pathogenesis (Hückelhoven et al. 2013). We did not observe any obvious developmental phenotype of our *HvADH-1* KD events under conditions applied. We assume, however, that HvADH-1 has additional important functions e.g. in barley response to oxygen deprivation. Moreover, KD of *HvADH-1* supported *Bgh*-induced single cell-death reminiscent of a hypersensitive reaction, which is otherwise typical for the response to avirulent *Bgh*. ADH activity might hence be important to maintain the primary energy metabolism and viability of the host cell under conditions of an increased metabolic flux at the site of infection. Such a function could be potentially important to stay alive and defend in interaction with hemibiotrophic or necrotrophic pathogens. Perhaps similarly, barley BAX Inhibitor-1 is a factor of susceptibility to powdery mildew (Eichmann et al. 2010) and BAX Inhibitor-1 proteins can prevent starvation induced program cell death (Bolduc et al. 2002).

One KD event (344-E12) and one OE event (346-E8) were chosen for deeper analysis. With these events we observed that application of chitin, considered as a fungal elicitor, on the first leaf of young barley plants can influence ADH activity and pustule formation in the second, systemic leaf. Chitin application elicits the generation of reactive oxygen species in barley and the phosphorylation of mitogen-activated protein kinases (Scheler at al., 2016), two canonical features of pathogen-associated molecular pattern (PAMP)-triggered immunity in plants (Macho and Zipfel, 2014). In Arabidopis, PAMPs are potent inducers of systemic acquired resistance (Mishina and Zeier, 2007), whereas for barley, corresponding information is sparse (Hückelhoven and Seidl, 2016). Faoro and coworkers reported that chitosan (deacetylated chitin) can induce systemic resistance in barley to *Bgh* (Faoro et al., 2008). We found that chitin treatment results in a reduced ADH-1 activity in the systemic leaf of empty-vector control plants

and the KD event, which correlated to a reduced number of pustule formation in the corresponding leaf. This effect does not manifest in the ADH-1 OE event, probably due to the very high level of ADH-1 activity, and the fact that systemic ADH-1 activity was not significantly modulated by chitin treatment. How to explain such a systemic effect on ADH activity? First, ADH could be a direct target of systemic signalling. Miller at al. (2009) showed that a plant NADPH oxidase mediates systemic signalling via ROS-signalling in response to diverse stimuli, including wounding. Additionally, PAMPs activate a calcium-dependent protein kinase/NADPH oxidase activation circuit in cell to cell signalling (Dubiella et al., 2013). Interestingly, Baxter-Burrell et al. (2002) described a NADPH oxidase-dependent rheostat, which is involved in the regulation of ADH expression. This regulates cell survival under oxygen deprivation and further requires the ROP GTPase Activating Protein 4 (ROPGAP4) for controling ADH activity in Arabidopsis. ROPGAP4 also limits susceptibility of Arabidopsis to the powdery mildew fungus *Erysiphe cruciferarum* (Hoefle et al. 2011; Huesmann et al. 2011). Here we show that barley epidermal strips respond quickly with an induction of ADH-1 activity to the treatment with *Bgh* spores (Fig. 3B). Together, one might speculate that such a local PAMP-induced signal could be transferred to a systemic leaf via a similar NADPH oxidase-dependent mechanism as described in Arabidopsis to regulate activity of ADH-1.

As we could show previously that the *HvADH-1* gene is primarily expressed in young, growing barley leaves, we speculate that ADH has a function in leaf growth. This might be associated with fatty-acid synthesis in growing barley leaves, as ADH holds a function in the “PDH bypass”, a metabolic pathway, which channels the precursor acetaldehyde, derived from glycolysis, into the fatty acid biosynthesis pathway (Strommer, 2011). According to this hypothesis, chitin treatment of the first leaf might result in alterations of resource allocation for growth/fatty acid biosynthesis into defence, which might primarily require hexose sugars (Proels and Hückelhoven, 2014a). Such changes in resource allocation are broadly discussed in plant pathology and insect interactions (e.g. Havko et al. 2016) but little understood in context of systemic immune signalling. Alternatively, ADH activity might directly or indirectly provide metabolic products, which *Bgh* can use as carbon source. Further analysis is thus required to clarify the molecular basis of the observed systemic down-regulation of ADH activity following chitin treatment. Independent of the mechanistic explanation of this phenotype, the down-regulation of ADH-1 activity associates with less pustule

formation in the systemic leaf and can be seen as an adaptation to a potential fungal attack resulting in an induced resistance status in the systemic leaf.

In summary, the presented data support a function of *HvADH-1* in the compatible interaction of barley and *Bgh*. The KD of *HvADH-1* coincides with a reduced susceptibility to *Bgh* on excised leaves and intact plants. Overexpression of *HvADH-1* results in increased susceptibility to *Bgh* only when excised leaves were inoculated, probably best explained in the context of accelerated leaf senescence. Moreover, chitin treatment induces a down-regulation of ADH-1 activity in the systemic (non-treated) leaf, which correlates with a reduced susceptibility to *Bgh*. This phenotype does not manifest in *HvADH-1* overexpressing events, which are unable to modulate ADH activity. ADH may hold a function in keeping upright the glycolytic/fermentative pathway or PDH bypass in pathogen-stressed plants, which could be crucial for biotrophy of the powdery mildew fungus.

MATERIALS AND METHODS

Plant and pathogen material, inoculation

Donor material and transgenic plants of the barley (*Hordeum vulgare* L.) cultivar 'Golden Promise' were grown in a growth chamber at 18˚C, 60% relative humidity, and a photoperiod of 16 h (150 µmol $s^{-1}m^{-2}$ photon flux density). *Blumeria graminis* (DC) Speer f.sp*. hordei* Em. Marchal, race A6 (Wiberg 1974), undergoes a compatible interaction with the barley lines used and was maintained on 'Golden Promise' under the same conditions. For microscopic evaluation, *B. graminis* f.sp. *hordei* was inoculated onto detached second leaves of 14-day old barley plants to give a density of 20 conidia mm^{-2}. For evaluation of microcolonies the density of inoculation was reduced to 5 conidia mm^{-2}. To determine the fast response to *Bgh* challenge in epidermal strips, the lower side of first leaves of 10-day-old barley (*Hordeum vulgare* L.) cultivar 'Golden Promise' plants were treated three times with a brush, which was saturated with *Bgh* spores (by coating the brush with fresh spores form an inoculated plant). Control leaves were treated with a spore-free brush to set the same mechanical stimulus. At the indicated time-points epidermal strips were harvested, immediately frozen in liquid nitrogen and stored at -80°C till protein extraction.

Cloning and stable plant transformation

The KD construct was generated by cloning a cDNA fragment of *HvADH-1* (accession AF253472) using the primers (in 5´-3´orientation) GACAGAGGTGTGATGATCGGGGATG and TGTATCATAGCATTGACGTTGCCAGTGC into the RNAi entry vector pIPKTA38. The entire sense-intron-antisense construct was then gained by clonase reaction with the pIPKb007 vector (Himmelbach et al., 2007) yielding ZmUbi::HvADH-1antisense:intron:HvADH-1sense:nos. The OE construct was generated by cloning the full length coding sequence of *HvADH-1* using the primers TGGATCCTGTGAAGTGAGAGATC and AGTCGACCGATGATCTGGTTCAGAAG, thereby introducing the restriction sites *Bam*HI and *Sal*I, respectively. The PCR product was first cloned in pGEM-T and sequenced to proof the identity of the sequence. The *HvADH-1* fragment was released from pGEM-T and cloned into pUbi-AB (DNA-Cloning-Service, Hamburg, Germany) using *Bam*HI and *Sal*I restriction sites. Subsequently, the ZmUbi::HvADH-1:nos cassette was subcloned into p6U via *Sfi*I restriction sites. Stable genetic transformation of barley cultivar 'Golden Promise' with corresponding constructs was performed by *Agrobacterium*-mediated gene transfer to totipotent cells of immature embryos followed by callus formation and plant regeneration under selective pressure using hygromycin (Hensel et al. 2008). Transgenic plants harboring the respective construct were identified by hygromycin resistance of leaf segements and PCR-based amplification of the hygromycin resistance gene from genomic DNA (Hensel et al., 2008; Eichmann et al., 2010).

Histological staining and microscopic analysis

The outcome of the interaction of transgenic barley plants with *B. graminis* f.sp. *hordei* was evaluated 30 h after inoculation. Penetration of attacked cells was ascertained by detection of haustoria formation. Penetration efficiency was quantified by counting haustoria at 100 interaction sites per leaf of four to seven leaves per event and experiment. Staining with 3,3-diaminobenzidine (DAB) was taken as reliable markers of H_2O_2 accumulation in hypersensitively reacting cells (Thordal-Christensen et al. 1997). Bright-field and fluorescence microscopy was performed as described by Hückelhoven and Kogel (1998). Because the rate of fungal penetration into short and long epidermal cells is different, only short cells (cell type A and B, see Koga et al.

1990) were evaluated. We exclusively evaluated interaction sites where only one fungus per cell attempted to penetrate.

Protein isolation and native-PAGE

Crude protein isolation from leaf material and epidermal strips, native-PAGE, and in-gel ADH staining was performed as described in Proels and Hückelhoven (2014b) with the exception that the DTT concentration in the extraction buffer was increased to10 mM. Gels were stained for 30 min, following the stain solution was replaced by water and gels were stored at 4°C till analysis. Gels were photographed using a high-resolution CCD camera and band intensities were analyzed using the Vilber Lourmat gel detection system.

cDNA synthesis and RTqPCR

Leaf material was harvested, extracted for total RNA and cDNA was synthesized from one µg of total RNA using the QuantiTec Reverse Transcription kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Reverse transcription quantitative real-time PCR (RT-qPCR) was performed in a Mx3005P Cycler (Stratagene, La Jolla, CA, USA). Individual PCR reaction contained 1 µl of cDNA, 10 µl Maxima SYBR Green qPCR master mix (2×) (Thermo Fisher Scientific) and 300 nM of a gene-specific forward and reverse primer in a total volume of 20 µl. To quantify *HvADH-1* (AF253472) expression the following primer pair (in 5´-3´orientation) was used: oligo-1 ATTCAAGGCGACGCGAAGCAC, oligo-2 TGAAGAGGATCTTGACGCGCA C. Expression data were normalized to the *HvUbc* gene (AK360562) using the primer pairs: oligo-1 TCTCGTCCCTGAGATTGCCCACAT, oligo-2 TTTCTCGGGACAGCAACACAATCTTCT. The program consisted of an initial step at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 sec, 55 °C for 60 sec, and 72 °C for 60 sec. The specificity of amplification reactions was verified by a melting curve analysis performed at 95°C for 60 sec, 60°C for 30 sec, and again to 95°C for 30 sec. The empty-vector control samples served as calibrator to calculate relative expression levels. Experiments were repeated in two biological replications. Primer efficiency was close to 100% and hence we applied the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Chitin treatment

The first leaves of 9 or 10-day-old barley plants were treated with doubled destilled water (control) or 200 µg/ml chitin by gently rubbing a solution-saturated paper cloth over the top half of the leaf. This treatment was repeated 3 times in 3 h intervals. 48 h after the treatment the second leaves were harvested for crude protein isolation or were put on agar plates for *Bgh* inoculations at a spore density of 5 conidia mm^{-2}. Pustule development was evaluated using a binocular three days after inoculation.

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Table 1. Relative expression of *HvADH-1* in selected transgenic lines

	Relative expression level[a] (±SD[b])
Empty vector control	1 (±0,13)[a]
HvADH-1 KD (334-E12)	0,34 (±0,064)
HvADH-1 OE (346-E08)	39,31 (±13,40)

[a]expression in individual samples was compared to the average empty vector control (set as 1). *HvADH-1* expression was normalized to the expression of the housekeeping gene *HvUbc* using the $2^{-\Delta\Delta Ct}$ method. Analysis of independent biological samples led to similar results. [b]Standard deviation of the mean of three individual cDNA samples.

Figures

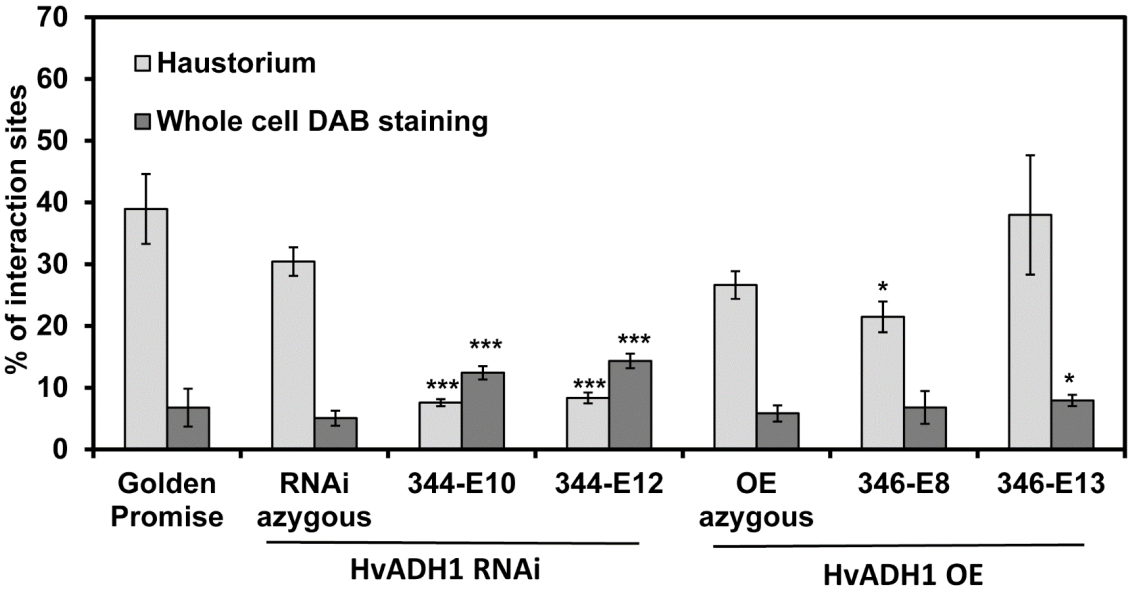


Fig.1. Knock-down of *HvADH-1* results in reduced susceptibility to *Bgh*. Second leaves of 14-day-old barley events were inoculated on-planta with *Bgh* spores at a density of 20 conidia mm^{-2}, harvested and DAB stained 30 h after infection and subsequently evaluated for penetrated epidermal cells (haustorium formation) and whole-cell DAB staining, which is indicative of *Bgh*-induced cell-death. Genotypes used are WT (cv. Golden Promise), two independent stable transgenic knock-down (RNAi) and overexpression (OE) events and corresponding azygous control (pooled segregants from the two events, which lost the transgene). Shown is the mean and the confidence intervals at $p<0,05$ of four to eight leaves per genotype. Asterisks indicate significant differences when compared to azygous controls according to Student`s t-test (* $p<0,05$; *** $p<0,001$).

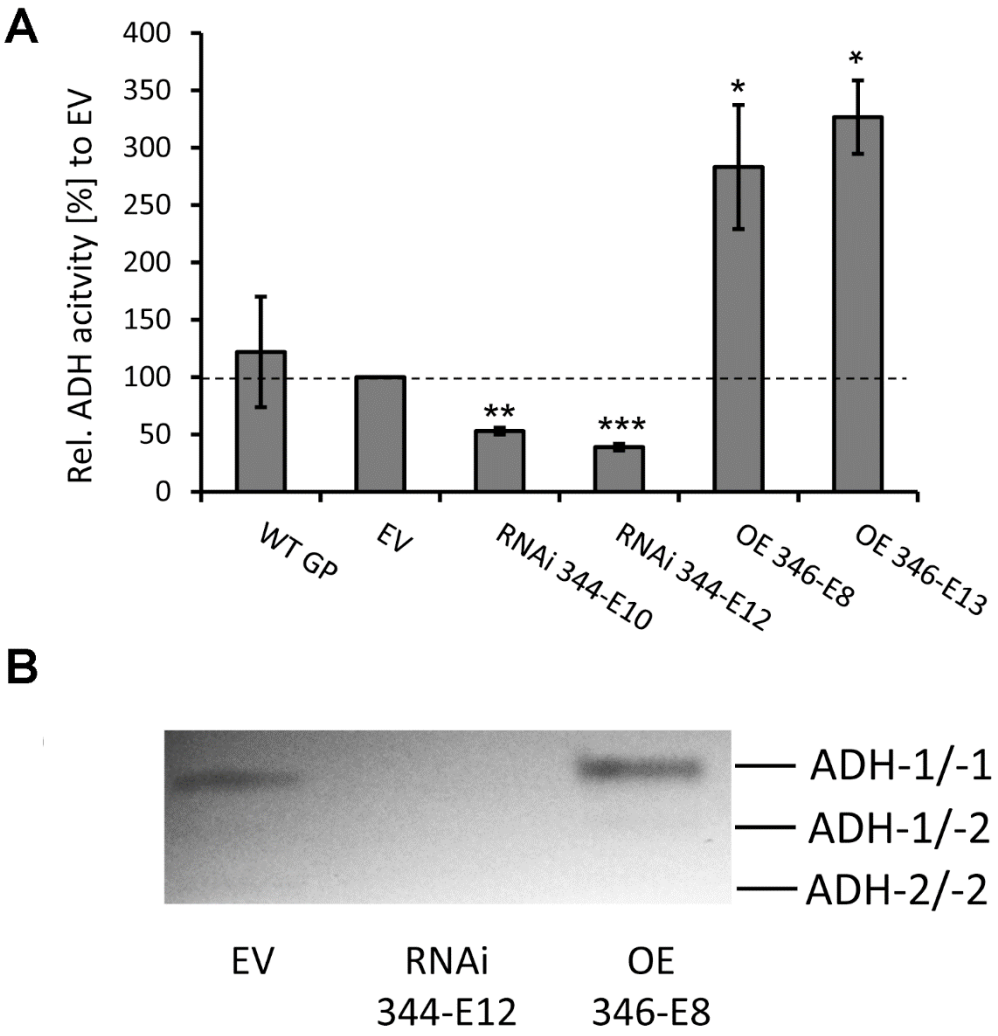


Fig. 2. Stable knock-down or overexpression of *HvADH-1* results in reduced or increased ADH-1 activity in leaves, respectively. (A) Second leaves of 10-day-old barley seedlings were harvested and ground in liquid nitrogen. Crude protein extracts were separated on native-PAGE and gels were stained for ADH activity. The predominant homodimeric ADH-1/-1 isoform band was quantified relative to the empty-vector control set to 100%. Genotypes used are WT (cv. Golden Promise), empty-vector control (EV), and two independent stable transgenic knock-down (RNAi) and overexpression (OE) events. Shown is the mean and SE of three to four biological replicates. Asterisks indicate significant differences according to Student`s t-test (* $p<0,05$; ** $p<0,01$; *** $p<0,001$). (B) Epidermal strips of 10-day-old barley leaves were harvested and native-PAGE and in-gel ADH staining performed as described in (A). ADH stained gels were photographed using a high resolution CCD camera. The experiment was repeated two times with similar results. Expected positions of homodimers (ADH-1/-1 or ADH-2/-2) and heterodimer (ADH-1/-2) of active barley ADH proteins are indicated.

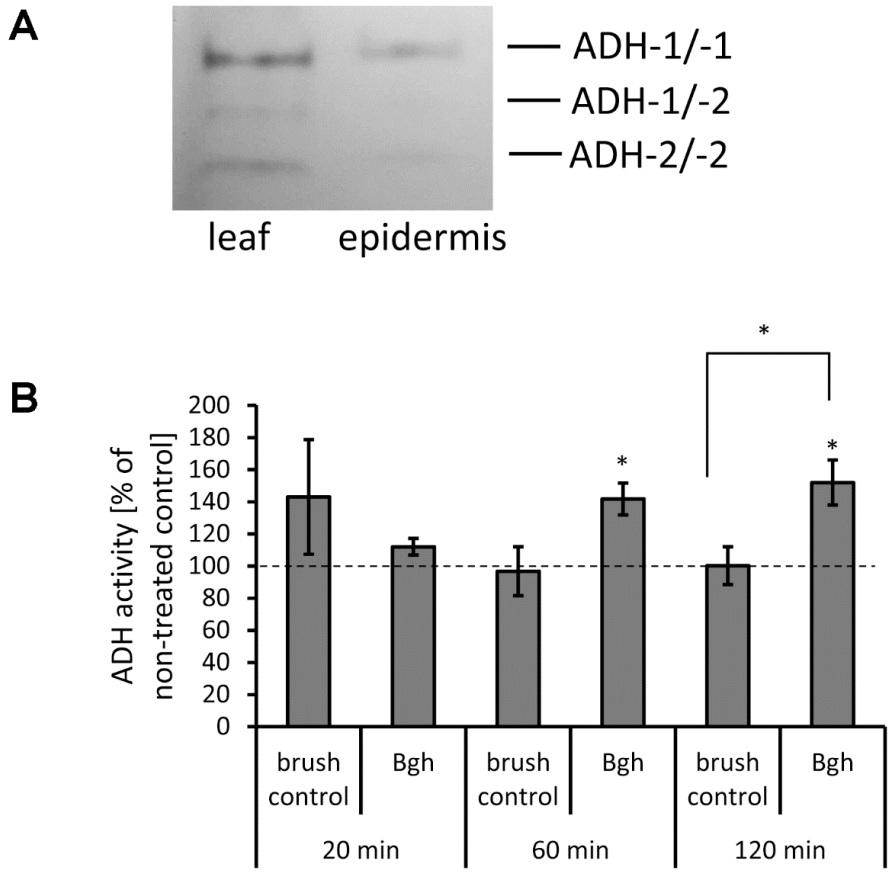


Fig. 3. (A) HvADH-1/-1 is the predominant ADH isoform in young barley leaves and epidermal strips. Whole leaves or epidermal strips of the lower side of first leaves of 11-day-old barley plants were harvested and ground in liquid nitrogen. Following, crude protein extracts were separated on native-PAGE and gels were stained for ADH activity. ADH stained gels were photographed using a high resolution CCD camera. Expected positions of homodimers (ADH-1/-1 or ADH-2/-2) and heterodimer (ADH-1/-2) of active barley ADH proteins are indicated. Shown data are representative for three biological repetitions. (B) HvADH-1/-1 is induced after *Bgh* challenge in epidermal strips. First leaves of 11-day-old barley plants were treated three times with a brush, which was saturated with *Bgh* spores. Control leaves were treated with a spore-free brush to set the same mechanical stimulus. Epidermal strips were harvested before treatment and 20, 60 and 120 min after treatment and ground in liquid nitrogen. Following, crude protein extracts were separated on native-PAGE and gels were stained for ADH activity. The relative ADH-1/-1 induction compared to non-treated zero-control of four independent biological replicates and SE are shown. Asterisks indicate significant differences to the 0 min control treatment or the individual brush control as indicated according to Student`s t-test (*$p<0,05$).

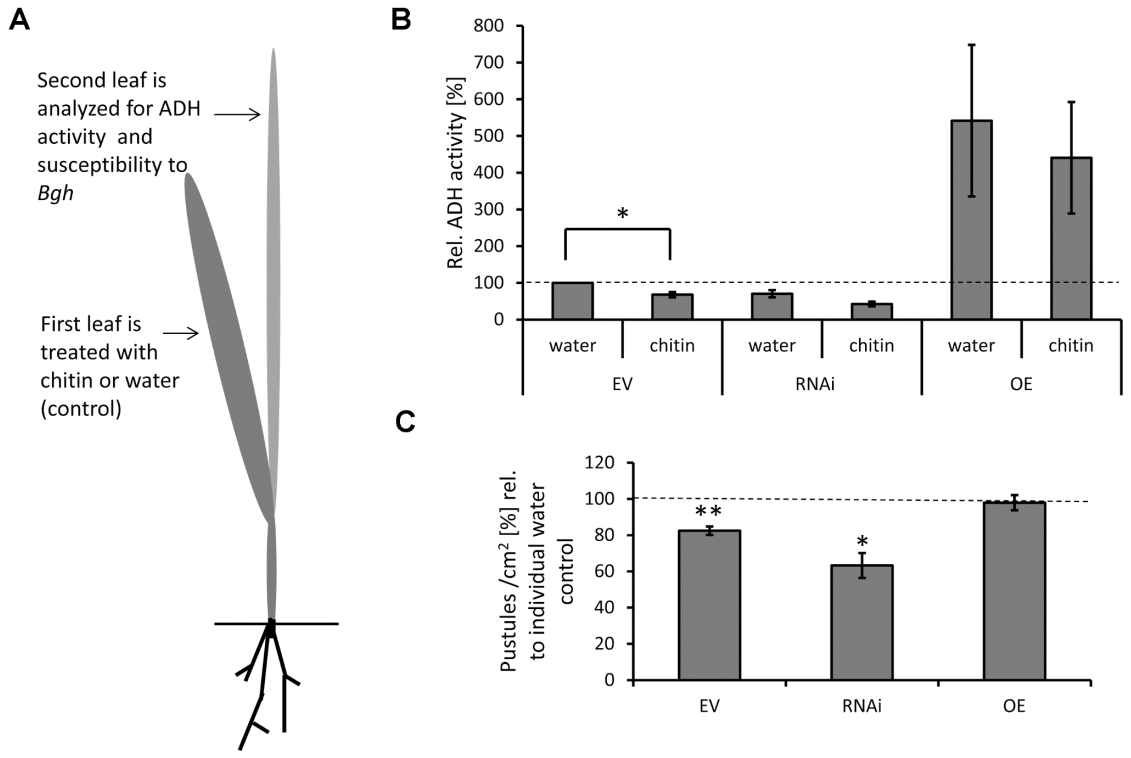
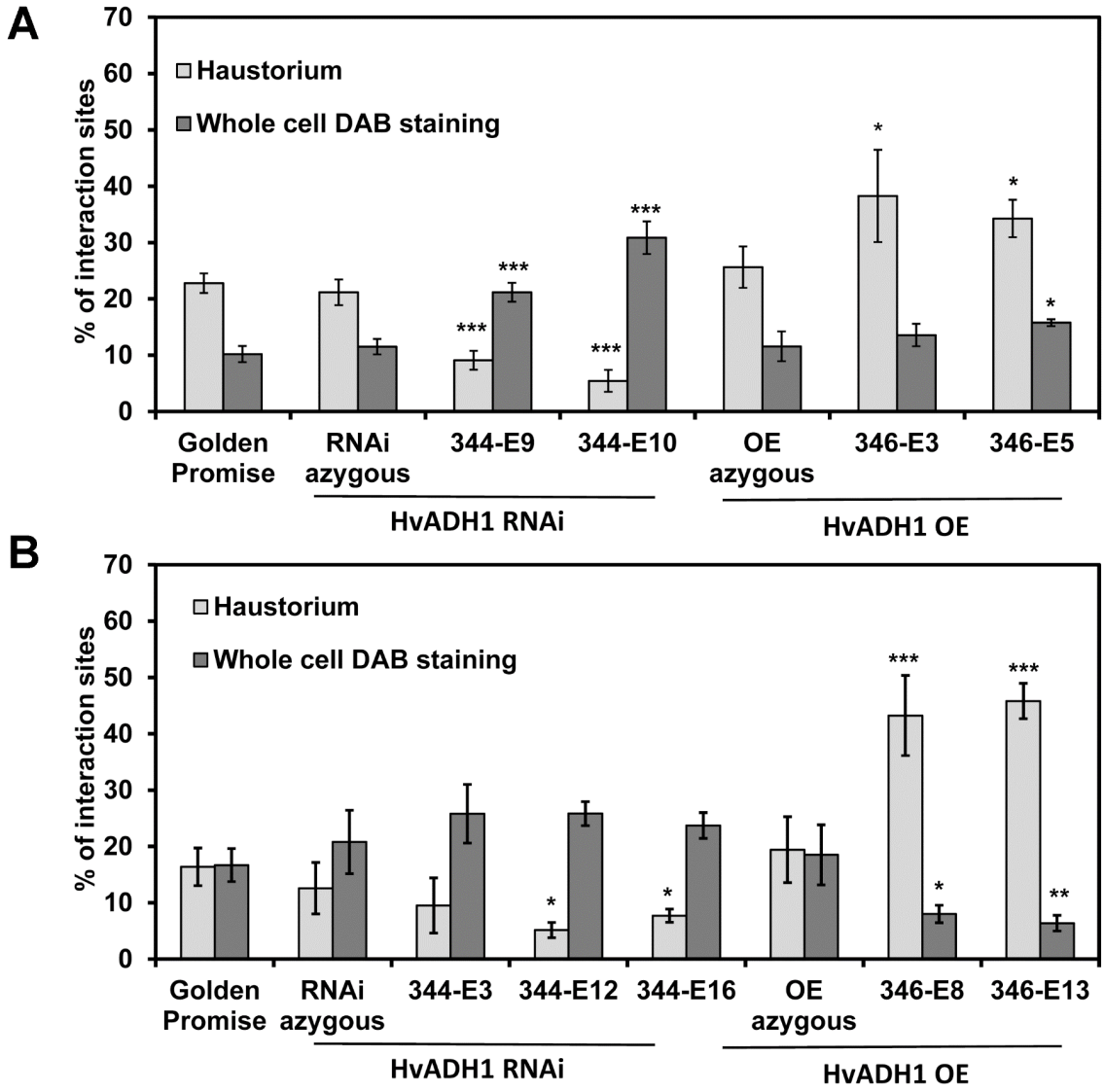
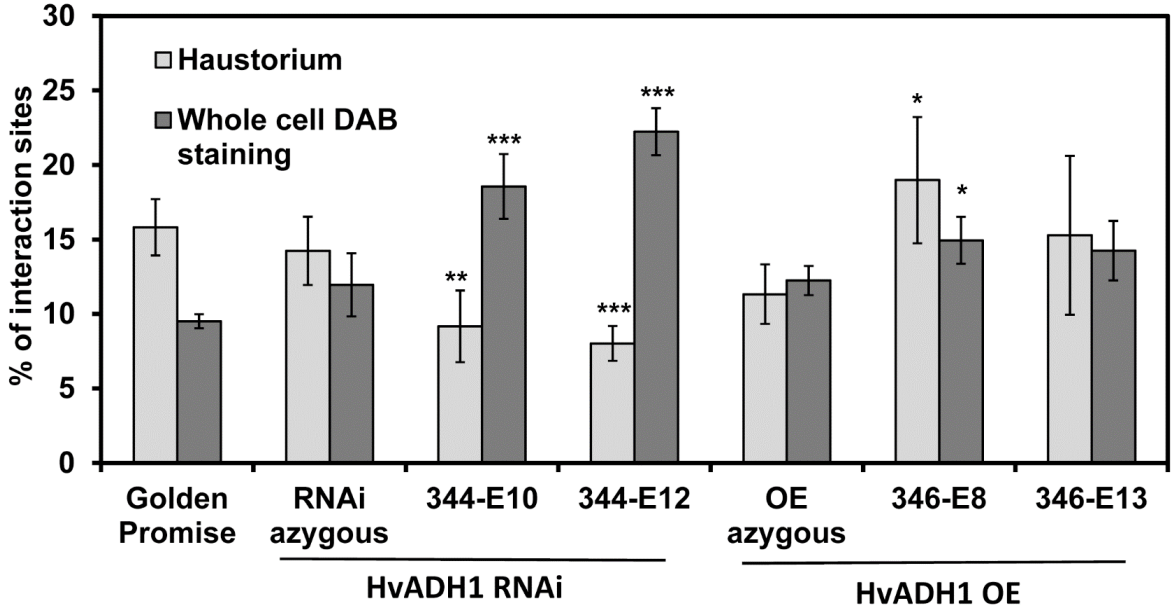


Fig. 4. HvADH-1 activity is systemically regulated in responds to chitin. (A) The first leaves of 9 or 10-day-old barley plants were treated with doubled destilled water (control) or 200 µg/ml chitin by gently rubbing a solution-saturated paper cloth over the top half of the leaf. This treatment was repeated 3 times with 3 h intervals. 48 h after the treatment the second leaves were harvested for crude protein isolation (B) or were put on agar plates for *Bgh* inoculations at a spore density of 5 conidia mm^{-2}(C). B) Second leaves of barley genotypes (treated as described in (A)) were harvested and ground in liquid nitrogen. Crude protein extracts were separated on native-PAGE and gels were stained for ADH activity. The predominant ADH-1/-1 band was quantified relative to the water-treated empty-vector control set to 100%. Genotypes used are empty-vector control (EV) and one KD (RNAi, 334-E12) and overexpressor (OE, 346-E08) event. Shown is the mean and SE of three biological replicates. Asterisks indicate significant differences according to Student`s t-test (* p<0,05).C) Second leaves of barley genotypes (treated as described in (A)) were harvested and inoculated with *Bgh* spores at a density of 5 conidia mm^{-2}. Three days after inoculation, pustule numbers per cm^2 leaf area was evaluated using a binocular. The water-treated control was set to 100% for each genotype analyzed. Shown is the mean and SE of three biological replicates. Asterisks indicate significant differences according to Student`s t-test when compared to water treated controls (* p<0,05; ** p<0,01).

Supplemental Figures



Supplemental Fig.1. In two experiments (A and B) second leaves of 14-day-old barley seedlings were excised and put on water-agar plates. 30 h after inoculated with *Bgh* spores at a density of 20 conidia mm^{-2} leaf segments were harvested and DAB stained and subsequently evaluated for penetrated epidermal cells (haustoria formation) and whole-cell DAB staining, which is indicative of defence induced cell-death. Genotypes used are WT (cv. Golden Promise), five independent stable transgenic knock-down (RNAi) and four overexpressor (OE) events and corresponding azygous contol offspring. Shown is the mean and the confidence intervals at p<0,05 of four to eight leaves per genotype. Asterisks indicate significant differences when compared to azygous controls according to Student`s t-test (* p<0,05; ** p<0,01;*** p<0,001).



Supplemental Fig. 2. Second leaves of 14-day-old barley seedlings were inoculated on-planta with *Bgh* spores at a density of 20 conidia mm^{-2}, harvested and DAB stained 30 h after infection and subsequently evaluated for penetrated epidermal cells (haustoria formation) and whole-cell DAB staining, which is indicative of defence induced cell-death. Genotypes used are WT (cv. Golden Promise), two independent stable transgenic knock-down (RNAi) and overexpression (OE) events and corresponding azygous control events. Shown is the mean and the confidence intervals at p<0,05 of four to eight leaves per genotype. Asterisks indicate significant differences when compared to azygous controls according to Student`s t-test (* p<0,05; ** p<0,01; *** p<0,001).