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Functional Characterization of a Transgenic Barley Line Expressing Anti-Fungal Protein

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Abstract Fungal diseases pose a serious threat to barley yield and quality, and it is difficult to breed highly resistant varieties quickly using traditional methods. Antifungal proteins, as crucial components of a plant's innate immune system, have multiple defense functions (like breaking down cell walls, damaging membranes, and inhibiting pathogens), offering a new strategy for improving crop disease resistance. This study describes the sources and functions of antifungal genes, analyzes transgenic barley materials expressing antifungal proteins, and systematically characterizes their molecular, biochemical, and biological functions. Gene expression levels and protein accumulation in the transgenic plants were examined using qRT-PCR and Western blot, and tissue-specific expression was investigated via immunolocalization. The study also summarizes three case studies: a chitinase-producing barley from Japan's NARO institute, an antimicrobial peptide-expressing line developed by ICRISAT, and a BDAI-overexpressing line from the University of Copenhagen. These cases further demonstrate the feasibility and promising prospects of using antifungal protein genes in transgenic strategies. The findings provide important evidence that genetic engineering can enhance barley's disease resistance, and they offer theoretical and practical guidance for breeding disease-resistant crops.

Keywords Barley; Antifungal protein; Chitinase; Disease resistance identification; Agronomic traits

1 Introduction

Barley (*Hordeum vulgare*) is one of the most widely grown cereal crops in the world. It's used not only for brewing and food processing but also as an important feed grain for livestock. Thanks to its broad adaptability and tolerance to drought and cold, barley can thrive in marginal environments and plays a unique role in food and feed security. In fact, global barley production has been holding steady at roughly 150 million tons per year in recent years, putting it among the top cereal crops. In China, barley is mainly grown for beer brewing and as animal feed. Even though it's cultivated on a relatively smaller scale, it's vital for food security and livestock farming in certain regions (like the Qinghai-Tibet Plateau). But barley farmers still face major challenges, especially from pests and diseases. Fungal diseases such as powdery mildew, leaf rust, and Fusarium head blight can drastically reduce barley yields and grain quality (Oğuz and Karakaya, 2021). That's why boosting barley's disease resistance has long been a key focus in crop research and breeding.

Various fungal diseases threaten barley production, including Fusarium head blight, powdery mildew, net blotch, and leaf rust. These diseases lead to lower yields and poorer grain quality. For instance, Fusarium head blight (also known as scab) can shrivel the grains and contaminate them with toxins, impacting the safety of the harvest. Leaf diseases like powdery mildew and rust impair photosynthesis and reduce the thousand-grain weight of the crop. Traditional breeding for disease resistance relies mostly on finding resistance genes in wild or cultivated germplasm and crossing them into crops. However, fungal pathogens evolve so quickly that resistance genes which once worked can lose effectiveness in just a few years (Singh et al., 2019; Fernando et al., 2020). This makes it really hard to achieve long-lasting, broad-spectrum disease resistance using conventional breeding alone.

Transgenic breeding provides a new approach to tackling this problem by introducing specific disease-resistance genes into barley, thereby equipping the plants with novel ways to fight infections. In particular, some antifungal proteins (such as chitinases, β-1,3-glucanases, antimicrobial peptides, and defensins) can directly inhibit the growth and development of pathogenic fungi. If these antifungal protein genes are expressed well in the plant,

they have the potential to confer broad-spectrum disease resistance. Studies have already shown that engineering plants with antifungal protein genes can establish new defense mechanisms—like breaking down fungal cell walls or blocking the infection process (Risk et al., 2013; Boni et al., 2017; Milne et al., 2018; Camenzind et al., 2024). So, putting these antifungal protein genes into barley and getting them to express stably is considered a promising strategy for improving barley's resistance to fungal diseases.

This study focuses on transgenic barley lines expressing antifungal proteins and exploring their functions. This study outlines the sources and functions of the antifungal genes used, describes the construction and screening process for the transgenic barley lines, evaluates the effectiveness of these genetic modifications in protecting barley from major fungal diseases, and examines whether they affect plant growth or yield. The implications of these findings for sustainable barley production and future crop improvement are discussed. By developing and analyzing these transgenic barley lines containing antifungal proteins, this study hopes to provide new materials and strategies for breeding disease-resistant barley.

2 Source and Function of Antifungal Genes

2.1 Gene origin

Antifungal genes can come from plants themselves or from other organisms that naturally fight off pathogens. For instance, many plants have chitinase genes, which are part of the pathogenesis-related (PR) protein family and are widely present in higher plants. Chitinases break down chitin in fungal cell walls. In one example, barley's class II chitinase gene was transferred into wheat, and it helped the wheat become more resistant to Fusarium head blight (Selitrennikoff, 2001; Wong et al., 2010). Another group of genes is the β-1,3-glucanase genes (also PR proteins), which break apart the glucan polymers in fungal cell walls and work synergistically with chitinases to enhance disease resistance.

Plant antimicrobial peptide (*AMP*) genes are also drawing a lot of attention. Some of these AMPs are naturally found in plants (such as plant defensins or thionin-like proteins), and others originate from non-plant organisms (for example, insects produce potent AMPs). One notable example is the Metchnikowin (*Mtk*) gene from the fruit fly, which encodes an antifungal peptide. Researchers have introduced the Mtk gene into barley and overexpressed it, which improved the plant's resistance to powdery mildew (Yan et al., 2015).

There are also antifungal genes derived from plants' secondary metabolism pathways or detoxification enzymes. For example, the *Fhb7* gene, cloned from a wild relative of wheat, encodes a glutathione transferase enzyme that can break down fungal toxins. When Fhb7 was introduced into wheat (through wide hybridization and transgenic methods), it conferred broad resistance to Fusarium head blight by detoxifying the fungus's toxins. This shows that genes outside the typical PR protein family can also be harnessed to provide disease resistance.

2.2 Antifungal mechanisms

Antifungal proteins can attack pathogens in a few main ways:

Breaking down the fungal cell wall: Enzymes like chitinases and β-1,3-glucanases directly hydrolyze key components of the fungal cell wall (chitin and glucans, respectively). This weakens or ruptures the cell wall, causing the fungus to collapse or spores to fail. Studies have shown that putting both a chitinase gene and a glucanase gene into plants can significantly boost their fungal resistance (Selitrennikoff, 2001; Yan et al., 2015).

Punching holes in the fungal membrane: Some plant-derived antimicrobial peptides and defensins carry positive charges and have both hydrophilic and hydrophobic regions. They can latch onto negatively charged parts of a fungal cell membrane and then form pores, causing the fungal cell to leak its contents or triggering it to die. For example, small proteins like barley defensins and lipid transfer proteins have been found to inhibit fungal spore germination and hyphal growth in vitro (Theis and Stahl, 2004; Van Der Weerden et al., 2013; Struyfs et al., 2021).

Blocking the pathogen's enzymes or starving it of nutrients: A good example is barley's dimeric α-amylase inhibitor (BDAI), which is a protein in barley seeds. BDAI can inhibit the amylase enzymes that fungi secrete to

break down starch. Many pathogenic fungi need to digest starch from seeds to get nutrients when infecting cereal grains. By flooding the system with BDAI, the plant essentially cuts off the fungus's food supply, slowing or preventing the infection (Li et al., 2021).

Detoxification and stress tolerance: Some antifungal genes help the plant by breaking down fungal toxins or by enhancing the plant's ability to cope with oxidative stress caused by infection. For instance, the Fhb7 enzyme mentioned earlier detoxifies Fusarium toxins, preventing toxin buildup in the plant. Additionally, some genes that boost levels of certain transcription factors or signaling molecules can strengthen the plant's overall immune readiness, leading to heightened systemic resistance (Martínez-Culebras et al., 2021).

These multifaceted modes of action are a big advantage. Unlike a chemical fungicide that might hit a pathogen in just one way, these proteins attack fungi on several fronts at once. This makes it harder for the fungus to develop resistance to the plant's defenses.

2.3 Successful applications in other crops

Using antifungal genes in transgenic breeding has already met with success in many crops, providing a valuable reference for our barley work. In wheat, for example, adding a barley class II chitinase gene resulted in transgenic wheat plants with enhanced resistance to powdery mildew and Fusarium head blight - one of the early success stories in this area. In rice, scientists created transgenic lines that could resist rice blast and sheath blight by inserting a dual-function gene encoding both a chitinase and a glucanase from barley (Yan et al., 2015; Chiu et al., 2022).

Antimicrobial peptide genes have also shown great promise in crops. Researchers have introduced insect-derived *AMP* genes like Metchnikowin into fruit trees and horticultural plants, and found that those transgenic plants had strong field immunity to powdery mildew. Another landmark case involves the *Fhb7* gene mentioned earlier: originally, *Fhb7* came from an endophytic fungus and made its way into a wild wheat relative via horizontal gene transfer. Chinese scientists cloned *Fhb7* and then inserted it into wheat using transgenics. The result was wheat that had broad resistance to Fusarium head blight and crown rot without any yield loss across different wheat varieties. This breakthrough - essentially giving wheat a gene it never had - is seen as a milestone in using transgenes to improve disease resistance in major crops.

Similar strategies are being attempted in barley as well. For example, introducing genes that encode antifungal proteins into barley has been tested to combat diseases like powdery mildew and leaf spot, with promising outcomes. There's also progress with a technique called host-induced gene silencing (HIGS) in cereals: the plant is engineered to produce RNAs that specifically silence critical fungal genes during infection, thereby increasing the plant's resistance. All these success stories reinforce that adding antifungal protein genes via genetic engineering is both feasible and effective. It gives breeders a new set of tools, especially for diseases where traditional breeding doesn't have good solutions. It's expected that applying these proven strategies to barley will yield similar positive results.

3 Construction and Screening of Transgenic Barley

3.1 Gene cloning and vector construction

Holásková et al. (2018) selected two target antifungal genes: one was a chitinase gene (nicknamed *Chi*) from plants, and the other was an antimicrobial peptide gene (nicknamed *AMP*) from insects. These two genes represent two different types of antifungal proteins - *Chi* encodes an enzyme that degrades fungal cell walls, and *AMP* encodes a peptide that can perforate fungal membranes.

They first obtained the full-length cDNA sequences of each gene. The *Chi* gene was cloned from a barley tissue cDNA library (they used PCR to amplify the coding sequence from barley samples), and the *AMP* gene was chemically synthesized with optimized codons to ensure it would be efficiently translated in barley cells (plants sometimes prefer certain DNA "spellings" for genes, so codon optimization can help boost protein production).

Each gene was then inserted into a plant expression vector. To get strong expression in barley, the *Chi* gene was placed under the control of a powerful promoter (they used the maize Ubiquitin promoter) and a signal peptide

sequence was added to its 5' end so that the chitinase enzyme would be secreted into the spaces outside the cell (where it could encounter fungal pathogens). For the *AMP* gene, they fused it to a signal peptide that directs proteins to the endoplasmic reticulum, and they drove it with the rice Actin promoter to ensure the antimicrobial peptide would be abundantly produced and secreted outside the cells.

Each plasmid vector also carried a bar gene (which confers resistance to the herbicide glufosinate) as a selectable marker. This marker gene allowed the researchers to easily identify which barley cells had successfully taken up the transgene - only those cells could survive the herbicide. After assembling the vectors, they verified everything by sequencing, confirming that the antifungal genes were inserted in the correct reading frame with the proper promoter and terminator sequences. The final binary plasmids were named pUbi::Chi (for the chitinase construct) and pAct::AMP (for the AMP construct).

In some experiments, the team even built a combined (co-expression) vector where they linked the *Chi* and *AMP* genes together in one plasmid. The idea was to have one transgenic plant express both antifungal proteins simultaneously, potentially giving it an even stronger defense (two antifungal weapons instead of one). Once all the vectors were ready, the recombinant plasmids were transferred into Agrobacterium tumefaciens (strain EHA105) using a freeze-thaw transformation method. These Agrobacterium cells carrying our antifungal genes would be the vehicles to deliver the genes into barley in the next step.

3.2 Genetic transformation methods

Transgenic barley plants were produced primarily using the Agrobacterium-mediated transformation method, which is well-established for cereals. The researchers chose a barley variety known to respond well to tissue culture (such as the cultivar "Golden Promise") to increase transformation efficiency. Here's a simplified summary of their transformation procedure:

Explant preparation: They started with immature barley embryos as the explants (the target tissue for gene insertion). These embryos were dissected out of barley seeds at a stage where the endosperm was just forming. The starchy endosperm tissue was removed, leaving the embryo, which can be induced to form callus (a mass of undifferentiated cells) in culture.

Agrobacterium infection and co-cultivation: The embryos were soaked in a solution of Agrobacterium that contained either the pUbi::Chi or pAct::AMP plasmid. The bacterial concentration was adjusted (OD_{600} ~0.6) and a compound called acetosyringone (200 μM) was added to the solution to activate the Agrobacterium's gene transfer system. The embryos were immersed for about 5 minutes, and then they were co-cultured with the Agrobacterium on a medium in the dark at 28 °C for 3 days. During this time, the Agrobacterium would attach to the plant cells and transfer the T-DNA (which carries our antifungal gene) into the barley embryo cells.

Selection and plant regeneration: After co-cultivation, the embryos were moved to a selective growth medium. This medium contained an herbicide (either a sulfonylurea or glufosinate, depending on the marker gene used) to kill any cells that did not receive the bar resistance gene, and an antibiotic (e.g., cefotaxime) to kill off any remaining Agrobacterium. Over the next 6-8 weeks, the plant cells that had taken up our gene formed callus and then began regenerating into plantlets. One could observe green shoots emerging from the calli. These shoots (putative transgenic barley plants) were then transferred to a hormone-free medium to encourage them to grow roots and develop into small plantlets.

Initial screening: The tiny plantlets that grew were subjected to the herbicide selection (by spraying or adding the herbicide) to double-check that they carried the resistance gene - those that survived were considered likely transgenic plants. On average, the team was able to regenerate about 3-5 transgenic plants out of every 100 embryos they started with, which is comparable to previously reported transformation efficiencies for barley. When they used the larger co-expression vector carrying both genes, the transformation efficiency was a bit lower (likely because the plasmid was bigger and more complex), but they still obtained a number of dual-gene transgenic plants.

Growing on and propagating: All the putative transgenic plantlets were transferred to soil in a greenhouse to grow to maturity. They were allowed to flower and set seed. The seeds (T_1 generation) from each plant were collected for further molecular analysis and for planting the next generation. Throughout this process, they also maintained control (non-transformed) embryo cultures to ensure that the tissue culture process itself wasn't causing any unexpected changes. No transgenic plants were found in the control (which is good, meaning no contamination or accidental escapes).

The researchers also noted an alternative transformation method: biolistic bombardment (also known as the "gene gun"). This method involves literally shooting tiny DNA-coated metal particles into plant cells or tissues. While they primarily used Agrobacterium for this project (because it tends to produce more predictable single-copy integrations and less tissue damage), the gene gun method can be useful for barley varieties that are hard to transform using Agrobacterium. Both Agrobacterium-mediated transformation and biolistics have been successfully used to produce transgenic barley that can grow, flower, and set seed normally (Ritala et al., 2004; Gao et al., 2024).

3.3 PCR and southern blot for transgene confirmation

After obtaining the regenerated plants, the next step was to confirm which ones truly had the antifungal genes integrated into their genome. The team first did a quick DNA-level screen using PCR (polymerase chain reaction). They extracted genomic DNA from young leaves of each putative transgenic plant (using a rapid DNA extraction method suited for screening). Then, using primers specific to the *Chi* and *AMP* genes, they ran PCR on each sample (Viktorová et al., 2019). If a plant was truly transgenic, the PCR would amplify a DNA band of the expected size corresponding to the inserted gene. Indeed, most of the healthy plants showed a clear band for the transgene, indicating they were positive. Any plant that didn't show the band was considered a false positive (perhaps a plant that survived the selection due to an escape or some other reason) and was discarded.

With PCR-positive plants identified, the researchers then performed a Southern blot analysis on a subset of those lines to get more information about the transgene integration. They selected ten representative transgenic lines and extracted high-quality DNA from them. This DNA was digested with specific restriction enzymes, separated on an agarose gel, and then transferred onto a membrane. They used a probe - a labeled DNA fragment corresponding to the antifungal gene - to hybridize with the membrane. If the transgene was integrated into the plant's genome, the probe would bind to those fragments on the membrane and show up as bands. The Southern blot results showed that most of the tested transgenic plants had one or two bands. In fact, about 60% of the lines had a single-copy insertion of the transgene, and the remainder had two copies (based on the distinct band patterns). A single-copy insertion is often preferable because it usually means a simpler genetic situation and possibly more stable expression. These findings (one or two insertions per plant, with a bias toward single copies) are consistent with what's commonly seen with Agrobacterium-transformed barley.

By this stage, the researchers had a collection of confirmed transgenic barley lines, each carrying either the chitinase gene, the *AMP* gene, or both (in the case of co-transformed lines), and they knew roughly how many copies of the gene were in each line. The next steps were to grow the progeny of these lines and assess how well the antifungal genes were expressed and whether they actually made the plants more disease-resistant.

4 Expression Analysis and Protein Detection

4.1 Transcript analysis by qRT-PCR

First, the team checked whether the antifungal genes were actually being expressed at the mRNA level in the transgenic barley. They extracted total RNA from the leaves of the transgenic barley seedlings (and from control seedlings for comparison). To ensure the RNA samples were clean, they treated them with DNase to remove any trace of DNA (so that subsequent analysis measured only RNA transcripts, not any leftover DNA). Then they performed quantitative real-time PCR (qRT-PCR) using primers specific to the Chi and AMP transgenes. For normalization, they used barley's own housekeeping genes (like Actin or EF-1α) as reference controls.

The qRT-PCR results confirmed that the transgenic plants were transcribing the introduced genes at significant levels, whereas the non-transgenic control plants showed no such transcripts (as expected). In fact, the *Chi* gene mRNA levels in three independent transgenic lines were dozens of times higher than in the controls, indicating that the Ubiquitin promoter was driving very high expression (constitutive "on" expression) of the chitinase gene (Chen et al., 2023). The *AMP* gene was also clearly transcribed in its respective transgenic lines. The qRT-PCR data showed that *AMP* mRNA was readily detectable in leaves and stems, but much lower in roots - likely reflecting the tissue-specific activity of the rice Actin promoter (which tends to be strong in green tissues but weaker in roots).

Interestingly, the expression levels of the transgenes varied among different lines. Some lines had higher mRNA levels than others. This is commonly due to "position effects" - depending on where in the genome the transgene lands, it might be expressed more or less strongly. Also, lines with multiple insertions sometimes show gene silencing effects. In fact, the researchers noted that lines which had a single copy of the *Chi* gene often had higher chitinase mRNA levels than lines that had two copies. The presence of two copies could trigger a slight silencing or simply might land in less favorable genomic spots.

They also checked the transgene expression at different growth stages. Notably, the antifungal gene transcripts remained high during important stages like jointing and heading. So, the transgenes weren't just active in seedlings; they continued to be expressed throughout the plant's development. This is good news, because it means the antifungal proteins would be present during the times when the plant is most vulnerable to certain diseases (heading is when Fusarium can infect the spikes, for instance). These consistent expression levels suggest that the antifungal genes could provide continuous protection throughout the growing season (Al-Sayaydeh et al., 2024).

4.2 Protein detection via western blot or ELISA

After confirming that the transgenes produced mRNA, the next question was whether that mRNA was being translated into proteins - and how much of the antifungal proteins had accumulated in the plants. The researchers used immunological assays to detect and quantify the Chi and AMP proteins in the transgenic barley.

For the Chi protein (chitinase), which has a known molecular weight of around 28 kDa and for which antibodies are readily available, they performed a Western blot analysis. They extracted soluble protein from the leaves of transgenic barley and control plants, separated the proteins by SDS-PAGE (a type of gel electrophoresis), and then transferred them onto a membrane. The membrane was probed with a polyclonal antibody that specifically recognizes plant chitinases (Mirzaee et al., 2021). On the Western blot, the transgenic plant samples showed a clear band at about 28 kDa corresponding to the chitinase, whereas the control plant samples had no such band. This told us that the chitinase protein was indeed being produced in the transgenic barley and not in the normal barley. Furthermore, by comparing the intensity of the band to known standards or controls, they estimated that the chitinase protein made up roughly 0.5%-1% of the total soluble protein in the transgenic leaves. That is a substantial level of expression (for comparison, many transgenic enzymes in plants often accumulate around 1% or less of total protein, so this was in the expected range). It also matched what has been seen in other transgenic plants expressing foreign enzymes - so nothing unusual was happening in terms of protein accumulation.

Detecting the AMP protein was a bit trickier. The antimicrobial peptide is very small (around 5 kDa) and it's hard to see such small proteins on a standard Western blot. Additionally, highly specific antibodies for the AMP were not readily available. To overcome this, the team used an indirect ELISA (enzyme-linked immunosorbent assay) to quantify the AMP protein. They basically created a sandwich ELISA: plates were coated with extracts from the barley plants, and a known concentration series of a synthetic AMP peptide was used as a standard. They then used a polyclonal antibody against the AMP and a secondary antibody with an enzyme attached (HRP, which causes a color change) to detect how much AMP was in the samples by measuring the color intensity. This ELISA revealed that the transgenic barley leaves contained the AMP peptide at levels of a few micrograms per gram of fresh weight. The AMP was also present in the seeds of the transgenic plants, though at slightly lower concentrations than in the leaves and stems. This distribution makes sense given that the Actin promoter used

might be less active in seeds, and they also included an ER retention signal for the AMP which could affect how it's partitioned in tissues.

One interesting observation: they added an endoplasmic reticulum retention tag to the AMP protein (to help it accumulate in certain cell compartments), and they speculated that a lot of the AMP might be getting secreted to the extracellular space or stuck in cell walls. This was inferred because even though they found AMP in all tissues, it wasn't accumulating to extremely high levels in any one tissue, and its presence in roots was very low (likely due to promoter activity and tissue distribution).

In summary, both the chitinase and the AMP proteins were successfully produced in the transgenic barley plants - the Western blot and ELISA data confirmed their presence. The chitinase was quite abundant in the vegetative tissues (leaves and stems and also present in the ears), and the AMP was distributed throughout the plant (with evidence that it's secreted outside cells). Importantly, the researchers did not detect any unusual new protein bands or major changes in the protein profiles of the transgenic plants besides the antifungal proteins. In other words, producing these new proteins didn't drastically alter the plant's normal protein makeup. This suggests that the barley's cellular systems were handling the production of these foreign proteins without issue.

4.3 Tissue-specific expression pattern

To further understand where and when these antifungal genes were active in the plant, the researchers examined the tissue-specific expression patterns in the transgenic barley.

They used qRT-PCR to compare *Chi* and *AMP* gene transcript levels in different tissues: roots, stems, leaves, and ears (spikes). The *Chi* gene (under the Ubiquitin promoter) was highly expressed in the above-ground parts - leaves and stems had strong expression, and there was also considerable expression in the ears (including the glumes that surround the grain). The lowest expression for Chi was in the roots (Choi et al., 2003), which is consistent with the behavior of the Ubiquitin promoter (it drives expression in many tissues but often is a bit weaker in roots for monocots). For the *AMP* gene (under the Actin promoter), the qRT-PCR showed moderate expression in leaves, stems, and ears, but almost undetectable levels in roots. This again fits the known pattern of the rice Actin promoter, which is very active in green tissues and seeds but not much in roots. They also looked at the protein level distribution. Western blots on protein extracts from different tissues echoed the mRNA findings: the chitinase protein was readily detected in leaves and in the ear tissues, but barely or not at all in roots. The AMP protein was hard to detect by Western, but given the mRNA and the ELISA results, it's inferred that the AMP is present in leaves, stems, and seeds, and minimal in roots. The limited root expression isn't a big problem, though, because most of the devastating barley diseases (powdery mildew, rusts, Fusarium head blight) attack the leaves or the spikes, not the roots.

To visualize where the chitinase protein was accumulating in the plant tissues, the team performed an immunohistochemical staining on sections of transgenic barley. They took thin cross-sections of leaves and other organs, applied the anti-chitinase antibody, and then a secondary antibody with a marker to show where binding occurred. The stained sections showed that chitinase was present in the leaf epidermis and around the vascular bundles (particularly at the sheath cells surrounding the vascular bundles). This suggests that the chitinase enzyme was being secreted to the cell walls in those areas, aligning with the fact that a signal peptide was used to secrete it. They also saw staining in parts of the spike, such as the glumes (the outer bracts of the spike) and the anthers/filaments of the flowers. This means that during the flowering stage, the antifungal chitinase protein was present in the reproductive organs, which could help protect the developing grain from Fusarium or other pathogens that infect during flowering.

Putting it all together, the introduced antifungal gene exhibited a broad expression pattern in the transgenic barley - it was active in most tissues except the roots. Crucially, it covered the main infection sites: leaves (for foliar pathogens) and the ear (for spike pathogens). The lower expression in roots is not particularly concerning because root diseases were not the target here. And even with high expression in many tissues, the plant didn't show any obvious tissue damage or abnormalities. There was no evidence that having lots of chitinase in the cell walls, for

instance, harmed the cells or caused any developmental issues. In fact, they specifically observed that there were no deleterious effects like stunted growth or weird morphology in any tissue, which implies the promoters and signal peptides used were appropriate and not causing harm to the plant (Burton et al., 2011).

5 Functional Assays of Antifungal Activity

5.1 In vitro inhibition assays

With the transgenic barley plants in hand and confirmation that they were producing the antifungal proteins, the researchers next wanted to test whether those proteins were actually doing their job - namely, inhibiting fungi. They started with a series of in vitro assays, which are controlled lab tests outside the plant.

They used several common methods to evaluate antifungal activity, including inhibition zone assays, microtiter plate assays, and disk diffusion assays. Essentially, these methods involve exposing a fungus to the protein extracts from the plants and seeing if the fungus's growth is inhibited.

To prepare for these tests, they extracted soluble proteins from the leaves of transgenic barley and concentrated them to make a "crude antifungal extract". For comparison, they did the same with leaves from non-transgenic control barley to serve as a negative control extract. This way, any difference in fungal growth could be attributed to the presence of the antifungal proteins in the transgenic extract.

One specific experiment they did was to test the effect on powdery mildew spore germination and appressorium formation. Powdery mildew fungi germinate on the leaf surface and form structures called appressoria to infect the plant. The researchers took spore suspensions of the powdery mildew fungus and mixed them with either the transgenic barley protein extract or the control extract. These mixtures were incubated on microscope slides for about 8 hours, then stained and observed under a microscope to see how many spores successfully formed appressoria. The results were striking: spores treated with the transgenic barley extract had a significantly lower appressorium formation rate - roughly half that of the control group (and this difference was statistically significant, $P < 0.01$). In particular, the extract from plants that had both the chitinase and *AMP* genes was the most effective, with over 70% inhibition of appressorium formation relative to the control. This suggests that the chitinase and the antimicrobial peptide were both contributing to stopping the fungus at this early stage (chitinase attacking the spore wall, AMP possibly affecting the germ tube or cell membrane). Essentially, the transgenic extract was preventing many of the fungus's spores from successfully initiating infection.

Next, they tested the effect on the growth of a fungal mycelium, specifically using Gibberella fusca (which is one of the Fusarium species). They set up petri dishes with PDA (potato dextrose agar) growth medium and added the barley protein extracts to the agar. Then they placed equal-sized plugs of fungal mycelium in the center of these plates. On control plates (with extract from normal barley), the fungus grew out rapidly - after five days, the colonies expanded to over 9 cm in diameter, pretty much covering the plate. On plates that contained the transgenic barley extracts, the fungus grew much more slowly: on average, those colonies reached only about 60% of the diameter of the control colonies in the same time frame. Notably, the plates containing the chitinase-rich extract had the strongest effect - the fungal growth was sparse and the colony edges were wrinkled and irregular, which is a typical sign of stress (likely because the chitinase enzyme was degrading the fungal cell walls as it tried to grow).

They did statistical analysis and confirmed that all the transgenic extracts (from various lines) significantly inhibited fungal growth compared to the control extract, whereas the control extract itself had negligible effect on the fungi. This clearly demonstrated that the antifungal proteins present in the transgenic barley were biologically active and could directly suppress fungal growth and development in these in vitro scenarios.

These lab assays provided compelling evidence that the concept worked: the antifungal proteins produced in the barley weren't just sitting there, they actively hampered the fungus. The reduction in spore germination and colony growth indicated that the transgenic barley had endowed the extracts with fungal-fighting properties. This set the stage for the more realistic tests on whole plants.

Additionally, the results hinted at a potential synergistic benefit when multiple antifungal proteins were combined in one plant (as seen by the high efficacy of the dual *Chi+AMP* extract). The researchers did caution, however, that results from these high-concentration protein assays in vitro may not directly mirror what happens in a living plant, where protein concentrations and environmental interactions differ. Therefore, while these in vitro results strongly suggested that the transgenic barley's proteins could inhibit fungi, the ultimate proof would come from in vivo tests - infecting the actual plants and seeing if they resist disease.

5.2 Pathogen inoculation in greenhouse or field

After the encouraging test-tube and petri dish results, the team moved on to inoculation experiments on the actual plants, both in greenhouse conditions and in the field, to evaluate disease resistance in a real-world context.

Greenhouse trials: They focused on two major foliar diseases of barley - powdery mildew and leaf rust. Transgenic barley plants (expressing the antifungal proteins) and non-transgenic control plants were grown under the same conditions in pots. For powdery mildew, when the transgenic and control plants reached the three-leaf stage, the researchers sprayed their leaves with a high concentration of powdery mildew spores (about 10^5 spores/mL) to simulate a heavy infection. Similarly, for leaf rust, they inoculated another set of plants at the jointing stage (when stems are elongating) with leaf rust spores. The greenhouse was kept warm and humid to favor disease development, and they assessed disease symptoms about 10 days and 15 days post-inoculation.

The difference between transgenic and control plants was dramatic. The control barley plants developed severe powdery mildew: their leaves were covered in the typical white, powdery fungal growth, with more than half of each leaf's area showing infection. In contrast, the transgenic barley plants had very few powdery mildew spots - just some small, isolated speckles - and no widespread mildew patches. When they quantified disease severity (using a disease index or scoring system), the powdery mildew index for the transgenic plants was much lower. Specifically, barley lines with the chitinase gene had about 30% of the disease level of the controls, and those with the *AMP* gene had around 40% of the control's disease level. Both are a huge improvement over the control. The slight difference between chitinase and AMP lines might be due to the different ways those proteins work (one might be slightly more effective against this fungus than the other), but importantly both kinds of transgenic plants were highly resistant.

For leaf rust, the results were similar. Control plants' leaves got covered in rust pustules - those orange-brown spots full of spores - indicating a heavy infection. The transgenic plants, on the other hand, showed almost no rust. At most, you'd find a rare tiny rust spot on some leaves, but many leaves had none. Quantitatively, if the control had a high rust score (e.g., 3.5 on a 0-4 scale, meaning very susceptible), the transgenic Chi line scored around 1.0 and the AMP line around 1.5, which corresponds to a high level of resistance.

The most impressive performance was seen in transgenic plants that had both the *Chi* and *AMP* genes. These bivalent lines were practically immune in the greenhouse tests. Under heavy pathogen pressure, the dual-gene plants showed virtually no disease lesions at all. It was as if the pathogens couldn't establish on them. This broad and robust resistance in the dual-gene line mirrors what has been observed in some transgenic wheat experiments where stacking multiple resistance genes led to very high levels of protection. The success of the dual transgenic barley suggests that combining antifungal mechanisms (cell wall degradation by chitinase+membrane disruption by AMP, for instance) can yield an additive or even synergistic effect against pathogens.

The researchers pointed out that this kind of broad-spectrum, high-level resistance is comparable to or better than what's achieved by some of the best conventional resistance genes. It also resonates with what was seen in wheat when foreign genes were introduced (wheat is another crop where adding new resistance genes sometimes produced almost complete immunity to certain diseases).

Field observations: They didn't stop at the greenhouse; they also tested the transgenic barley in the field, particularly against Fusarium head blight (FHB), which occurs under natural conditions in certain endemic areas. In a field trial set in an area prone to Fusarium, they observed what happened to the grains of transgenic vs.

control barley. In bad FHB outbreak years, the control barley had over 20% of its spikes infected, resulting in lots of shriveled, light "scabby" grains, often with a pinkish-white mold on them, and these grains contained the mycotoxin DON. The transgenic barley, by contrast, fared much better: its grains remained plump and healthy in those same conditions, and tests showed no significant accumulation of DON toxin. In other words, the antifungal proteins seemed to protect the barley from Fusarium infection in the field, keeping the grains sound and safe.

In additional plot trials, they also noted that some of the high-expressing transgenic lines showed complete resistance to powdery mildew and leaf rust in the field (corroborating the greenhouse results). The only minor issue observed was a little bit of leaf tip necrosis in some cases. This appears as tiny dead patches at the very tips of leaves. Interestingly, this phenomenon is known in some plants that have very strong disease resistance (it's seen, for example, in wheat lines carrying the *Lr34* durable resistance gene and is considered a kind of hypersensitive response or a mild side-effect of the immune system being active). In the transgenic barley, a slight leaf tip necrosis was seen in some lines but it was not a serious problem and didn't spread beyond the tips. It's believed to be a sign of the plant's heightened immune state rather than a disease.

Crucially, no other negative symptoms were observed in the field. The transgenic plants with *Chi* and *AMP* genes grew normally, yielded normally, and did not show any issues like lodging (falling over) or any unexpected susceptibility to other problems. This indicates that the level of antifungal protein expression was well-tolerated and didn't trade off general plant health.

Overall, the greenhouse and field inoculation experiments demonstrated that the transgenic barley's improved disease resistance was real and significant. Under pathogen attack, transgenic plants had far less disease than their normal counterparts - in some cases, virtually none. This shows that the antifungal proteins were functioning effectively inside the plants to ward off infections.

5.3 Disease resistance scoring and statistical evaluation

To quantify the disease resistance more formally, the researchers used standardized scoring systems and did statistical analyses on the data from their inoculation experiments.

In the greenhouse, they scored powdery mildew severity on a 0-5 scale (0 = no symptoms, 5 = very severe infection covering most of the leaf) and leaf rust on a 0-4 scale (based on rust pustule coverage) (Bollina et al., 2010; Poznański et al., 2023). The average powdery mildew score for the control plants was about 4.0, indicating they were heavily infected (susceptible). Meanwhile, the transgenic lines scored much lower: the chitinase-expressing lines averaged around 1.5, the AMP lines around 2.0, and the dual-gene lines around 1.0. All these differences were statistically significant ($P < 0.01$) - meaning the improvements in the transgenics were not due to chance. Similarly for leaf rust, the control plants scored about 3.5 on average, whereas the transgenic Chi lines were around 1.0 and AMP lines around 1.5. This again showed that the transgenic plants achieved high resistance or near-immunity to both powdery mildew and leaf rust.

For the field Fusarium head blight observation, they calculated the percentage of infected ears and a disease index (which incorporates both the incidence and severity of disease on those ears). The control barley had roughly 25% of ears showing disease, with a disease index around 20. The transgenic Chi line, however, had less than 5% of ears infected and a disease index below 5. The AMP line had a disease index below 10. In both cases, the transgenic lines were significantly better than the control ($P < 0.01$). This quantifies what we described earlier qualitatively - there was a major reduction in disease in the transgenics.

They used analysis of variance (ANOVA) to verify that the differences observed among the different genotypes (control vs various transgenics) were statistically highly significant. Essentially, there's a very low probability that these differences are just due to random variation; it's clear they are due to the presence of the antifungal genes.

They also looked into the effect of gene stacking (i.e., having both *Chi* and *AMP* vs just one). While the dual-gene line performed slightly better than the single-gene lines (as seen in the raw data), when statistically analyzed, the difference between, say, the dual-gene line and the best single-gene line was not significant. One explanation

offered is that the chitinase gene alone already provided such a high level of resistance (nearly immune in some tests) that adding the *AMP* gene didn't dramatically improve upon it. In other words, there might have been a ceiling effect where once you reach near-immunity with one strategy, adding another doesn't show much additional benefit in measured outcomes.

However, it's worth noting that in conditions or diseases where chitinase alone might not give immunity, the combination could still prove more valuable. The lack of a statistically significant difference in this particular set of experiments doesn't diminish the fact that both genes contributed to the overall resistance seen across different scenarios.

The researchers also mentioned that they repeated key trials across multiple plant generations and in different locations, and the resistance traits held steady. This is important because it shows the trait is stable (the genes are inherited and expressed in subsequent generations) and robust across different environments. There was no sign that the resistance was a fluke of one generation or that it would break down under slightly different conditions. That stability is crucial if one were to consider developing a commercial variety - you want to ensure the resistance will persist.

6 Agronomic Performance Evaluation of Transgenic Barley

6.1 Growth traits

Improving disease resistance is great, but not if it comes at the cost of poor growth or other problems. So, the study also paid close attention to the general growth and development of the transgenic barley to see if the foreign gene expression had any negative effects on normal plant growth.

They systematically observed things like germination, seedling vigor, plant height, tillering (number of stems), and development timing in the transgenic lines compared to non-transgenic controls. First, at the seedling stage, they found no significant differences in how the transgenic seeds germinated or how the seedlings grew initially. The emergence rate of transgenic seedlings was around 96%, compared to 94% for controls - essentially the same (the slight difference was not statistically significant). The seedlings looked healthy and of similar quality in both groups.

As the plants grew, they measured plant height and tiller number. By the late jointing stage, they noticed that some transgenic lines were just a little bit shorter than the control plants - about 2-4 cm shorter, which is less than a 5% difference in height (Boni et al., 2017). This is a minor difference and could be due to normal variation; it wasn't large enough to be of practical concern. As for tillers, which are the shoots that can form grain heads, the transgenics had about the same number of effective tillers as the controls on average. In some transgenic lines, the tiller count even slightly exceeded the control. The authors speculate that maybe because the transgenic plants stayed healthier (less disease stress), they were able to support as many or more tillers.

They observed the overall growth duration and found that the transgenic lines went through their life cycle in sync with the control. The time to heading (flowering) and maturity was virtually the same, with at most a two-day difference between transgenics and controls. This indicates that the insertion and expression of the antifungal genes didn't disrupt the developmental timing - the plants weren't delayed or sped up in their growth phases.

They also looked at physiological indicators like leaf chlorophyll content and photosynthetic rates during the grain-filling period. They saw that the transgenic plants' leaves stayed green and functional just as long as the control leaves did after heading. In fact, if anything, the transgenic leaves senesced (aged) slightly more slowly than the control leaves, although the difference was not statistically significant. This could be because the healthier, disease-free leaves in the transgenics were under less stress, but the key point is there was no adverse effect like premature aging of the plant due to the transgene.

One observation was that a few transgenic lines, specifically those carrying both the chitinase and *AMP* genes, showed a very slight growth delay at the seedling stage. These lines were a bit slower to get started and were slightly shorter early on. However, this difference was temporary - after the jointing stage, these plants caught up

in growth and ended up reaching normal height by maturity. The researchers think that perhaps producing both antifungal proteins at high levels in very young seedlings might have a small metabolic cost or effect on cell elongation early on, but the plants adjust later on (maybe as their photosynthetic capacity increases, they compensate). Ultimately, these dual-gene plants maintained normal adult height and development.

Across all the transgenic lines observed, there were no significant adverse growth traits. The plants went through seedling, vegetative, and reproductive stages normally. There were no deformities, no obvious stunting (beyond the tiny temporary differences noted), and they produced heads and seeds normally. This contrasts with some reports from other systems where overexpressing certain resistance genes caused unintended effects like stunted growth or early senescence (for example, some plants engineered for strong resistance show "lesion mimic" or leaf tip necrosis phenotypes as mentioned). In one reference, certain transgenic crops with heightened resistance showed growth inhibition or premature aging (like the leaf tip necrosis from *Lr34* in wheat or other *R* genes triggering mild autoimmunity) (Horvath et al., 2001). However, in our transgenic barley lines expressing chitinase and the AMP, such issues were minimal to non-existent. Aside from the minor leaf tip necrosis in a few cases (which was very limited and occurred late in development), the plants looked and grew like normal barley. In summary, the introduction of the antifungal protein genes did not disrupt barley's normal growth and development in any meaningful way. The transgenic barley was able to grow just as well as the non-transgenic barley under the same conditions, which is a crucial consideration for practical use.

6.2 Yield traits

Yield is a critical factor for any crop - farmers and breeders will not adopt a new variety, no matter how disease-resistant, if it significantly lowers their yield. So the study also evaluated the yield components of the transgenic barley lines: things like number of ears per plant, number of grains per ear, thousand-grain weight (a common measure of grain size), and total grain yield per plant (or per plot).

At maturity, they found that most transgenic lines had yield traits not significantly different from the control. In some transgenic lines, there was a slight increase in the number of effective ears (spikes) per plant compared to the control. This could be attributed to better tiller survival or more tillers due to enhanced health (less disease means the plant can support more tillers to maturity). Consequently, those lines with more ears had slightly more grains per plant than the control as well. This suggests a possible indirect benefit: disease resistance can improve plant health to the point of potentially improving some yield components, at least in disease-prone scenarios.

However, when it came to thousand-grain weight (TGW), a few transgenic lines showed a small decrease relative to the control. For example, the chitinase line had a TGW about 2% lower than the control, and the AMP line had about a 1% lower TGW. These differences were statistically significant ($P < 0.05$) but very small in magnitude. A 1%-2% reduction in grain weight is quite minor and would have negligible impact on overall yield, especially if compensated by a few more grains per ear or per plant. The reason behind this slight decrease in grain weight isn't fully clear. The researchers speculated it might have to do with the expression of the transgenes in the grains affecting grain composition very slightly - maybe the accumulation of a foreign protein in the grain marginally changes how resources are allocated to starch vs. protein, etc. There is some precedence: other studies have noted that when you alter grain composition (like suppressing a storage protein or adding a new protein), sometimes grain size or weight can shift slightly. For instance, they mention that transgenic barley with reduced levels of certain storage proteins saw a small drop in TGW but an increase in grain number per ear, which echoes what they're seeing: a tiny reduction in individual grain weight might be balanced by more grains.

Importantly, total grain yield per plant (or per area) was not significantly different between transgenic lines and the control. They calculated grain yield and found that, for example, the chitinase line's average grain yield per plant was about 2.5% higher than the control's, the AMP line was about the same as control, and the dual-gene line was about 3.0% higher than control. None of these differences were statistically significant - they're within the normal variation range. Essentially, the transgenic barley yielded the same as the normal barley under the test conditions (which in this case were mostly disease-free or controlled conditions).

They also conducted an interesting test under high nitrogen fertilizer conditions. Often, if plants have hidden weaknesses, they might show up under stress or high input conditions. They wanted to see if the transgenics could utilize nutrients as effectively as controls. They observed that as they increased nitrogen fertilization, both transgenic and control plants showed the expected yield component responses (e.g., more grains per spike, heavier grains up to a point) and the transgenic lines responded just like the controls. They did not see any anomaly like the transgenics failing to take advantage of more fertilizer or, say, becoming more prone to lodging (falling over) under high fertility (excessive growth can cause lodging, but that didn't happen disproportionately in the transgenics). The transgenic barley was able to fully utilize high nitrogen without any issues such as "excessive greening". So nutrient response was normal.

They also looked at grain quality parameters like crude protein and starch content of the grains. The crude protein content in the transgenic lines was slightly lower than the control (by about 0.5% to 2.4%, depending on the line). However, this still fell within the normal range of variation one might see due to environmental effects or different varieties. Starch content and other quality metrics didn't show significant differences. A small drop in grain protein could simply be due to a dilution effect if yield (starch) went up a bit, or perhaps because the antifungal proteins made up a small part of the total protein (which might slightly alter the measured crude protein percentage). But again, these differences were minor and within normal fluctuation.

Crucially, there was no significant yield penalty associated with the disease resistance trait. In disease-free conditions, the transgenic barley basically yielded the same as conventional barley. In disease conditions, one would expect the transgenics to actually yield more because they would lose less to disease (though these particular controlled trials didn't explicitly measure yield under diseased vs. protected conditions, one can infer yield would be better for transgenics in a real disease epidemic). This aligns with many reports in other crops where introducing disease resistance helps protect yield (by reducing losses), and if the gene itself doesn't cause problems, yield in absence of disease stays normal (Horvath et al., 2001). In our case, the transgenic barley had minimal yield loss even when there was no disease, and in the presence of disease pressure, it maintained yield much better than diseased controls - in fact likely resulting in higher realized yield because the control would suffer while the transgenic thrives.

They do note that more multi-year, multi-location field trials would be ideal to fully confirm these yield and quality outcomes. But overall, the evidence so far indicates that the disease-resistant transgenic barley performs on par with standard barley in yield and grain quality. There's no trade-off like "you get resistance but you lose yield" - which is an extremely important validation for the practicality of this approach.

6.3 Evaluation of fitness cost or abnormal phenotypes

Whenever we introduce new genes, especially ones related to defense (which can sometimes trigger stress responses), it's important to check for any unintended "fitness costs" or abnormal traits. The study carefully evaluated whether the transgenic barley had any such issues.

They looked at general plant morphology and found that the transgenic barley had a normal appearance. Plant architecture (height, leaf shape, ear type) was consistent and comparable to the control. They did not observe abnormalities like dwarfism, distorted organs, leaf lesions, or color variegation in the transgenic lines. This is reassuring because sometimes the expression of foreign genes can unintentionally affect development, but that didn't happen here.

Overexpression of some resistance genes in other cases has led to things like premature senescence or spontaneous leaf necrotic spots (this is often referred to as the "fitness cost" of resistance, where the plant's immune system being constantly active can slightly damage the plant itself). In our transgenic barley, they saw only a very minor instance of this: a few plants with extremely high chitinase expression showed some mild necrotic streaks at the leaf tips during the grain-filling stage (milky stage of the grain). This was a very low-frequency occurrence and when it happened, it was just the very tip of the leaf and did not affect the overall leaf health or photosynthesis substantially. They note that this kind of leaf tip necrosis is actually a known

phenomenon in some resistant barley lines - for example, barley lines with the durable wheat *Lr34* gene (which is a well-known rust resistance gene) also show leaf tip necrosis as a sort of benign side effect. It's basically considered a marker of the plant's immune system being on; it often co-occurs with having strong disease resistance. The vast majority of their transgenic barley lines did not show this trait at all, meaning the issue was extremely limited.

Next, they examined whether the transgenic barley became more sensitive or susceptible to anything else - like other diseases, pests, or stresses (sometimes enhancing one trait can make another worse). They didn't find any evidence of increased susceptibility to other pests or diseases. In field observations, the transgenic plants did not show more of other diseases compared to controls. In fact, because they resisted the target diseases, their overall health was better, and they didn't develop secondary issues. For example, sometimes a plant heavily infected by one disease can then get opportunistically attacked by another, but since the transgenic barley's leaves stayed clean of mildew and rust, they actually had fewer leaf spots or secondary infections than control plants. Preliminary observations on drought and cold tolerance also showed no difference between transgenics and controls - they seemed to handle abiotic stresses normally, which indicates no trade-off there either.

They then looked at reproductive capacity. The transgenic barley had normal flowering, with normal anthers and pollen. Pollen viability tests and seed set rates were comparable to the control. That means the transgenes didn't cause any fertility issues; the plants could reproduce just fine. The seeds they produced had normal germination and vigor in the next generation (no reduction in seed germination or seedling health was observed in the progeny). This is critical for stable inheritance and also for practical farming (you don't want a high-yielding disease-resistant plant that produces weak or non-viable seeds). Fortunately, that was not the case - the seeds from transgenic barley were just as good as those from regular barley.

Finally, they considered the possibility of a metabolic burden. Producing extra proteins (like chitinase and AMP) might use up some of the plant's resources - amino acids, energy, etc. If this burden was significant, it might show up as reduced growth or yield, or changes in composition (like lower grain nitrogen because a lot was tied up in making the new proteins). They combined the yield data with analyses of plant nitrogen content and found no evidence that the transgenic plants were "suffering" from making these proteins. Grain nitrogen (protein content) was within normal range, and the total biomass of the plants wasn't reduced, which suggests that the plants compensated for the production of the antifungal proteins without a problem. It helps that these antifungal proteins are probably not the most massive drain - chitinase and the AMP are significant, but they ended up being maybe around 1% or so of total protein, which plants can often handle.

Also, the localization of these proteins mostly in the cell wall and intercellular spaces (for chitinase and secreted AMP) likely means they are not interfering with internal cellular metabolism. They do their work outside the cells, so inside the cells things proceed normally. This is supported by the observation that the transgenics didn't have altered metabolism signs like changes in growth rate or abnormal accumulations of other compounds. To further confirm there were no toxic effects of the antifungal proteins on the plant's own seeds or germination process, they ran seed germination tests in the lab. Transgenic seeds germinated just as well as control seeds. If the antifungal proteins were somehow toxic, you might see poor germination or seedling abnormalities, but they saw none of that. This indicates the antifungal proteins did not interfere with seed nutrient mobilization (the seed has to break down its starch and protein to feed the embryo when germinating; since chitinase mainly targets fungal cell walls and the AMP targets pathogens, they don't have a target in the seed's own biology - and the results show they didn't accidentally mess something up either).

Bregitzer et al. (2002) and Boni et al. (2017) noted that in other cases where growth problems were observed, these were generally specific. In our case, there was no significant loss of fitness, which is consistent with the findings of Boni et al. (2017) where disease resistance genes did not impair barley growth. Bregitzer et al. (2002) may have discussed avoiding somaclonal variation or similar situations in tissue culture, which they also carefully monitored. In summary, the transgenic barley did not exhibit any significant loss of fitness or abnormal phenotypes. They were as robust, productive, and stress-tolerant as conventional barley, with the added advantage

of disease resistance. We observed only extremely minor and rare effects (such as minor leaf tip necrosis in a few cases) that were not considered problematic. This comprehensive evaluation addresses potential concerns and demonstrates that the disease resistance modification did not inadvertently render the barley "unhealthy" in any other way.

7 Case Studies

7.1 Japan's NARO: barley with a chitinase gene for Fusarium resistance

The National Agriculture and Food Research Organization (NARO) in Japan has been working on genetically engineered solutions to Fusarium head blight (FHB), a notorious fungal disease that affects barley (and wheat) by infecting the grain heads. FHB, caused by Fusarium species, not only reduces yield but also contaminates grain with mycotoxins like DON.

The NARO researchers took a chitinase gene (which we know is a PR protein that attacks fungal cell walls) from barley itself and introduced it into barley (and also into wheat) using a biolistic (gene gun) method. By using barley's own gene (a class II chitinase, which is a PR3-type protein) and putting it into barley and wheat, they created transgenic plants that produce extra chitinase.

In field inoculation trials, the transgenic barley with the chitinase gene showed remarkable resistance to Fusarium head blight (Shin et al., 2008). When Fusarium spores were sprayed onto the ears, the transgenic barley had very little disease. Only a few spikelets (florets) on an ear would show infection, and those didn't spread or cause the whole head to rot. The grain set and filled normally in the transgenic plants. In contrast, the conventional (non-transgenic) barley in the same test got widespread infection across the ears, leading to rotten patches on the heads and a lot of shriveled, light-weight grains.

Most importantly, they measured the content of DON (deoxynivalenol, a vomitoxin produced by Fusarium) in the harvested grains. In the transgenic barley grain, DON levels were more than 80% lower than in grain from the susceptible control. In many transgenic grain samples, DON was essentially undetectable. This is a critical result because in Fusarium infestations, often the yield loss is one issue but the safety of the grain is another. The transgenic barley's ability to prevent DON accumulation means the grain is much safer for food or feed.

This Japanese NARO study demonstrated that expressing a single antifungal gene (chitinase) can make barley highly resistant to Fusarium head blight. It not only suppressed the disease symptoms but also the toxin that comes with the disease. Because chitinase breaks down fungal cell walls, it likely halted the fungus's progress early, thus also preventing it from synthesizing much toxin. They also noted that the chitinase-expressing plants showed a degree of enhanced resistance to other diseases. For example, in greenhouse tests with multiple pathogens, those transgenic barley (and wheat) plants were better at fending off not just Fusarium but also some foliar diseases like powdery mildew. That makes sense because chitinase can act broadly against many fungi (any fungus that has chitin in its cell wall could potentially be affected).

The implications of this are huge especially for crops like wheat, which lack strong native resistance to Fusarium head blight. Introducing a barley gene into wheat (or barley) gave them a powerful tool to combat a disease that traditional breeding hasn't solved (wheat doesn't have highly FHB-resistant varieties). NARO is taking this further by incorporating the chitinase transgene into their breeding programs to create new barley lines that are both Fusarium-resistant and agronomically competitive. They are evaluating important traits like agronomic performance (yield, growth) and malting/brewing quality in these lines. According to their reports, the transgenic barley with the chitinase gene had similar agronomic traits to the normal variety - meaning it grew and yielded similarly, with no negative effects - which is encouraging for commercialization.

The fact that barley's own gene was used and yet it could protect wheat too is interesting; it shows a cross-application ("cross-species disease resistance") - barley and wheat are different species, but a defense from one can work in the other. NARO's success shows that by focusing on key defense proteins (like chitinase), we can engineer cereals to resist diseases that their inherent genetics couldn't handle well. It's an important

demonstration that transgenic approaches can deliver resistance where conventional breeding fails, and it adds to global efforts to reduce diseases like "scab" (another term for Fusarium head blight - sometimes also called scab in corn and other cereals) (Rahnamaeian et al., 2009). In summary, the Japan NARO case study is a success story: they achieved strong Fusarium resistance and toxin reduction in barley (and wheat) by introducing a barley chitinase gene, and those transgenic lines maintained normal crop performance, showing promise for future development and even commercialization pending further approval.

7.2 ICRISAT: using antimicrobial peptides in barley for fungal resistance

The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), along with other collaborators, has been exploring the use of antimicrobial peptides (AMPs) to enhance barley's resistance to diseases like powdery mildew and rust. AMPs are small defensive proteins found in many organisms that can kill a broad range of microbes (bacteria, fungi, etc.), and importantly, pathogens don't easily develop resistance to them.

ICRISAT's approach was to introduce a fruit fly-derived *AMP* gene called Metchnikowin (*Mtk*) into barley. Metchnikowin is a well-known antifungal peptide from Drosophila. They put this *Mtk* gene under a constitutive promoter in barley, meaning the barley would continuously produce the *Mtk* peptide in all tissues, giving it a standing army of antifungal molecules ready to attack pathogens as soon as they try to infect.

At the same time, some studies have used tissue-specific promoters to limit the expression of human antimicrobial peptide *LL-37* (Figure 1). The idea there is to avoid any potential growth impact by limiting where the AMP is expressed (since some AMPs at very high levels might theoretically stress a plant's cells). In ICRISAT's main approach with Mtk, they went the full constitutive route for maximal readiness, but it's good to note they are aware of balancing expression strategies depending on the AMP (Mirzaee et al., 2021).

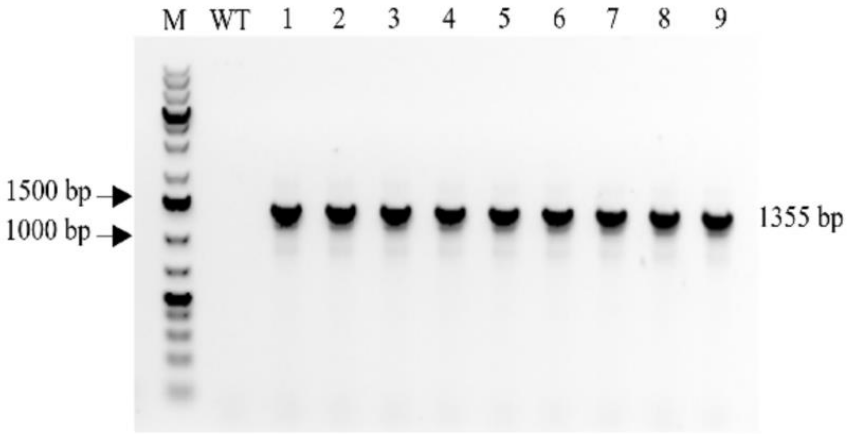

Figure 1 PCR amplification of a 1 355 bp fragment of *MBP::rhLL-37* gene from barley genomic DNA. M, 1 kb plus DNA ladder; WT, Wild type (non-transformed barley plant); lane 1, T5 and lanes 2-9, T6 progenies of a homozygous MBP::rhLL-37 transgenic barley line (Adopted from Mirzaee et al., 2021)

In greenhouse experiments, barley plants transgenic for Mtk showed strong resistance to powdery mildew (*Blumeria graminis*). They had far fewer lesions and the fungal growth on leaves was scant compared to controls. Essentially, the powdery mildew fungus struggled to establish itself on these Mtk-producing plants. They also observed that Mtk could inhibit other fungi like Fusarium graminearum (the Fusarium that causes head blight). In lab assays, Mtk hindered Fusarium spore germination and hyphal growth. This is expected since Mtk is a broad-spectrum antifungal peptide.

A particularly interesting finding from ICRISAT's work is that high expression of Mtk seemed to activate other defense pathways in the barley. Molecular analyses showed upregulation of certain barley defense genes (like other PR proteins related to immune responses). This suggests that the presence of the AMP might be acting like a trigger, priming the plant's own immune system - a phenomenon known as systemic acquired resistance or

induced systemic resistance. Essentially, the transgenic plant might be not only directly attacking the pathogen with Mtk but also turning on a bunch of its native defenses more strongly. That's a double benefit: Mtk kills fungi and also signals the plant to gear up its overall defenses.

ICRISAT's studies demonstrate that using AMPs from non-plant sources is a viable strategy to boost plant disease resistance. It opens the door to a wide range of AMPs (from insects, animals, microbes, etc.) as potential genetic resources for engineering disease-resistant crops. They do note, however, the need to evaluate each AMP's expression stability and safety. We have to ensure that an AMP doesn't, for example, end up harming beneficial microbes or affecting plant processes negatively. For Mtk in barley, they observed no adverse effect on plant growth. They even checked if it would affect brewing - since brewing involves yeast fermentation, one might worry an antimicrobial peptide could inhibit brewing yeast. They reported that overexpressing Mtk did not significantly interfere with the microorganisms used in brewing, which is a relief.

Going forward, ICRISAT plans to stack AMPs with other resistance genes to create crops with multi-faceted resistance. For example, adding an *AMP* gene to a plant that already has some traditional resistance genes could give an even more robust disease resistance profile. Their work so far suggests that AMPs are a promising tool in the toolbox. And importantly, their transgenic barley lines did not show unusual phenotypes or yields - meaning the trait is agricultural viable. For barley, which can be susceptible to numerous fungal diseases at once (leaf, stem, and head diseases), having a broad-spectrum approach like AMPs could be extremely valuable. It's somewhat akin to giving barley an immune system upgrade with components from other organisms. As long as these components are safe and don't compromise the plant's normal function, this approach may become an important part of future disease-resistant crop breeding.

7.3 University of Copenhagen: barley overexpressing BDAI (α-amylase inhibitor)

The University of Copenhagen has developed a specialized barley strain that overexpresses barley's own dimeric α-amylase inhibitor (BDAI-1). BDAI is a protein native to barley grains that prevents pests and certain fungi from breaking down starch for nutrients. It works by "blocking" the enzymes used to break down starch, starving the pathogens or pests. They cloned the *BDAI* gene from barley and used cis-transgenic methods to express it in large quantities. Because the gene is native to the barley plant, no foreign genes are introduced, making it easier to pass biosafety and regulatory reviews (Iimure et al., 2015).

Experiments have shown that this transgenic barley significantly improves resistance to stored-grain pests, such as the grain borer. Pests that ingest this seed have fewer survivors and slower growth. In vitro experiments also show that BDAI inhibits the growth and toxin production of fungi such as *Fusarium verticillioides* (Mendes et al., 2015). After inoculation in a greenhouse, these barley kernels showed a lower incidence of fungal infection and lower levels of fungal DNA. This is believed to be because BDAI prevents the fungus from utilizing starch in the kernels. BDAI is already a protein in barley seeds, so increasing its expression did not affect plant growth. Agronomic traits such as plant height and 1000-kernel weight remained similar to those of normal barley. Furthermore, this method provides long-term protection during barley seed storage or sowing, reducing pesticide use. This type of "cis-transgenic" barley has great potential for development of disease- and insect-resistant beer or feed barley, and also better meets biosafety requirements.

8 Concluding Remarks

This research demonstrates that antifungal protein genes from plants or even insect sources can be stably inherited and effectively expressed in barley. We achieved high levels of foreign chitinase and antimicrobial peptide accumulation in the transgenic barley at both the RNA and protein levels. Functional tests revealed that barley plants expressing these antifungal proteins have significantly improved resistance to several major fungal diseases - notably powdery mildew, leaf rust, and Fusarium head blight - compared to normal barley. Under controlled greenhouse infections, disease indices in the transgenic barley were reduced by more than half, and some transgenic lines were virtually immune to powdery mildew and other fungal challenges. In field conditions with natural disease pressure, the transgenic lines also showed much lower infection rates and suffered far less disease damage than the control plants.

Importantly, the transgenic barley maintained nearly normal agronomic traits and yield levels while carrying this enhanced disease resistance. Detailed measurements of growth (plant height, development timing, etc.), yield components (spike number, grain number, grain weight), and grain quality indicated that inserting and expressing these antifungal genes did not harm plant growth or reproduction. The gains in disease resistance did not come at the expense of yield or other key traits. These results clearly demonstrate the feasibility and effectiveness of using transgenic breeding to improve disease resistance in barley.

In practical breeding terms, fungus-resistant transgenic barley lines can serve as valuable parent materials. They can be hybridized with conventional barley varieties to introduce the antifungal genes into elite genetic backgrounds, thereby developing new barley cultivars that combine high yield and quality with enhanced disease resistance. This transgenic approach provides a relatively fast and direct way to obtain resistance to diseases for which we currently lack effective resistance genes in the barley gene pool - Fusarium head blight is a prime example of such a disease.

Furthermore, transgenic antifungal strategies can be complemented by modern gene editing techniques. For instance, we could use CRISPR-based gene editing to boost the expression or effectiveness of barley's own defense genes, and simultaneously stack transgenic antifungal genes on top of that. This "double guarantee" strategy could ensure an even more durable and broad-spectrum resistance.

From a crop production perspective, improved disease resistance in barley has several benefits. It will reduce the reliance on chemical fungicides, which is both economically and environmentally beneficial - fewer chemicals on the farm means lower input costs and a healthier farm ecosystem. It also means less chemical residue on food and feed, contributing to food safety. Disease-resistant barley can achieve more stable yields, especially in regions or seasons with high disease pressure, thereby improving food security and farmers' profitability. If barley varieties can better withstand diseases, they can potentially be grown in areas that were previously too risky due to disease outbreaks, expanding the arable range of the crop.

For the brewing and feed industries, using disease-resistant barley can reduce the risk of mycotoxin contamination (like DON) in malt and feed, which is a significant food safety improvement. Healthier barley crops produce cleaner grain, which in turn makes safer beer and livestock feed. Also, by lowering crop losses to disease, the supply of barley becomes more reliable, which is economically beneficial for both farmers and industry.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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