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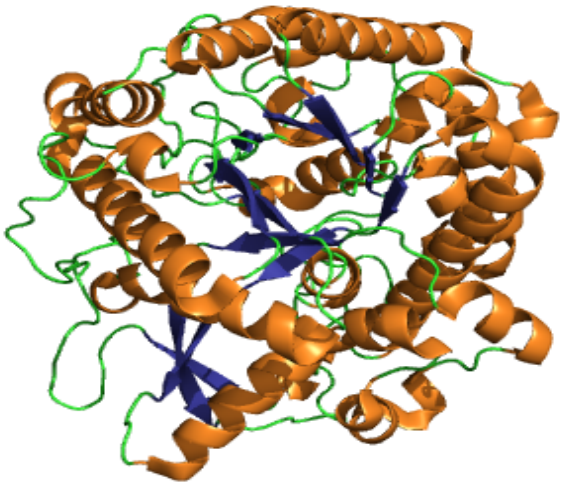

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Abstract

Myrosinases (Thioglucoside glucohydrolase, EC 3.2.3.147) are present in Brassicales plants. This enzyme can hydrolyze glucosinolates into various products some of which are toxic against pathogens and insects, thus serving as an important defense mechanism. Many vegetables and oil crops contain the myrosinase-glucosinolate system making studies on myrosinase very significant to understand how this type of plant protection has evolved. The performed experiments concerned expression of myrosinases in *Pichia pastoris* aiming to test their functionality based on recombinant enzyme activity and to compare different expression and cell breakage systems. Two myrosinase cDNAs from *Arabidopsis thaliana* (*TGG1* and *TGG4*) had been cloned earlier into the pPIC3.5 vector behind an alcohol-inducible promoter and with a histidine-tag added to the 3´-end (C-terminus of the protein). *Pichia* cells had earlier been transformed with the constructs and the transgene integrated into *Pichia* genome by homologous recombination. The purity and functionality of the *TGG1* wild-type enzyme and *TGG4* wild-type and mutated enzymes were analyzed by a coupled colorimetric activity assay, protein determination, and SDS-PAGE. In addition, the expression of *TGG1* and *TGG4* were determined in *Arabidopsis* transformants containing GUS fusions with myrosinase gene promoter. GUS staining in these plants lines was investigated after treatment with beneficial *Bacillus* bacteria and challenge with pathogens. The basis of the study was to analyze the myrosinase activity and function necessary for plant protection and from the analysis we found under stress condition plants showed a more tissue specific myrosinase expression compared with control.

Key Words: *Pichia pastoris,* Myrosinase, Glucosinolate, Brassicaceae, Myrosin cells, Ascorbic acid, Thioglucoside glucohydrolase (TGG), β-glucuronidase (GUS).

Introduction

Plants constitute a diverse group of living organisms that are found all over the world and are exposed to a wide variety of herbivores, pathogens and other biotic stresses (1). Plants are directly or indirectly, the most important source of nutrients for all living organisms (2). Compared with animals, plants lack an adaptive immune system, but share large parts of an innate immunity system, to improve protection against attackers, plants have evolved a stunning array of structural, chemical, and protein-based defense mechanisms which can detect invading organisms and stop them before they are able to cause extensive damage (2). The glucosinolate–myrosinase system is an example of such a defensive mechanism that has evolved against insect pests and pathogens.

The glucosinolate-myrosinase system is a significant defense-associated feature of *Brassicales* and has been most studied in *Brassicaceae* family species (3, 4). Myrosinases (Thioglucoside glucohydrolase, sinigrinase, EC 3.2.3.147) are present in specialized cells known as myrosin cells which are distributed throughout certain plant tissues (5, 6, 7). Myrosinase has been observed only in plants that contain glucosinolates, but different opinions concerning their localization in plant tissues (8). A special kind of idioblast cells, myrosin cells, seems to harbor the enzyme in certain plant tissues. It is also clear that the activity of myrosinase varies depending on the species, cultivar, developmental stage and plant organ (10). High activity is normally found in seeds and seedlings (10). The major function of myrosinases are as defense-associated enzymes that hydrolyze glucosinolates into various compounds, some of which are toxic and involved in plant defense against pathogens and insects (8). There are indications that myrosinases also may involved in the control of plant growth and development, though (43).

The secondary metabolites glucosinolates (mustard oil glucosides) are sulphur-linked sugar conjugates that are present in *Brassicales* plants (9). Glucosinolates share a structure consisting of a *β-D*-glucopyranose linked as a sulfur ether to a (*Z*)-*N*-hydroximinosulfate ester, but vary in the side chain

(R group) derived from different amino acids, and more than 100 glucosinolates are known. These compounds are present in certain organs throughout the plant, but the nature and level depends on the organ, the developmental stage and some external factors (11, 12). Special giant cells (S-cells) seem to contain high levels of glucosinolates at least in certain plant tissues. The hydrolysis products of glucosinolate provide defense against various attackers (9). When an herbivore or a pathogen damages plant tissues, myrosinases catalyze the glucosinolate degradation that in turn produces toxic compounds (Fig. 1) like nitriles, isothiocyanates, epithionitriles and thiocyanates (8, 9). These toxic compounds have potent deterrent activities (8, 9). The glucosinolate-myrosinase system thus plays a very important role in *Brassicaceae* plants to prevent damage caused by most pathogens and insects (8, 9). However, a few insects and pathogens have through co-evolution overcome this defense and glucosinolates may also serve as feeding cues creating a considerable problem in crop production.

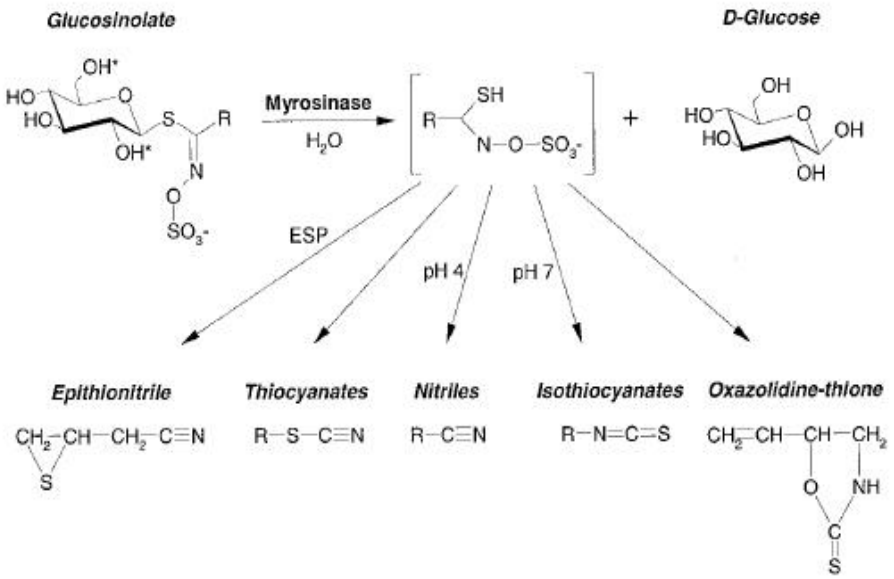


Fig. 1. Hydrolysis of glucosinolates by myrosinase produces D-glucose and a thiohydroximate-O-sulfate molecules that will rearrange to various products depending on the local environment.

Generally it seems that glucosinolates and myrosinases are stored located apart from each other in the intact plant (6). Accordingly glucosinolate breakdown products are produced only when insects or pathogens are

wounding tissues, where the resulting mechanical damage enables myrosinases and glucosinolates to interact (6).

Myrosinases catalyze the hydrolysis reaction of glucosinolates by cleaving the thio ether bond, resulting in D-glucose along with an aglycone (Fig. 2). This aglycone is unstable and will rearrange to produce various products like isothiocyanates (10). It is known that some additional factors may direct the degradation into certain directions. Proteins like epithio specifier protein and nitrile specifier proteins have been identified that under certain conditions can influence the product formation (13). The hydrolysis products are often toxic due to their electrophilic nature causing alkylation of nucleophiles present in membranes, protein and DNA (14).

Interestingly, certain hydrolyzing products of glucosinolates for example nitriles, isothiocyanates (ITC) and thiocyanates may be propitious as drug candidate in various diseases (15). Accordingly, all *Brassica* (mustard/cabbage) plants contain functional food properties. The consumption of these nutritive plants can contribute with health benefits if consumption increases (15).

A diet containing fruits and vegetables that contain vitamins and glucosinolates can counteract cardiovascular diseases and other degenerative diseases in humans (16, 17). Epidemiological studies have demonstrated that a diet rich in *Brassica* vegetables can reduce the risk of getting heart diseases, diabetes and some types of cancer such as colon, lung, rectum and bladder cancer (16, 18). On the other hand, high levels of the glucosinolate derived isothiocyanates may disturb iodine metabolism in thyroid glands leading to goiters (19). In higher concentrations isothiocyanates may also cause liver damage (20).

The hydrolysis reaction of glucosinolates may be affected by different non-protein factors like temperature, pH, the presence of cofactors etc. (12). Ascorbic acid (AA) may influence the catalytic activity of myrosinases. In low concentrations, AA activates the enzyme while in high concentrations inhibitory effects are observed (20). On the other hand, dehydro-ascorbic acid, the oxidation product of ascorbic acid, does not stimulate myrosinase

activity (20). Myrosinases can hydrolyze glucosinolates in the absence of ascorbic acid but the reaction is then generally slower. Ascorbic acid stimulates e.g. radish myrosinases activity ten-fold as compared to the control (20).

The yeast *Pichia pastoris* expression system was formulated by scientists at Salk Institute Biotechnology/Industry Associates (SIBIA). This expression system was used for high-level expression of recombinant proteins. As an expression system, it has many benefits such as protein processing, protein folding and post-translational modifications typical of eukaryots. It is simple, quick and less expensive to use as compared to other eukaryotic expression systems like Baculovirus and insect cells or mammalian cell cultures. As *P. pastoris* is yeast, it has in common advantages of molecular and genetic manipulations with *Saccharomyces* but with the advantage of 10- to 100-fold higher heterologous protein expression levels. These characteristics make *Pichia* very useful as a protein expression system.

A reporter gene system known as GUS (β-glucuronidase from *E. coli*) is often used to analyze the activity of a promoter of a gene of interest by fusing the promoter with GUS and transforming plants with the construct. The activity of the promoter can then be measured in the transformants either in a quantitative way or via visualization of its activity in different tissues on the basis of the β-glucuronidase activity producing a colored product from specific substrates (21). The myrosinase genes have been shown to exist in multiple forms (i.e. gene families) in various *Brassica* species (22). In *Arabidopsis thaliana* four functional myrosinase-encoding genes (*TGG1*, *TGG2*, *TGG4* and *TGG5*) have been reported (23, 24). In addition, other proteins like PEN2 and pyk10 has been claimed to be myrosinases (25, 26). Table 1 shows the different myrosinases and gene identifiers characterized from *A. thaliana.* In this study, the expression pattern of *TGG1* and *TGG4* myrosinase in Arabidopsis was investigated using promoter: GUS reporter plants.

Table 1. Six myrosinase genes in *Arabidopsis thaliana* as indicated by their common abbreviations and gene identifiers.

Myrosinase gene	Gene identifier	Reference
TGG1	*At5g26000*	TAIR (www.arabidopsis.org)
TGG2	*At5g25980*	TAIR (www.arabidopsis.org)
TGG3	*At5g48375*	TAIR (www.arabidopsis.org)
TGG4	*At1g47600*	TAIR (www.arabidopsis.org)
TGG5	*At1g51470*	TAIR (www.arabidopsis.org)
TGG6	*At1g51490*	TAIR (www.arabidopsis.org)

Aim of the project

The aim of this project was to increase the knowledge about myrosinases. This was done by expressing recombinant *Arabidopsis* myrosinases in *P, pastoris,* to better understand the enzyme function of myrosinases and the role of different myrosinases. Moreover, the expression of *TGG1* and *TGG4* upon *Bacillus* treatment and subsequent challenge with the plant pathogen *Alternaria* were investigated. Mutant activity studies were done using TGG4 variants mutated at the active site and substrate binding site to compare the stability and functionality of the enzyme.

Materials and Methods

Recombinant protein expression in *P. pastoris*

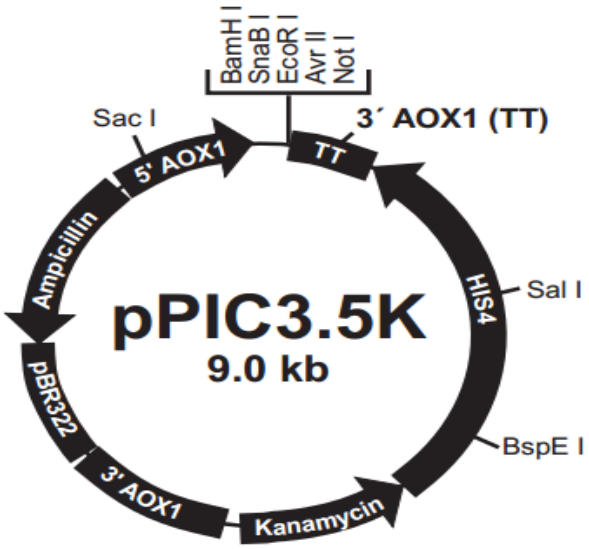
P. pastoris is methylotrophic yeast, often used for expression of recombinant proteins as it can perform post-translational modifications and express a reasonable amount of protein (27). The expression system utilized in the present study contains a strong inducible promoter which is activated by alcohols like methanol. This promoter drives alcohol oxidase enzymes that end up into peroxisomes (28). The peroxisomes can account for 80 % of the total cell volume and the alcohol oxidase enzyme can constitute up to 30 % of the total protein in the yeast cell when yeast are grown on methanol (29). *P. pastoris* contain two types of alcohol oxidases abbreviated as AOX1 and AOX2. The brunt of alcohol oxidase activity is due to AOX1, thus the protein to be conveyed is often coupled to the *AOX1* promoter (30). As an expression vector *P. pastoris* has a drawback in that the protein expressed is heavily glycosylated with N-linked oligosaccharide chains of mannose (8-14 mannose residues per side chain) but this is much less than the yeast *Saccharomyces cerevisiae* (50-150 mannose residues per side chain) (31).
The *P. pastoris* strain used was GS115, and in combination with the pPIC3.5 (design by invitrogen^{TM}, life technologies) expression vector. It comprised the *AOX1* promoter and leads to intracellular expression of the recombinant protein, unless it contains a secretory leader sequence.

P. pastoris inoculation and biomass accumulation

The yeast culture was grown to high density before the expression of recombinant protein was initiated. As a carbon source glycerol granted a faster growth and did not show any negative effect on transcription compared to glucose (32). Thus it is easier to induce expression of recombinant proteins when changing to methanol containing media (32).
One clone from *TGG1* (AT5G26000) and *TGG4* (AT1G47600) both wild type and three mutants of TGG4 such as E418Q (active site), Q211A (sulphate binding site) and I278A (ascorbate binding site) were added in 5 ml BMGY medium, a glycerol containing media prepared by 10 gm yeast

extract (Dickinson and company, USA) and 20 gm peptone (Duchefa Biochemie, USA) in 700ml distill water and then autoclaved. Just before inoculation 100 ml of 1M sodium phosphate (E. Merck, Germany) buffer pH 6.00, 100 ml of yeast nitrogen base solution, 2ml of 0.02% biotin (Duchefa Biochemie, Netherland) and 100 ml of 10% glycerol were added. Then the culture was grown overnight at 28°C in a shaking incubator. When a satisfying turbidity was achieved, 5 ml was transferred to a 500 ml Erlenmeyer flask containing 150 ml BMGY and grown at 28°C in the same shaking incubator.

In the project we used the pPIC3.5K plasmid (Fig. 2) was used for the constructs. The plasmid was linearized by digesting with *Sal1* restriction enzyme to stimulate recombination into the host chromosome after transformation into *Pichia*. Multiple integrations of the target gene may occur in the *Pichia* genome and this can be tested for using increasing antibiotic level.



Comments for pPIC3.5K:
9004 nucleotides

5´ *AOX1* promoter fragment: bases 1-937
5´ *AOX1* primer site: bases 855-875
Multiple Cloning Site: bases 938-968
3´ *AOX1* primer site: bases 1055-1075
3´ *AOX1* transcription termination (TT):
bases 981-1314
HIS4 ORF: bases 4242-1708
Kanamycin resistance gene: bases 5471-4656
3´ *AOX1* fragment: bases 5850-6607
pBR322 origin: bases 7689-7016
Ampicillin resistance gene: bases 8694-7834

Fig. 2. The above figure showed the plasmid pPIC3.5K to construct TGG4 mutants.

In the project mustard myrosinase purified from *Sinapis alba* seeds was used as template for TGG4 mutants constructs. The three dimensional structure of the mustard enzyme has been resolved at high resolution and

```
1MYR          -------------------------DEEITCQENNPFTCGNTDGLNSSSFEADFIFGVAS 35
TGG4          MAIPKAHYSLAVLVLLFVVVSSSQKVCNPECKAKEPFHCDNTHAFNRTGFPRNFTFGAAT 60

1MYR          SAYQIEGTIGRGLNIWDGFTHRYPDKSGPDHGNGDTTCDSFSYWQKDIDVLDELNATGYR 95
TGG4          SAYQIEGAAHRALNGWDYFTHRYPEKVP-DRSSGDLACDSYDLYKDDVKLLKRMNVQAYR 119

1MYR          FSIAWSRIIPRGKRSRGVNQKGIDYYHGLIDGLIKKGITPFVTLFHWDLPQTLQDEYEGF 155
TGG4          LSIAWSRVLPKGRLTGGVDENGITYYNNLINELKANGIEPYVTIFHWDVPQTLEDEYGGF 179

1MYR          LDPQIIDDFKDYADLCFEEFGDSVKYWLTINQLYSVPTRGYGSALDAPGRCSPTVDPSCY 215
TGG4          LSTRIVEDYTNYAELLFQRFGDRVKFWITLNQPFSLATKGYGDGSYPPGRCTG----CEL 235

1MYR          AGNSSTEPYIVAHHQLLAHAKVVDLYRKNYTHQG-GKIGPTMITRWFLPYNDTDRHSIAA 274
TGG4          GGDSGVEPYTVAHNQLLAHAKTVSLYRKRYQKFQGGKIGTTLIGRWFAPLNEFSELDKAA 295

1MYR          TERMKQFELGWFMGPLTNGTYPQIMIDTVGARLPTFSPEETNLVKGSYDFLGLNYYFTQY 334
TGG4          AKRAFDFFVGWFLDPLVYGKYPTIMREMVGDRLPEFTPEQSALVKGSLDFLGLNYYTTQY 355

1MYR          AQPSPNPVNATNHTAMMDAGAKLTYINASGHYIGPLFESDGGDGSSNTYYYPKGIYSVMD 394
TGG4          ATDAPPPTQLNAITDARVTLGFYRNGVPIGVVAPS------------FVYYPPGFRQILN 403

1MYR          YFKNKYYNPLIYVTENGISTPGS--ENRKESMLDYTRIDYLCSHLCFLNKVIKEKDVNVK 452
TGG4          YIKDNYKNPLTYITENGVADLDLGNVTLATALADNGRIQNHCSHLSCLKCAMKDG-CNVA 462

1MYR          GYLAWALGDNYEFNNGFTVRFGLSYINWNNVT-DRDLKKSGQWYQKFISP---------- 502
TGG4          GYFAWSLMDNYEFFGNGYTLRFGMNWVNFTNPA-DRKEKASGKWFSKFLAK---------- 511
```

Fig. 3. Amino acid residues in TGG4 suggested to be involved in different functions as suggested from crystallized mustard myrosinase ("1MYR", PDB) are shown in color given as: Disulfide bridges, Glucose binding, Aglycone binding, Zn^{2+} binding, Ascorbate/SO_4^- binding, Active site

is the only known plant myrosinase structure. Myrosinase is a member of the glycosidase family I (http://www.cazy.org) where many proteins have been crystallized, and the reaction mechanism studied in more detail. The high degree of sequence identity between the mustard and Arabidopsis myrosinases was used for homology modelling and prediction of the relevant amino acid residues in TGG4 using Modeller. The sequence alignment for the TGG4 used for designing mutagenesis experiment is shown in the Fig. 3.

TGG1 and TGG4 induction in *Pichia pastoris*

To induce expression of the *AOX1* promoter coupled protein it is necessary to remove the BMGY medium and exchange it to a methanol-containing BMMY medium which was same like BMGY medium but it contains 50 ml 5% methanol (E. Merck, Germany) and 50 ml of autoclaved water instead of 100ml glycerol (33, 34). When the culture reached approximately OD_{600} of 1.3-1.5, the culture was centrifuged at 5,000 rpm for 10 min at room temperature in 50 ml Falcon tubes, the supernatants discarded and the remaining pellet was resuspended in BMMY to OD_{600} ~1. High concentrations of methanol might have toxic effects or decrease the growth of the yeast, thus 0.5% methanol (final concentration) was applied in the culture for induction (33, 34). Fresh methanol was added on a daily basis to a concentration of 0.5 % to recover the loss due to evaporation and consumption. The culture was grown in a Fernbach flask at 28°C in a shaking incubator. The mouth of flask was covered with cheesecloth and fresh methanol was injected using a syringe. It is crucial to maintain good aeration of the culture because alcohol oxidase has poor oxygen affinity (30).

Harvesting of yeast cells

It was known that the recombinant protein showed intracellular expression. Hence yeast cells were harvested by centrifugation at 5,000rpm for 10 min using 250 ml or 500 ml plastic bottles. The supernatant was removed and the pellet resuspended in sterile water. Once again cells were centrifuged at

5,000rpm for 10 min and the supernatant removed. After that the pellet was stored at -20°C until used.

Crude cell extract preparation

The cell pellet was weighed and suspended in start buffer (1: 1.5 w/w). The start buffer also contained protease inhibitors like 1 mM PMSF (Sigma, USA) and 1% of protease inhibitor cocktail (Thermo Scientific) to stop protein degradation. The resuspended cells were transferred to 2 ml tubes half filled with a bio spec product zirconium beads (Ø 0.5 mm). The cells in the tubes were disrupted using a bead beater. Shaking the tubes at maximum thrust made the beads rupture the tough yeast cells and homogenize the solution. The process was switched between 1 minute with beat beating and 1 minute sample cooling in an ice bath. This step was repeated 5 times to assure good disruption of the yeast cells. The tubes that contained disrupted yeast cells were centrifuged at 12,000 rpm for 10 min and the supernatant collected in plastic tubes. The supernatant was centrifuged at 15,000 rpm for 10 min at 4°C using a SS-34 rotor. This step was performed in order to remove cell debris that precipitated at the bottom of the tube. This process was repeated until all visible debris was removed. To remove smaller debris, the supernatant was filtrated through a 0.45 µm filter. After filtration 200 µl of crude extract sample was saved for activity assays while the rest was stored at 4°C.

Immobilized metal-ion affinity chromatography (IMAC) for purification of poly-histidine tagged recombinant proteins

IMAC is a chromatography technique used to separate proteins on the basis of their reversible binding between certain amino acids and metal ions (35). Histidine, tryptophan and cysteine have natural affinity to metal ions, but one can also generate affinity by combining histidine residues to a protein using a so called his-tag (35). The histidine residues can also bind to bivalent cations such as Zn^{2+}, Co^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+} and Ni^{2+} (35). These bivalent cations containing matrixes can be used to separate the recombinant protein from native proteins due to metal-affinity. Imidazole is one of the chemicals that can be used in reverse protein binding to the ligand because it

also has metal-ion affinity (35). Purification of recombinant protein was done by using 1 ml HisPur^{TM} Cobalt spin column (Thermo Scientific). These columns were pre-packed with cobalt as ligand and were stripped and recharged after each use.

A Gilson pump with a flow rate of 1 ml min^{-1} was used for the purification of crude protein samples. The crude extracts were collected from approximately 250 to 300ml of Pichia cell culture where the mass of cells was around to 3 to 5 gm. The column was equilibrated with 5 ml of equilibration buffer before running the pre-treated yeast crude extract. Then the column was washed with 7 ml of wash buffer again. Unspecific proteins were eluted with 5 ml elution buffer A. The flow-through was collected in a 15 ml falcon tube. The cobalt ligand affinitive proteins were eluted with elution buffer B and collected in 1 ml fractions. In this way 10 fractions were collected for every sample. The whole procedure was performed at room temperature. These fractions were preserved at 4°C and later enzyme activity and protein concentration was analyzed. After use the column was washed with 5 column volumes equilibration buffer and stored at 4°C.

Myrosinase enzyme activity assay employing sinigrin as substrate

When myrosinase hydrolyzes the substrate (here sinigrin (Sigma Aldrich, Germany), 2-propenyl glucosinolate) the sulphur ether bond is cleaved resulting formation of aglycone and glucose (36). The products are usually unstable but these vary and depend on the glucosinolate type and environment. An enzyme activity assay was chosen that depended on the more stable product glucose using a GOD-POD method. The glucose oxidase (GOD) oxidized the released glucose into gluconic acid and hydrogen peroxide. Hydrogen peroxide reacts with 4-aminophenazone and phenol in the presence of peroxidase (POD) to produce a red/violet quinineimine dye (Glucose GOD/PAP manual, RANDOX). This means, both the catalyzing agents GOD, POD plus 4-aminophenazone and phenol were included in the glucose reagent from RANDOX.

Glucose + O2 + H_2O === GOD===> Gluconic Acid + H_2O_2

$2H_2O_2$ + 4-aminophenazone + Phenol ==POD==> Quinoneimine + $4H_2O$

The protein sample was blended with 50 mM Na-citrate (Duchefa Biochemie, Netherland) pH 6.0 to a make final volume of 25 µl and incubated at 37°C for 5 min. The sample was heated to 37°C before adding 62 µl sinigrin (12.5 mM) then vortexed and incubated at 37°C again for 30 min. To stop the reaction, the sample was heated at 95°C for 5 min and cooled. 250µl of Glucose reagent (Eli Tech clinical system, France) was added to each sample after cooling and then incubated at 37°C for 15 min. A 200 µl aliquot from each sample was transferred to a 96 well plate to analyze absorbance at 550 nm with a microplate reader (FLOUstar OMEGA). Using glucose from a glucose test kit, a standard curve was made to calculate the amount of released glucose from the sample. To calculate the myrosinase activity the following formulas were used:

Total activity in X units (mmol glucose min^{-1}) = average of A550*F*dilution factor/30 min

Specific activity in Y mmol glucose min^{-1} mg $protein^{-1}$ = total activity / total protein

F = obtained from standard curve.

Protein concentration determination by the Bradford method

In the Bradford assay the color of the test sample depends on the protein concentration. The dye Coomassie brilliant blue G-250 (Merck, Germany) changes its absorbance maximum from 465 nm to 595 nm when it binds to basic amino acids (arginine). This binding with protein converts the color of the dye from reddish brown to blue. The intensity of blue color reflects the protein concentration in the sample (37).

The sample was analyzed using a microtiter plate protocol at 595 nm with a microplate reader (FLOUstar OMEGA). Before performing analysis with samples, Bovine serum albumin (Fermentas) was used to make a standard

curve with known protein concentration. The standards concentrations were 0.5, 1, 2, 4, 6 and 8 μg ml^{-1}.

SDS-PAGE analysis of proteins

The SDS-PAGE technique is used to separate proteins on the basis of their subunit molecular mass (38). Proteins are blended with sodium dodecylsulfate (SDS) to form negatively charged denatured complexes that are size fractionated through the gel (38). Dithiothreitol was added to the samples to break inter- and intra-molecular disulphide bridges and thus disrupt any quaternary structure and release subunits. This also allows the protein to form more uniform complexes with SDS (1.4 gram SDS for each gram of protein) (39).

Protein samples were mixed with 5 μl loading dye (200 μl loading buffer supplied by Novex, USA + 10 μl 1M DDT product of Invitrogen). The mixture was heated at 95°C for 5 min to achieve denaturation and adequate binding in the presence of excess SDS. Then samples were centrifuged briefly and loaded on a (4-12 %) readymade gel supplied by C.B.S Scientific which was fixed on a vertical running chamber. The running chamber was filled with 1X running buffer. A voltage of 150 mV was run through the gel for 1.5 hour. The gel was then fixed in fixing solution for 5 min and incubated in Coomassie blue solution for at least 4 hours to visualize the protein. The gel was de-stained with de-staining solution to get visible and clear bands. For better visualization the gel was washed with distilled water and stored in water.

Determination of myrosinase activity in the presence of ascorbic acid

Ascorbic acid serves as an activator-inhibitor of myrosinase and is probably utilized as a nucleophile in the hydrolysis reaction (36). Ascorbate has dual effect functioning as a stimulating or inhibiting agent depending on concentration (40). It also has some adverse effects on the GOD-POD assay by inhibiting the formation of quinineimine dye when it reacts with

hydrogen peroxide (34). Although we cannot get reliable absolute values in the presence of high concentrations of ascorbate, still some information about the relative differences between myrosinases can be obtained.

For assays 50 mM Na-citrate pH 6.0 was added to the 2ul (0.2% corresponds to IMAC purified fraction) of protein sample and incubated at 37°C for 5 min. Pre-heated different concentrations of ascorbic acid (Sigma Aldrich, China) were added to the sample to a final volume of 25 µl before 62 µl of pre-heated 12.5 mM sinigrin were added. After adding the sinigrin, the samples were mixed and incubated at 37°C for 30 min. Further handling was as described above. Each time fresh ascorbic acid solutions were made before performing the assay and the concentration of ascorbic acid utilized in the assay were 0.25, 0.5, 0.75, 1, 1.5 and 2 mM.

Seed sterilization and treatment of plants with *Bacillus* and *Alternaria*

In this project we also used *TGG1* (1-3-12) and *TGG4* (4-2-4) *Arabidopsis* lines for expression analysis of these myrosinase genes. The respective promoter regions of up to 2kb was cloned pJIT166 after removal of the 2x35S promoter sequence by digesting with *Kpn*1 and *Hind*III restriction sites to produce the expression cassettes. The map of plasmid used for TGG GUS constructs was developed by kunling et al. but still unpublished (Kuenling et al. unpublished).

Seeds from transformants containing *TGG1*and *TGG4* were wrapped with Miracloth and tied with different color clips for individual identification. About 50-70 seeds from each line were immersed in 70 % ethanol (Solveco, Sweden) for 1 minute before transfer to 10% bleach (10 ml chlorine (Colgate-Palmolive S) + 90 ml water+5 µl Tween-20 supplied by Merck, Germany) for 5 minutes. The bleach solution was removed and the seeds rinsed in sterile water 5 minutes three times. For growing seeds on solid medium, seeds were kept on MS agar plates in growth chamber for 10 days. Roots should be visible after 3 days. When plants had grown a little bit bigger in size, 4 plants from each line were lined up in square MS-Agar plates and kept in the growth chamber. Four square MS-Agar plates for

TGG1 (1-3-12) and four plates for *TGG4* (4-2-4) were prepared. An aliquot (10 µl) of *B. amyloliquefaciens* (10^7 ml^{-1}) bacterial stock solution was added to each plant approximately 2 cm below the root tip. The fungal pathogen *Alternaria brassicicola* was grown on PDA plates at 22^{O}C, 16/18 light and dark photoperiod for 3-4 weeks. The spores were scraped into water and filtered through Miracloth to remove mycelium. The spore count was determined by use of a haemocytometer under a compound microscope. Fresh fungal spore suspension 5×10^5 ml^{-1} was used to inoculate plant leaves. To each leaf 5 µl of fungal suspension was added.

Two plates were treated with *Bacillus amyloliquefaciens* when the plants were 10 days old and placed back in the growth chamber again. Ten days later one plate with bacteria and one plate without bacteria from each line were challenged with the pathogen *Alternaria*. For the experiments one plate lacked any treatment as control, one plate with *Bacillus*, one plate with *Bacillus* and *Alternaria* and one plate with only *Alternaria* for both *TGG1* (1-3-12) and *TGG4* (4-2-4). All plants were transferred for staining separately according to treatment after one day of pathogen treatment. The alterations in the plant tissues caused by myrosinases expression with or without treatment were observed by microscopy.

GUS histochemical staining with X-gluc

TGG1 (1-3-12) and *TGG4* (4-2-4) plants were immersed in staining solution prepared by 0.1M sodium phosphate (E. Merck, Germany), 10mM EDTA (VWR International, Belgium), 0.1% Triton X-100 (Sigma Chemical Company, USA), 1mM potassium ferricyanide (Sigma, USA), and 0.1M X-gluc. The chemical form of X-gluc is 5-bromo-4-chloro-3indoxyl-beta-D-glucuronide cyclohexylammonium salt prepared by Gold Biotechnology, (http://www.goldbio.com/open.htm). The plants were incubated overnight with staining solution at 37^{O}C and then washed with several changes of 50 % ethanol until tissues become clear. Approximately 12 hours incubation period was used between each 50% ethanol change in room temperature.

Results

Expression analysis of myrosinase enzyme in *Pichia pastoris*

The main objective of this project was to express TGG1 and TGG4 wild type and three TGG4 mutant myrosinase enzymes in the host *P. pastoris* to analyze the properties of the enzymes. For this reason wild-type and mutant myrosinase were isolated from *Pichia* cells, and purified using an IMAC gravity column. After purification, myrosinase activity was measured as the release of glucose from the substrate sinigrin and the specific activity mentioned in (Table. 2).

Table 2. Myrosinase activity of IMAC purified fractions and crude extracts of TGG1and TGG4 wild-type and TGG4 mutant1 (E418Q) and mutant2 (Q211A) are presented. The table shows the specific activity of myrosinase of TGG1 and TGG4 wild-type crude but not for various IMAC fractions of wild-type and mutant strains because the protein values obtained from Bradford assay were below the standard. The fractions with serial numbers such as 8 and 9 correspond to the IMAC fractions that showed myrosinase activity.

Sample	IMAC fraction	Released glucose ($\mu M\ ml^{-1}$)	Specific activity ($\mu M\ min^{-1} mg\ protein^{-1}$)	Protein ($mg\ ml^{-1}$)
TGG1 (wt)	crude	46.5	2	24.5
TGG1 (wt)	8	4.26		
TGG1 (wt)	9	2.81		
TGG4 (wt)	crude	58.02	32	1.82
TGG4 (wt)	4	23.58		
TGG4 (wt)	5	24.14		
TGG4 (E418Q)	crude	40.45		
TGG4 (E418Q)	8	32.46		
TGG4 (E418Q)	9	14.43		
TGG4 (Q211A)	crude	24.975		
TGG4 (Q211A)	7	5.83		
TGG4 (Q211A)	8	22.48		

Separation and characterization of myrosinase by SDS-PAGE

The SDS-PAGE analytical technique was used to estimate the relative subunit molecular mass of the proteins. The position of bands in the gel given by various fractions and crude extract of TGG1 and TGG4 (wild-type and mutants) were compared with a protein size standard to determine the size and purity of myrosinase in various samples (Figures 4 and 5). The clear bands in the identified location determine myrosinase on the basis of theoretical myrosinase mass, but minor bands found in other locations proved that the collected IMAC fractions were not highly purified. Alternatively there may be a degradation of myrosinase, or alternative post-translational modifications, that result in minor bands. Thus further purification is needed for getting the highly purified protein needed, e.g. for crystallization. The purity of the peak fractions was estimated to 95% or more which is more than sufficient for the catalytic assay conducted here.

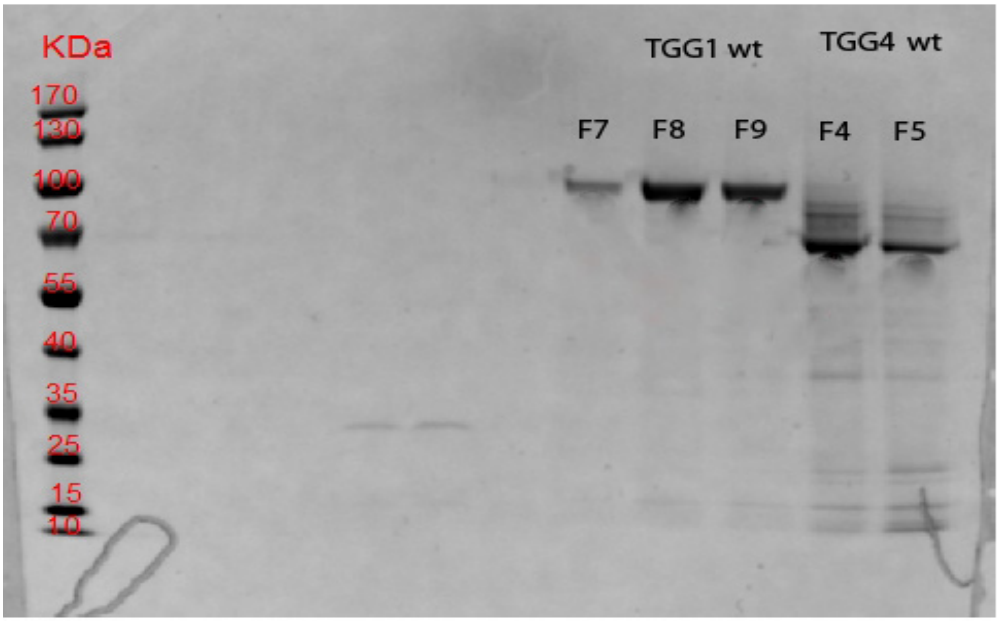


Fig. 4. SDS-PAGE analysis of wild type TGG1 and TGG4 IMAC fractions. In the picture fraction 7, 8 and 9 represent the wild type TGG1 IMAC whereas fraction 4 and 5 represent the TGG4 IMAC.

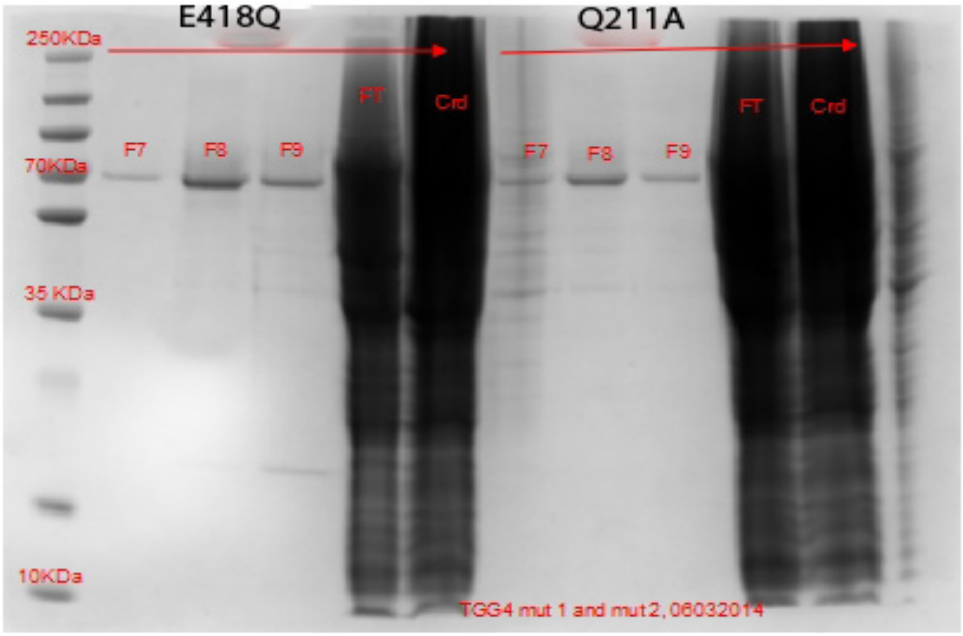


Fig. 5. SDS-PAGE gel picture of various IMAC fractions (F7, F8 and F9), flow through (FT) and crude extract (Crd) of TGG4 mutant1 (E418Q) and mutant2 (Q211A). The major bands in the purified fractions represent TGG4.

Analysis of effects of ascorbic acid on TGG4 mutant enzymes

Ascorbic acid effect on myrosinase was used to study the ability of various TGG4 to interact with ascorbate. In the project we made two unique preparations for the TGG4 wild-type and TGG4 mutants (E418Q, Q211A). IMAC fractions of TGG4 wild-type and mutant forms were tested with different ascorbic acid concentrations (Fig. 6). The results showed a small effect of ascorbic acid on the mutant forms compared with the wild-type enzyme, and that high concentration of ascorbic acid inhibited myrosinase as expected.

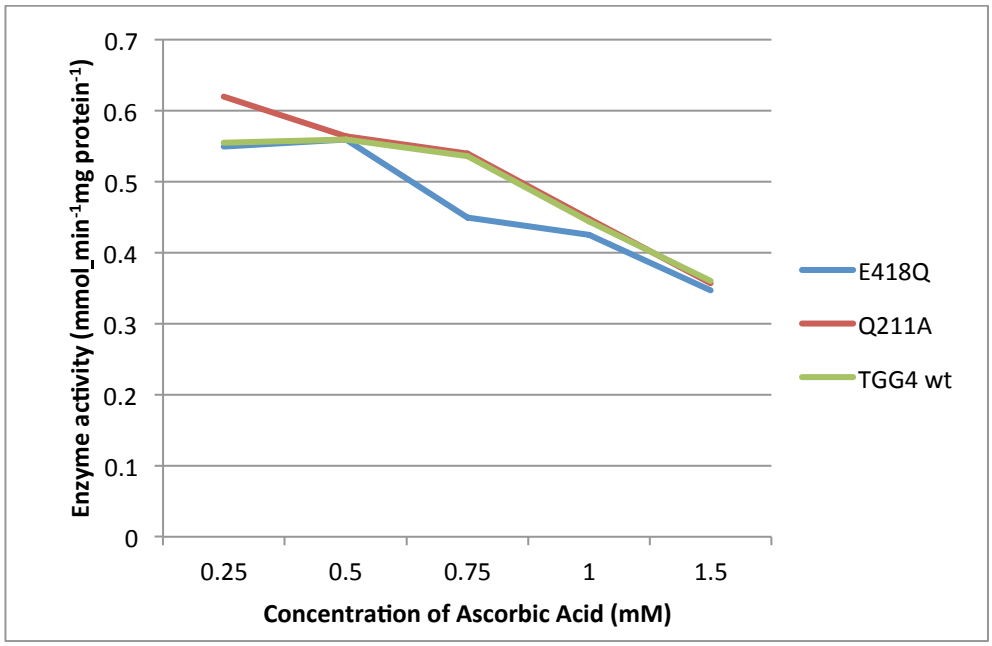


Fig. 6. Myrosinase-ascorbic acid assay of wild-type and mutant TGG4 enzyme. Two unique enzyme preparations were used for the assay gives similar results, one of which is depicted.

Myrosinase genes expression in TGG1 and TGG4 upon *Bacillus* treatment and subsequent challenge with pathogens

To study the expression of the activity of myrosinase genes in different tissues, a promoter: GUS histochemical staining was performed. *TGG1* and *TGG4* GUS lines were treated with *B. amyloliquefaciens* and then challenged with the pathogen *Alternaria* to study if beneficial and pathogenic microorganisms affect the myrosinase expression. The expression of myrosinase was found in roots of TGG4 GUS lines and leaves of TGG1 GUS lines (Figures 7-10). Figure 7 and 8 show myrosinase expression in leaves of TGG1 plants treated with *Alternaria*, *Alternaria* + *Bacillus*, *Bacillus,* and without any treatment, respectively. Alternaria-treated leaves gave high expression compared with other treatments (Figure 8). TGG4 expression was confined to root tips and adjacent areas (Figure 9 and 10). In this case *Alternaria* and Bacillus-treated TGG4 plants showed good GUS expression but other two treatments did not show any GUS activity.

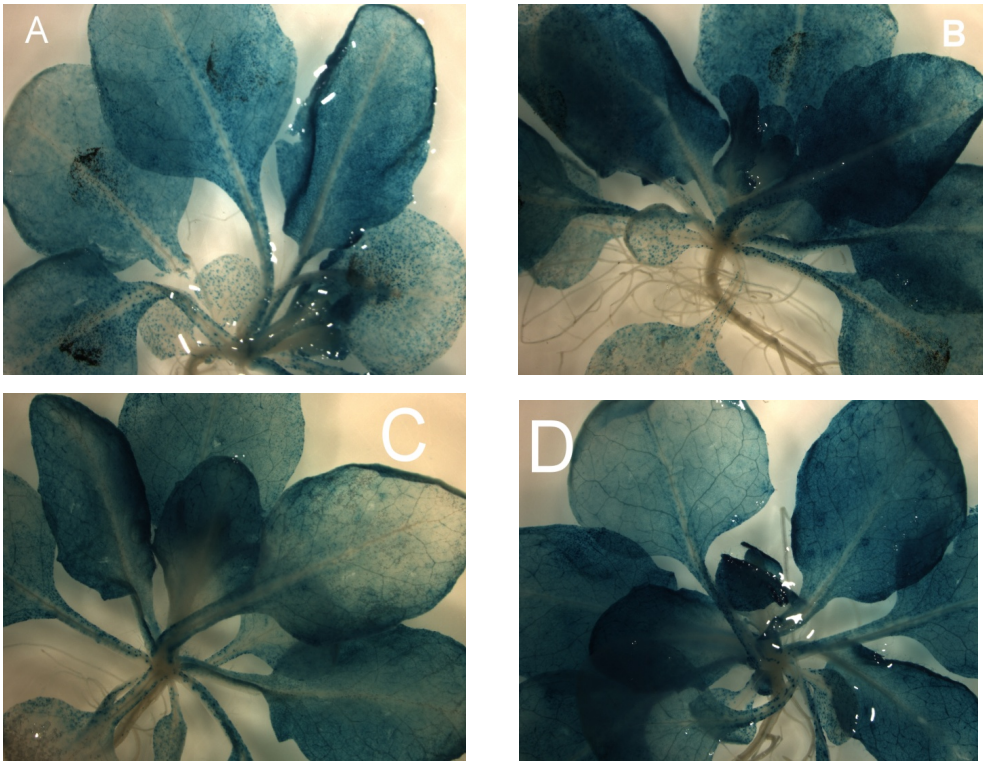


Fig. 7. Expression of TGG1: GUS line. A, B, C and D represent leaves treated with Alternaria, *Alternaria* + *Bacillus*, *Bacillus,* and without any treatment, respectively.

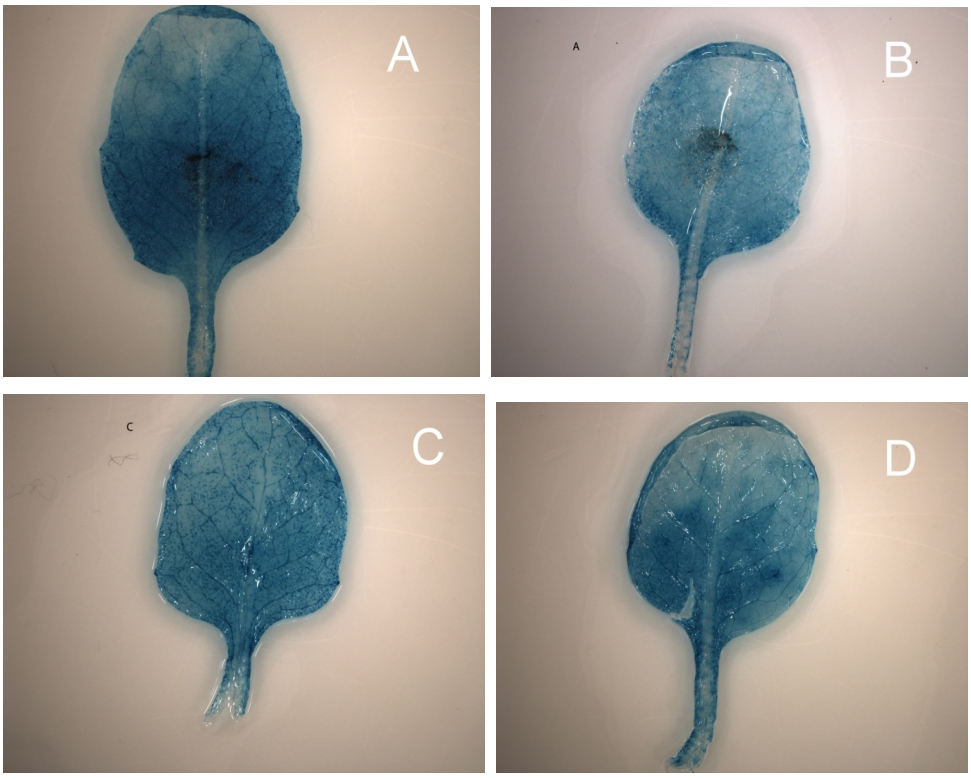


Fig. 8. Single leaf from each treatment collected from TGG1: GUS plants shown in figure 8 using the same order as Fig.7.

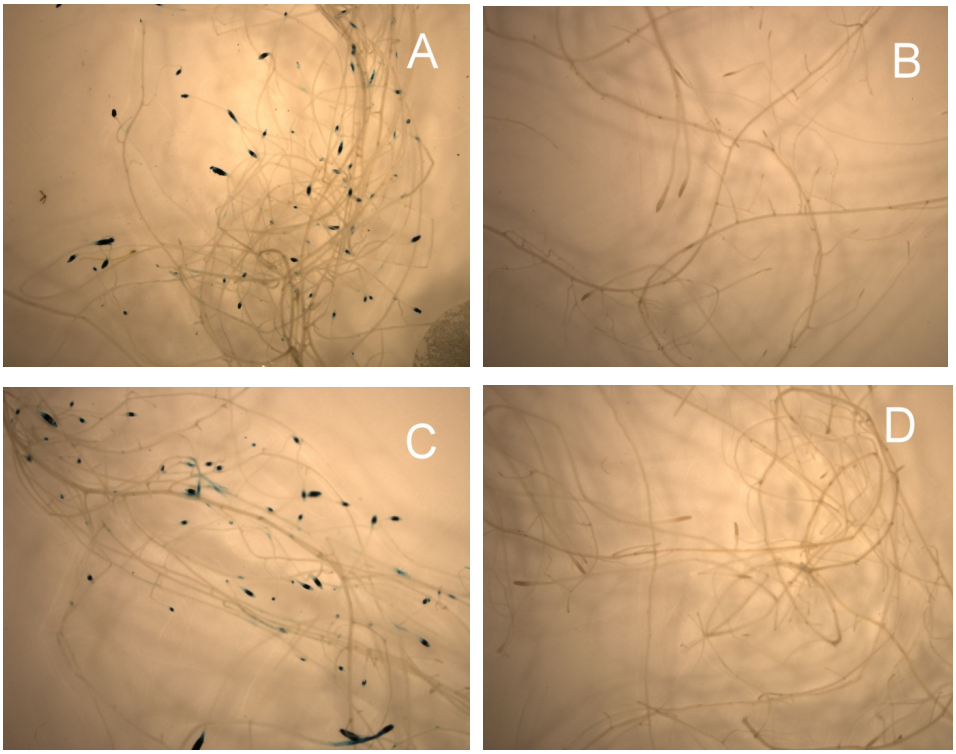


Fig. 9. Expression of TGG4: GUS line. A, B, C and D represent root pictures of TGG4: GUS line treated with *Alternaria*, *Alternaria* + *Bacillus*, *Bacillus,* and without any treatment, respectively.

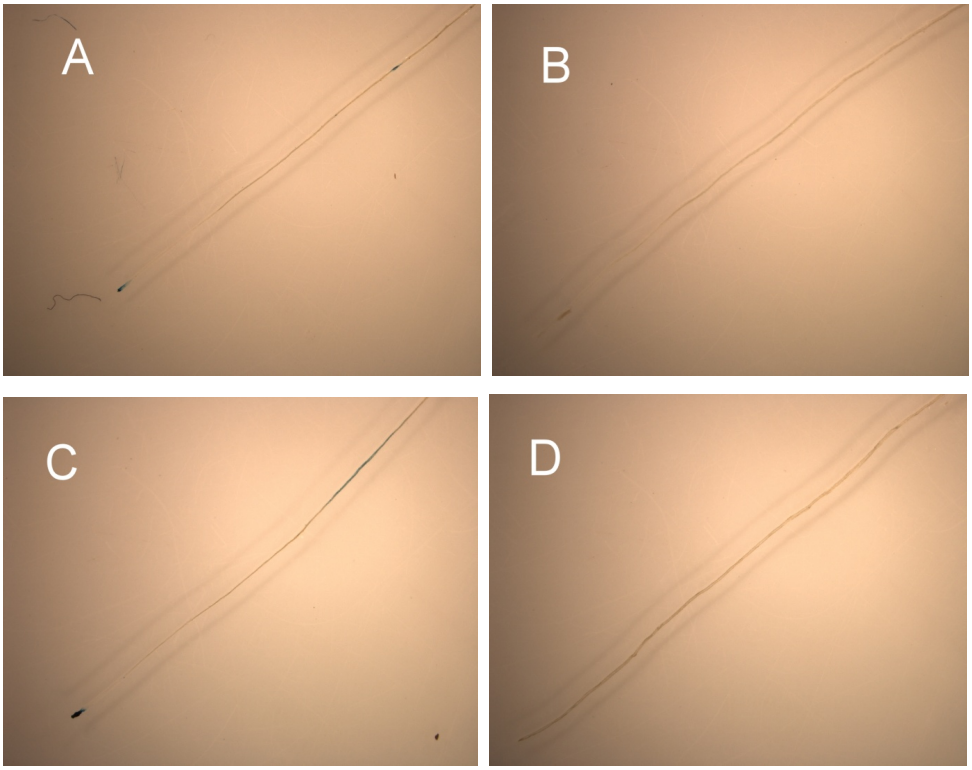


Fig. 10. Image of single root tip from each treatment of TGG4 ordered as in Fig. 9.

Discussion

Goal of this study were to express wild-type and mutant myrosinase in the host *P. pastoris* to test their functionality based on activity and to identify the role of different myrosinases.

The myrosinase enzyme activity was measured using a coupled enzyme assay in which release of glucose from the glucosinolate was determined using a fixed incubation time. The incubations were performed at a fixed pH and temperature, where incubation time was adjusted according to the research protocol. TGG1 wild-type and TGG4 wild-type showed good myrosinase activity on the basis of released glucose, such as 46.5 μmol ml^{-1} for TGG1 and 58.02 μmol ml^{-1} for TGG4, where as TGG4 mutant1 (E418Q: active site) and TGG4 mutant2 (Q211A: sulphate binding site) showed some activity but TGG4 mutant3 (I278A: ascorbate binding site) activity was below the sensitivity of the assay. The TGG4 mutants suggested to be involved in different functions as suggested from crystallized mustard myrosinase were shown in (Figure 3). The protein value of TGG1 wild-type and TGG4 wild-type were measured as 24.5mg/ml and 1.82mg/ml respectively, but the protein values from all IMAC fractions were below the standard. It might be due to the lack of maximum recovery of protein elution from the column. In column purification approximately 5ml of crude samples were loaded in the column and collected similar amount as eluted. Here it is also necessary to mention that the enzyme assay from wash and flow through collected from IMAC column purification did not show enzyme activity. Thus less protein obtained in eluted fractions might be due to target protein still stuck on the column. Because of the unstable nature of TGG4 mutant proteins there were problems in protein determination, especially in the case of purified fractions of mutants that were either below the standard or sometime no enzyme activity. In this experiment wild type TGG1 and TGG4 crude extract protein levels could be measured, but all other fractions and mutants were below the standard for Bradford. It might be due to losing high amounts of protein during the column purification. Thus in this study I could not measure the specific activity of the enzyme but in SDS-gel analysis the presence of myrosinase was identified (Figure. 4 and 5) by comparing with the theoretical mass of TGG1 and TGG4.

The SDS-PAGE analysis demonstrated the myrosinase protein quality and size. The position of tentative TGG1 band in SDS-PAGE corresponded to approximately 82 kDa, while 76 kDa is the theoretical mass of TGG1 amino acid chain. On the other hand TGG4 (wild-type and all mutants) showed SDS-PAGE bands just below 70 kDa, and the theoretical mass of TGG4 is 62 kDa. These discrepancies are probably due to glycosylation of the TGG proteins. Myrosinases are produced in the secretory pathway involving glycosylation in the ER by addition of sugar trees. The mustard enzyme also showed several sugar trees to be present in the crystal structure (41). The bands smaller than myrosinase in the gel pictures may be due to unspecific binding of proteins to the cobalt column that become eluted with imidazole. To avoid unspecific protein binding on the IMAC column alternative or additional purification seem to be needed.

The ascorbic acid- myrosinase assay was used to study the effects of two mutations in TGG4. The results showed that low concentration of ascorbic acid stimulates activity of myrosinase, whereas higher concentration of ascorbic acid inhibited the activity of the enzyme, similar to what has been reported for a radish myrosinase (20). In my study the TGG4 mutant1 (E418Q) and TGG4 mutant2 (Q211A) showed similar enzyme activities as the wild-type form, in the presence of varying concentration of myrosinase. Thus, these two mutations have no major effect on the ascorbate interaction with the enzyme. The TGG4 mutant 3 (I278A) did not show any activity in the ascorbic acid assay. The mutant may be unstable, or cause a change to the substrate binding site leading to an inappropriate positioning or fixation so catalysis cannot be initiated. This study also found significant enzyme activity in the TGG4 mutant 1 (E418Q) which was expected to be much less active based on the homology analysis with the mustard enzyme (40, 41).
However, myrosinase catalysis has not been studied before using mutants, and there is no good crystal structure with a substrate or inhibitor in the active site that better would show the actual distance and orientation of various amino acid side chains in the catalytic reaction. Compared to O-β-glucosidases myrosinases have lost an acidic residue (postulated to give

charge repulsion with the sulfate group in glucosinolates) in the catalytic triad (40, 41). It may be that mutant 1 result indicates that the reaction mechanism involves somewhat other mechanisms and residues than implied from the analysis of O-β-glucosidases (http://www.cazy.org/).

Finally, the GUS Histochemical staining of TGG1 and TGG4 promoter activities depicted the effect on myrosinase gene expression at the cellular level after treatment with a beneficial (*Bacillus*) or a detrimental (*Alternaria*), microorganisms. In wild-type plants TGG1 staining was confined to above ground tissues while TGG4 staining was evident in below ground tissues. Myrosinase expression analysis using RT-PCR and other assays in *Arabidopsis* also supports a similar segregation in expression pattern for different myrosinases (42, unpublished). Huseby et al. (43) reported guard cell and phloem idioblast-specific expression of TGG1 in wild-type *Arabidopsis* plants, further supporting a specific above-ground location for TGG1. In this study promoter TGG1: GUS activity in leaf tissue varied according to treatment, where plants treated with the fungal pathogen *Alternaria* represented the highest staining compared with the other treatments. In the case of promoter TGG4: GUS, plants that were treated with *Alternaria* or *Bacillus* showed GUS activity, whereas plants treated with *Bacillus* plus *Alternaria,* or without treatment, did not show any GUS activity. From the GUS study it was clear that under stress condition the TGG1 clear that under stress condition the TGG1 and TGG4 promoter: GUS plants showed a more tissue-specific GUS expression compared with the control plants.

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