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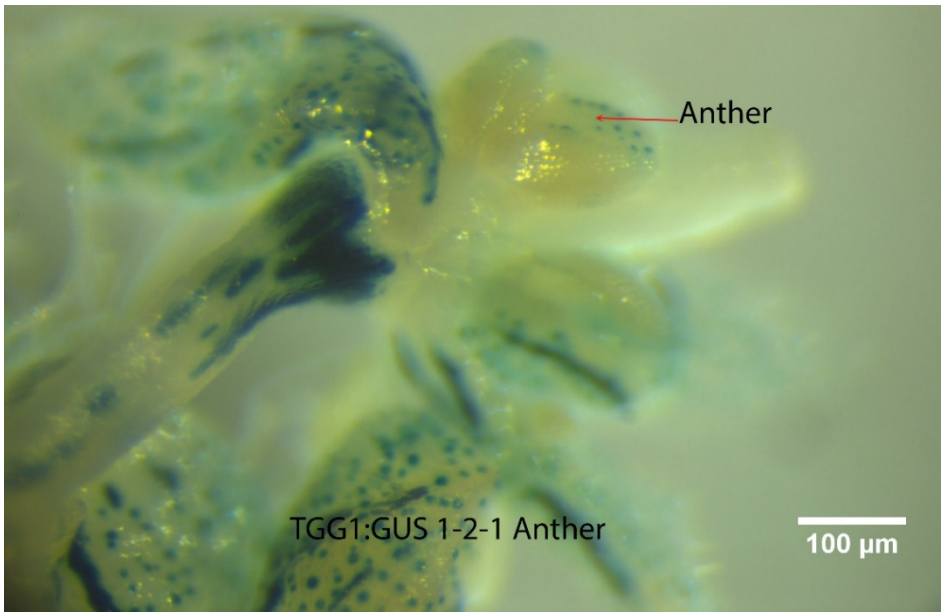
Arabidopsis myrosinases; analysis of developmental expression and effects of microorganisms

Mohammad Atikur Rahman



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Arabidopsis myrosinase; analysis of developmental expression and effects of microorganisms

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Abstract

Glucosinolates (GSL) are secondary metabolites found in most studied Brassicaceae family. When plant tissue is wounded, GSL undergoes a hydrolysis process initiated by the enzyme myrosinase (EC 3.2.1.147) producing toxic compound such as isothiocyanates, epithionitriles, and thiocyanates. The GSL-myrosinase system is a major defence against insect herbivores and pathogens. The GSL profile and myrosinase activity depend on the developmental stage and the nature of the plant tissue. Myrosinase is found in idioblast cells called myrosin cells in *Arabidopsis thaliana* and also in phloem parenchyma cells. The distribution of myrosin cells varies from plant to plant and organ to organ. In *Arabidopsis* Col-0 plants six myrosinase genes were found and referred to as thioglucoside glucohydrolase (*TGG*). We studied five different TGG promoter:GUS reporter lines to study the expression pattern of *TGG1* and *TGG4* at different developmental stages of *Arabidopsis*. *TGG1* expression was found in vascular tissues, cotyledon, leaves, rib, petiole, sepal, petal, anther, and gynoecium at different developmental stages. *TGG4* was only expressed in roots. *TGG1* expression was found in mature seeds but not in young seeds while *TGG4* was not expressed in seeds. The effect of plant growth regulating substances and signalling as a result of colonization by the beneficial bacterium *Bacillus amyloliquefaciens* UCBM5113 on *Arabidopsis* wild type and TGG promoter-GUS reporter plants were tested**.** Significant differences were observed in different parts of *Arabidopsis* at different developmental stages when the plant was stressed with the pathogen *Alternaria brassicicola*.

Keywords

Alternaria brassicicola, Arabidopsis thaliana, Bacillus amyloliquefaciens, Brassicaceae, Glucosinolates, Myrosinase, Myrosin cells, Thioglucoside glucohydrolase (*TGG*).

SAMMANFATTNING

Glukosinolater är sekundärmetaboliter som studerats främst I växter av familjen Brassicaceae. När växtvävnad skadas genomgår glukosinolater en hydrolysprocess initierad av enzymet myrosinas (EC 3.2.1.147) som producerar toxiska produkter, såsom isotiocyanater, epitionitriler och tiocyanater. Glukosinolat-myrosinas systemet är ett viktigt försvar mot skadeinsekter och patogener. Glukosinolat-halten och profilen samt myrosinasaktiviteten beror på växtens utvecklingsstadium och vävnadens natur. Myrosinas förekommer i idioblastceller som kallas myrosinceller och i backtrav (*Arabidopsis thaliana*) förekommer dessa bl.a. i floem och parenchym. Fördelningen av myrosinceller varierar mellan olika växter och även mellan olika organ I samma växt. I *Arabidopsis thaliana* finns sex huvudsakliga myrosinasgener så kallade tioglukosid-glukohydrolaser(TGG). I projektet studerades reporterlinjer där promotorer för TGG1 och TGG4 kopplats till β-glukuronidas (GUS) genen för enkel visualisering av genuttryck. Här studerades expressionsmönstret av TGG1 och TGG4 vid olika utvecklingsstadier av *Arabidopsis thaliana* ekotyp Col-0. TGG1 uttryck hittades I kärlvävnad, cotyledon, blad, bladnerver, petiol, foderblad, kronblad, ståndarknapp och gynoecium vid olika utvecklingsstadier. TGG4 uttrycktes endast i rötter. TGGI uttryck hittades även i mogna frön men inte i unga frön medan TGG4 inte uttrycktes i frön. Effekten av tillväxtreglerande ämnen och signalering som ett resultat av rotkolonisering av den gynnsamma bakterien *Bacillus amyloliquefaciens* UCBM5113 studerades på *Arabidopsis* vildtyp och TGG-GUS reporterväxter. Betydande skillnader observerades i olika delar av *Arabidopsis* vid olika utvecklingsstadier när växten stressades med patogenen *Alternaria brassicicola.*

Introduction

Glucosinolates (GSL) are secondary metabolites enriched in sulphur and nitrogen. These compounds are present in plants that belong to the order of Brassicales, e.g. the *Brassicaceae* family which comprises crop plants such as oilseed rape (*Brassica napus*), white mustard (*Sinapis alba*), broccoli (*Brassica oleracea* var. Italica), cabbage (*Brassica oleracea* var. botrytis), horseradish (*Armoracia rusticana*) and the model plant *Arabidopsis thaliana*. In 1831 the first glucosinolate, sinalbin, was identified from the seeds of white mustard [1] and since then almost 132 GSL have been identified [2] from species within the *Brassicaceae* family. GSL vary in the side chain derived from different amino acids and are classified as aliphatic, benzylic or indolylic [2]. The biosynthesis of GSL is divided into three phases, amino acid chain elongation, core structure development, and secondary refinement [2]. In *A. thaliana* over 40 GSL have been described and most of them contain aliphatic or indole side chains [2].

GSL undergoes hydrolysis by thioglucosidase enzymes denoted as myrosinases that result in the formation of reactive compounds such as nitriles, isothiocyanates, epithionitriles, and thiocyanates when plant tissue is damaged [4]. The nature of these product depends on pH, several other proteins and cofactors present where the degradation occurs [5]. The GSL-myrosinase system constitutes an important defence against insect herbivores and pathogens. The variety of defence compounds as GSL found in different plant species depends on the evolutionary arms race occurring with their natural enemies [6]. GSL are mobilized through different metabolic pathways in plant tissues to respond to different specialist and generalist insect pests and pathogens. Many of the genes involved in GSL metabolic pathway has been identified in *Arabidopsis* and several research groups have worked to find out the distribution, localization and degradation process of glucosinolates in *Arabidopsis* as well as different *Brassicaceae* species, especially *B. napus* and *S. alba* [48].

Sulphur-rich cells denoted as S-cells, found in phloem vessels, contain high amounts of GSL and serve most probably as storage sites before translocation via phloem to developing seeds or for wound induced defence [7]. The elongated S-cell which is located in the outer margin of *Arabidopsis* rosette leaves was originally identified due to the high sulphur concentration. The total amount of GSL, determined as total sulphur, present in the S-cells revealed more than 19 times higher sulphur than the surrounding tissue [7]. The cell walls of S-cells are comparatively thinner than the surrounding cells which may relate to the release of GSL.

The amount of GSL is higher in the peripheral part of *Arabidopsis* rosette leaves than in mid-lamina. S-cells present in the phloem parenchyma of *Arabidopsis* showed a specific action of programmed cell death (PCD) at an early stage of meristematic cell differentiation [8]. PCD is a

complex process where cells are degraded in an organized manner and the cellular contents released and absorbed by other cells. PCD is crucial for multicellular organism development, survival and defence against natural enemies [9]. The GSL profile and myrosinase activity depend on the developmental stage and the nature of the plant tissue as shown extensively for *Arabidopsis* [3, 10]. The amount of GSL in oilseed rape leaves is relatively low [11]. However, the inflorescence of oilseed rape contains large amounts of GSL. At seed maturation stage, large amounts of GSL are found in siliques while roots contain low amounts of GSL [12]. Environmental conditions play important roles in GSL accumulation, e.g. drought conditions increase GSL assembly in cultivated *Brassica* species [13]. Under moist soil conditions, the GSL level also increased as compared to dry soil [14].

The enzyme that degrades GSL is myrosinase (EC 3.2.1.147). At least 20 myrosinase encoding genes were found in *B. napus* and classified into three subfamilies denoted as MA, MB and MC [15]. In *Arabidopsis* Col-0 plants only six myrosinase gene were found and they are called thioglucoside glucohydrolase (*TGG*) [15]. According to western blot analysis, *TGG 1* is highly expressed in rosette leaves, flowers and less in silique, flower stalk, and seedlings. On the other hand, *TGG2* is mainly expressed in flower and silique [16]. *TGG3* is a pseudo gene and has no known myrosinase activity [17]. The three other myrosinase genes were denoted as *TGG4*, *TGG5*, and *TGG6* [18]. *TGG4* and *TGG5* are more likely root-specific and *TGG6* is a pseudo gene without known activity.

Myrosinase is found in idioblast cells called myrosin cells in both *A. thaliana* and *B. napus* but the location of the cells are different. Myrosin cells of *A. thaliana* are present in phloem parenchyma while in *B. napus* the idioblasts are found in ground tissue and phloem [19, 20]. The distribution of myrosinase varied from plant to plant and organ to organ. In *Arabidopsis* plants, the young seeds do not display myrosinase activity while the opposite feature occurs in *B. napus* and *S. alba* [19-21]. In *Arabidopsis*, myrosinase is present next to vascular tissues, phloem and is distributed on the abaxial side of the leaf [19, 20]. Myrosinase seems to be localized to myrosin grains that form continuous reticular multi membrane structures inside cells [19].

Myrosinases and GSL are separated from each other to avoid untimely hydrolysis within the cells of the plant. But this compartment should not be too far from each other because of immediate interaction needed to produce toxic compounds to protect the plant cells after herbivore attack or any damage of plant cells [22]. The pathway and mechanism behind this hydrolysis, as well as the location of the myrosinase and GSL, is not clear yet [23]. A high level of myrosinase activity was found in seed and seedlings of *Arabidopsis* [24]. The transcription factor FAMA plays a vital role in the early stage of myrosin cell differentiation and ground

meristem cell development [24]. The myrosin cell arises from ground meristem cells and this process is regulated by auxin [24]. Another gene that encodes the protein PEN2 with myrosinase activity has been described [25]. PEN2 is an atypical myrosinase with an acid/base catalyst glutamic acid in its catalytic site, characteristic for β-O-glycosylhydrolases but not myrosinases that have a glutamine possibly to avoid a charge clash with the GSL sulphate group [4]. PEN2 is localized both to peroxisomes and mitochondria of epidermal cells and forms homodimers or pathogen-induced multimeric aggregates of infected cells [26].

GLS and their degradation products are effective in deterring many pathogens and most herbivorous insect pests, so-called generalists, and thus protect the plant. However, certain insect pests, so-called specialists, use GSL and their degradation products to find the host plant for feeding or egg laying [4]. There is plentiful evidence about the role of isothiocyanates (ITCs) as biopesticides against various pathogens [27]. Sinigrin and its degradation products were shown to hamper growth to various degree depending on the microorganism tested [28]. Volatile ITCs derived from *Brassicaceae* plant extracts have been shown to affect yeast, bacteria, and fungi [27, 29]. The aromatic ITC products show a stronger effect on pathogens than aliphatic ITCs. The action is dose-dependent and varies with the situation. There is no rigid rule of how ITCs act towards various kinds of pathogens [29, 30]. Interestingly, certain GLS metabolites have an anti-inflammatory and anticarcinogenic effect on human beings indicating that *Brassica* plants can serve as functional foods [31]. However, a high GSL content in fodder plays a negative role for animals resulting in e.g. goiter (thyroid enlargement).

Soil-borne diseases are deleterious for agriculture. The surrounding environment of roots and aerial parts of the plant are totally different from each other. The micro biota community in below-ground of plant is different from other micro biota and creates continuous pressure on roots [32]. Roots have different composition and higher concentration of GSL than shoots due to high pathogen pressure from the soil micro biota [30].

In modern agriculture practice, the use of chemical pesticide has increased extensively because of e.g. less use of crop rotation. For sustainable agriculture practices, bio products are often preferred over chemical products. The use of GSL and GSL metabolites is considered as a source of bio based pesticides [33]. The refinement of GSL content of the plant could be a different source of the use of this bio chemical pesticide.

Aims

This study aimed to improve knowledge of *Arabidopsis* myrosinases (TGGs) by:

Studies of the expression pattern of *TGG1* and *TGG4* promoter-GUS reporter lines at different developmental stages.

Attempts to locate myrosin cells in different *Arabidopsis* plant organs at the cellular level.

Investigate the function of TGG1 and TGG4 upon treatment with the beneficial bacterium *Bacillus amyloliquefaciens* UCMB5113 and the pathogen *Alternaria brassicicola*.

Study myrosinase enzyme activity and potential complex formation in *Arabidopsis* leaves.

Materials and methods

Plant materials

Two different TGG promoter GUS constructs were tested, pTGG1 (1-3-12, 1-3-1, and 1-2-1) and pTGG4 (4-7-5 and 4-4-5) transformed into *A. thaliana* Col-0. seeds from a T4 (Homozygous) generation. Different age group (2days, 5days, 8days, 11days, 14days, 21days, 28days, 41days, and 51days old) of two different TGG promoter GUS constructs *Arabidopsis* plant were taken to analyse for developmental expression. Moreover, for stress development expression analysis different age group (10, 11, 12, 14, 19, and 21days old) of two different TGG promoter GUS constructs *Arabidopsis* plant were tested.

Bacterial materials

Bacillus amylolyquifacienes subsp. *plantarum* UCMB5113 (derived from UCM, Kiev, Ukraine) were used as a bacterial materials in the experiment. Bacteria were grown in LB 28 ◌̊C to stationary phase (usually around 7 days), after then the sample were heatshocked at 65 ◌̊C for 10 min to kill active bacteria and surviving spores collected by centrifugation and washed once in PBS before cfu analysis. Spore solution stored at 4C. An aliquot of 10 µl bacteria ($9.35x10^7$ ml^{-1}) was used to inoculate the each single plant.

Pathogen

Alternaria brassicicola (strain 20297) samples (5x5 mm) were cut with a sharp knife from previously prepared *Alternaria* plates and placed at the middle of a PDA (Potato Dextrose Extract and Bacto agar) plate. The plates were sealed with parafilm and stored at room temperature. The *Alternaria* spores were counted using a Hemocytometer (Burker, Knittel GLASER) in a Leica DFC 295 microscope (West Germany). A mechanical injury was made with the help of iron bar of plant leaves and inoculated with fungus culture ($17.3x10^4ml^{-1}$) of each leaf.

Developmental expression of *Arabidopsis* myrosinases

Two different TGG promoter GUS constructs were tested for developmental expression study [36]. Plant materials were sterilized with 10% chlorine and rinsed several times in MilliQ quality water. The sterilized seeds were germinated on 0.5x Murashige-Skoog (MS) medium, including vitamins (MS0222, Duchefa, Netherlands) and 0.8 % bacto agar. Later, the dishes were shifted to a growth chamber at 22° C with 16/8 h photoperiod. Two days and five days old plants of

each line were used for GUS staining [34]. After 5 days, the seedlings were transplanted to square Petri dishes containing 0.5xMSA. The plates were placed vertically in a growth chamber (four plants in each pate at the same inter-distance). Eight days, 11 days, 14 days, 21 days and 28 days old plants were then analysed by the GUS assay [34]. After 28 days the plants were transplanted to normal plant soil (S-soil, Hasselfors Garden, Örebro) and the plants shifted to the phytoton and kept at 22°C, 200 uE $m^{-1}s^{-1}$. Later, 41 days and 51 days old plants were taken for GUS staining. The processes were repeated two times and pictures were taken for each sample with Leica DFC490 (West Germany) digital camera

Square plates (22x22 cm) were prepared for the developmental expression analysis of the pTGG1:GUS1-3-1 and pTGG1:GUS1-2-1 plants on 0.5xMSA. Sterilized seeds of the lines were first germinated on 0.5x MSA small petri dishes and plants were transplanted five days later to big size square plates (four plants on each plate with same inter-distance). The plates were shifted to a growth chamber and placed vertically. The GUS test was done with 51 days old plants.

Mature seeds of five different GUS lines were imbibed 16 hrs with distilled water at room temperature in darkness. The seed coat was removed from the seeds with a forceps under a microscope. The young seeds were collected from siliques of 37 days old plant. The GUS staining test was done and pictures were taken with a digital camera.

Myrosinase coupled enzyme assay

Myrosinase catalyzes the cleavage of glucosinolates releasing the glucose moiety. The amount of glucose detached under a specific time period can be determined as a measure of the myrosinase activity [37]. In the subsequent glucose oxidase reaction, the glucose is oxidized by glucose-oxidase (GOD) and oxygen to gluconate and hydrogen peroxide [49]. The hydrogen peroxide is then oxidized by peroxidase (POD) to form a leuco dye in the presence of 4-aminophenazone and phenol and quantified spectrophotometrically at 505 nm.

D-Glucose + H_2O +O_2 ---- (GOD) ------> Gluconate + H_2O_2

H_2O_2 + DH_2 – (POD) ----> 2H_2O + D (DH_2 =Leuco dye, D=Dye)

GUS histochemical staining

The plant of two different TGG promoter: GUS lines were assayed for β-glucuronidase gene expression [34]. 0.1M (5-bromo-4-chloro-3-indolyl β-D-glucuronid, “X-gluc”) was used as a

substrate. The substrate was dissolved in NN-Dimethylformamide (DMF) and stored at -20 °C. GUS solution (50 mM Na-phosphate buffer, pH 7.2, 0.01 % Triton X100, 0.25 mM ferro-cyanid, 0.25 mM ferri-cyanid, 0.25 mM X-gluc) was prepared before the experiment. The plant material was transferred from the petri dish to an Eppendorf tube and destained with cold 90% acetone (15-20 mins) at room temperature followed by rinses with distilled water. The staining solution was added to the sample which then was covered with aluminium foil and incubated at 37°C for 24 h. The plants were then rinsed with distilled water. The samples were incubated with 70 % ethanol overnight and then studied with a stereo microscope and pictures were taken with a digital camera.

Fixation and embedded sectioning

The GUS stained plant lines were fixed with fixation solution [43]. The 21days old plant fixed with freshly prepared fixation solution (3 % paraformaldehyde and 0.5 % glutaraldehyde in 50 mM Na-phosphate buffer, pH 7.2, in fume hood) for 4 h and then the samples were rinsed with 1 % PBS and again fixed with 30 % ethanol, 50 % ethanol, and 70 % ethanol 30 min each. Then, the fixed plant samples were dehydrated through a series of alcohol and embedded with wax [51]. The samples were placed in an automatic embedding machine (TP1020, Leica) and treated with 70 % ethanol, 90 % ethanol, 100 % ethanol, and xylene and embedded in paraplast x-tra. The samples were then stored in a freezer. The embedded plant materials were cut with an HM 355S Microtome (West Germany) with R35 microtome blades (Feather). Slides with sections were incubated at 42°C for 24 h. The samples were again incubated with histoclear 10 min x 2 for deparaffination. Samples were then analysed with a Leica DFC 295 stereo microscope (Leica, West Germany) and pictures were taken with a camera.

After embedding of the roots with the embedding machine, root samples were embedded in 5% ultra-low gelling agarose (dissolved 2.5 g ultra-low gelling agarose in 50 ml PBS) [35]. Then root samples were embedded in paraffin and stored in a freezer and then followed the same procedure as above.

Bacillus* treatment and challenge with *Alternaria

The plant of two different TGG promoter: GUS lines were inoculated with *Bacillus amylolyquifacienes* subsp. *plantarum* UCMB5113 (derived from UCM, Kiev, Ukraine) [44]. An aliquot of 10 µl bacteria ($9.35x10^7$ ml^{-1}) was used to inoculate each single plant root in a square plate (0.5 % MSA, four plants in each plate with same distance). The plants were treated with distilled water as a control. After 4 h and 24 h the GUS test was performed. Then, the *Bacillus* inoculated plants were stressed with *Alternaria brassicicola* spore suspension [50]. The 12 days old plants previously inoculated with UCMB5113 were inoculated with *Alternaria brassicicola*

spore suspension and the distilled water pretreated plant was inoculated with the *Alternaria* culture as a control for non-primed plants. A mechanical injury was made with the help of T-shaped hollow iron bar of two opposite leaves of the plant and inoculated with 5 µl of fungus culture ($17.3x10^4 ml^{-1}$) of each leaf. Four hours later, the GUS test was done. Followed the same procedure for 14 days, 19 days and 21 days plant and the GUS test was done.

Fluorometric GUS assay of the plant

The plant samples were analysed quantitatively for MUG activity of tissue extracts using a fluorimetric assay of GUS enzyme activity [34]. The plants (21 days old plants inoculated with distilled water, inoculated with UCMB5113, inoculated with *Alternaria*, inoculated with both 5113 and *Alternaria*, and *Arabidopsis* Col-0 WT plant as control) were grinded with autoclaved plastic pestles and 200 µl of GUS extraction buffer (50mM NaH_2PO_4, pH7, 1mM DTT, 10mM EDTA, pH 8.0, 0.1% sarcosyl, 0.1% Triton) added into the Eppendorf tube. The grind plant materials were centrifuged 15 min, 13,000 rpm at 4°C. An aliquot (50 µl) of the supernatant of each sample was taken to a new Eppendorf tube and 50 µl of GUS assay buffer (2 mM MUG in GUS extraction Buffer) added. The samples were then incubated at 37°C for 60 min. The fluorescence was measured with the help of plate reader at 15 min and 60min.

Protein extract fractionation by size exclusion chromatography

Myrosinage were fractionalized by size exclusion chromatography to measure the complex forming ability [52]. As a result, protein were extracted from *Arabidopsis* plants. Sterilized *Arabidopsis* Col-0 seeds were germinated on 0.5x MSA in a growth chamber. Five days old plants were transplanted into normal soil and shifted to the phytotron. Leaves of 28 days old plants were collected and immediately ground with extraction buffer (20mM imidazole, pH 6.0, 150mM KCl, 0.1mM benzamidine and 0.1mM PMSF). Samples were centrifuged at 15,000 rpm, 15 min at 4°C. The supernatant was filtered using a 0.45 µm sterile filter and 50 mM Na-phosphate buffer and 1 mM sinigrin as substrate added. The samples were incubated at 37°C for 4 hrs and the reaction stopped in a heat block at 95°C for 5 min. The absorbance of the samples was recorded at 505 nm with a plate reader after addition of a glucose test reagent and incubated for 15 min.

The filtered supernatant was analyzed using a Superose 6 column (Amersham, Uppsala) on an FPLC instrument [52] and absorbance of fractions measured with a plate reader to record protein. The experiment was done two times and the samples were incubated at 37°C for 24 hrs to assay myrosinase activity.

Results and Discussion

Developmental expression pattern of *Arabidopsis* myrosinases

To identify spatial and temporal expression of myrosinase at different developmental stages of *Arabidopsis* plants, TGG promoter: GUS lines were used. There are many ways in which gene expression is regulated in a plant. A reporter gene produces a protein that is easily studied since it can be easily visualized as such or because it forms a product that is visible and can be quantified. β-Glucuronidase is an example of such a reporter. The reporter gene is fused to a promoter of the gene-of-interest and the reporter will thus only be expressed when and where the particular gene is expressed. GUS staining uses 5-bromo-4chloro-3-indolyl beta-D-glucuronide (X-gluc) that produces an insoluble intense blue indigo. Ferrocyanides protect indigo from further oxidation and reduce diffusion of the product and provide more accurate localization.

The plants were grown on 0.5xMSA plates vertically and 2, 5, 8, 11, 14, 21, 28, 41 and 51 days old plants were evaluated with the GUS test to identify where the gene is expressed throughout the life cycle. The pTGG1: GUS (1-2-1) (Fig. 1A-B), pTGG1: GUS (1-3-1) and pTGG1: GUS (1-3-12) 2days old plants showed a positive response in the GUS test. The distinct blue indigo was seen in stomata of the leaves in both abaxial and adaxial parts of the leaves when examined under a microscope. The pTGG4: GUS (4-4-5) (Fig. 1D) and pTGG4: GUS (4-7-5) lines retained the blue indigo in the root tip of the plant in 2days old plants.

In a case of 5days and 8days plant, only pTGG1: GUS (1-3-12) plants did not produce any blue indigo in one GUS experiment but showed stain in a repeat experiment. Moreover, the blue indigo was seen in petioles of 8days old plant of pTGG1: GUS (1-2-1). The pTGG1: GUS (1-3-1) and pTGG1: GUS (1-3-12) lines showed that *TGG1* was expressed in vascular tissues of the petiole while the *TGG4* gene was not expressed in the petiole. For later time points, 11, 14, 21, and 28days, the different lines stained positively in the GUS test (table 1). The *TGG1* gene was then active in petiole and leaf periphery at 11, 14, 21, and 28 days old seedlings. The *TGG4* gene was expressed in root tips and in axillary root tips but not in leaves or petioles.

Table 1. GUS expression among the different TGG promoter: GUS reporter line at different developmental stages. TGG1 and TGG4 expression is indicated as +/- (positive/negative) and * marks are used to denote any additional findings.

Plant / **Age (days)**	**pTGG1:GUS 1-2-1**	**pTGG1:GUS 1-3-1**	**pTGG1:GUS 1-3-12**	**pTGG4:GUS 4-4-5**	**pTGG4:GUS 4-7-5**
2	+ Stomata	+ Stomata	+ Stomata	+ Root	+ Root
5	+ Stomata	+ Stomata	–	+ Root	+ Root
8	+ Stomata, petiole	+ Stomata, petiole	–	+ Root	+ Root
11	+ Stomata, vascular tissue	+ Stomata, vascular tissue	+ Stomata, vascular tissue	+ Root	+ Root
14	+ Stomata, leaf vein, vascular tissue	+ Stomata, leaf vein, vascular tissue	+ Stomata, leaf vein, vascular tissue	+ Root	+ Root
21	+ Stomata, leaf vein, vascular tissue	+ Stomata, leaf vein, vascular tissue	+ Stomata, leaf vein, vascular tissue	+ Root	+ Root
28	* + Stomata, leaf vein, vascular tissue, root	* + Stomata, leaf vein, vascular tissue, root	+ Stomata, leaf vein, vascular tissue	+ Root	+ Root
41	* + Stomata, leaf vein, vascular tissue, root	* + Stomata, leaf vein, vascular tissue, root	–	+ Root	+ Root
Young seed	–	–	–	–	–
Mature Seed	+ Cotyledon	+ Cotyledon	–	–	–

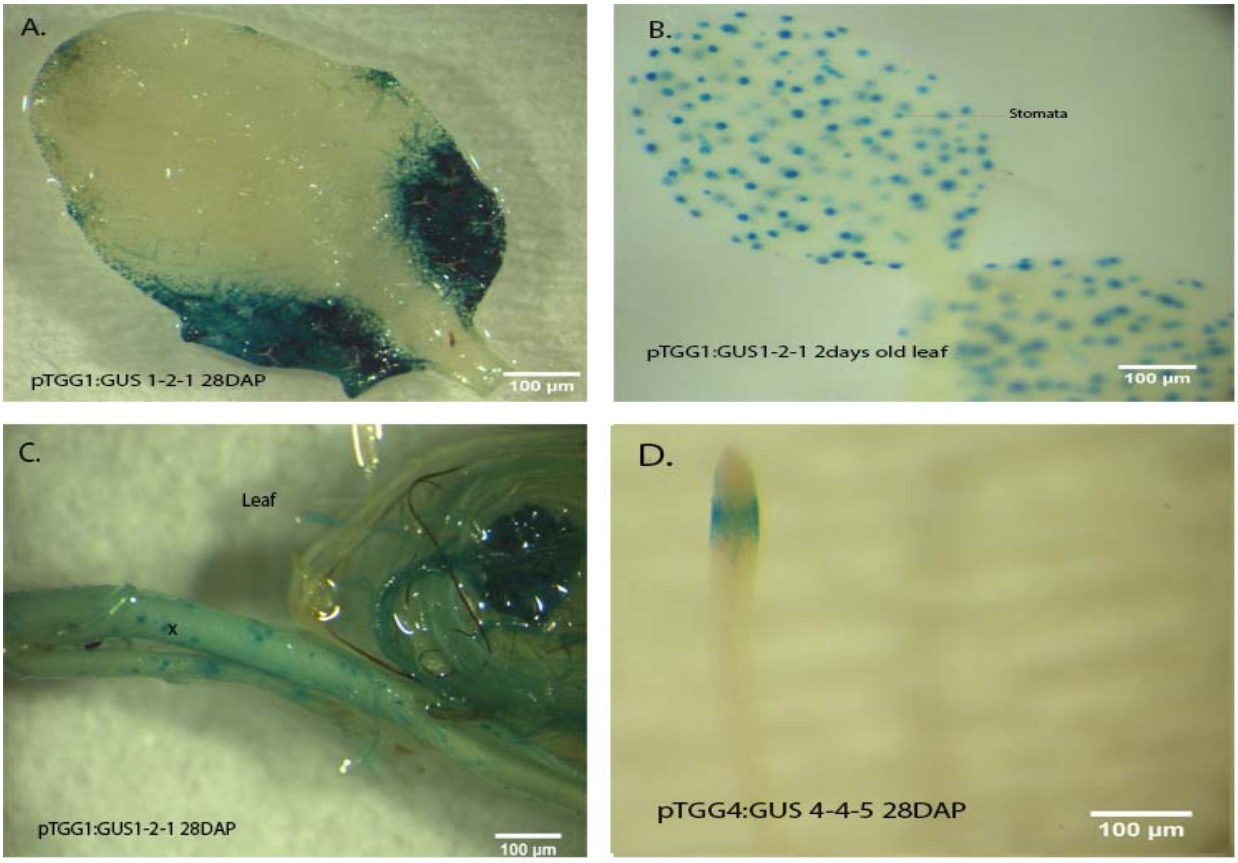

Figure 1. GUS expression of TGG1 and TGG4 *Arabidopsis* lines at different stages of plant development. A. pTGG1: GUS (1-2-1) 28days old plant observed blue indigo at the periphery of the leaf and no indication at middle mesophyll of the leaf. B. pTGG1: GUS (1-2-1) 2days old plant observed a clear indication of *TGG1* gene expression in leaf guard cells. C. pTGG1: GUS (1-2-1) 28days plant observed a clear indication of rectangular shaped blue indigo in plant root. D. pTGG4: GUS (4-4-5) 28days old plant observed blue indigo at the root tip.

When plants were transplanted to soil after 28 days of germination, the pTGG1: GUS (1-2-1) and pTGG1: GUS (1-3-1) plants showed *TGG1* expression in leaves and produced rectangular shaped blue indigo on plant root (Fig. 2A) but no blue indigo at the root tip. This phenomenon was also observed at 41 days and 51 days of these two plant lines but no *TGG1* expression on pTGG1: GUS (1-3-12) plant root. The pTGG1: GUS (1-2-1) and pTGG1: GUS (1-3-1) plant leaves showed blue indigo at the periphery of the leaves in contrast to the whole leaf and more at the vascular tissue of the plants at 41 days (Fig. 2D). However, the pTGG1: GUS (1-3-12) in 41days old plants did not produce any blue indigo on plant root and leaves and inflorescence (Fig. 2C). After transplantation of the pTGG4: GUS (4-4-5) and pTGG4: GUS (4-7-5) plants on soil, there was no difference observed in *TGG4* gene expression compared to a previous developmental stage.

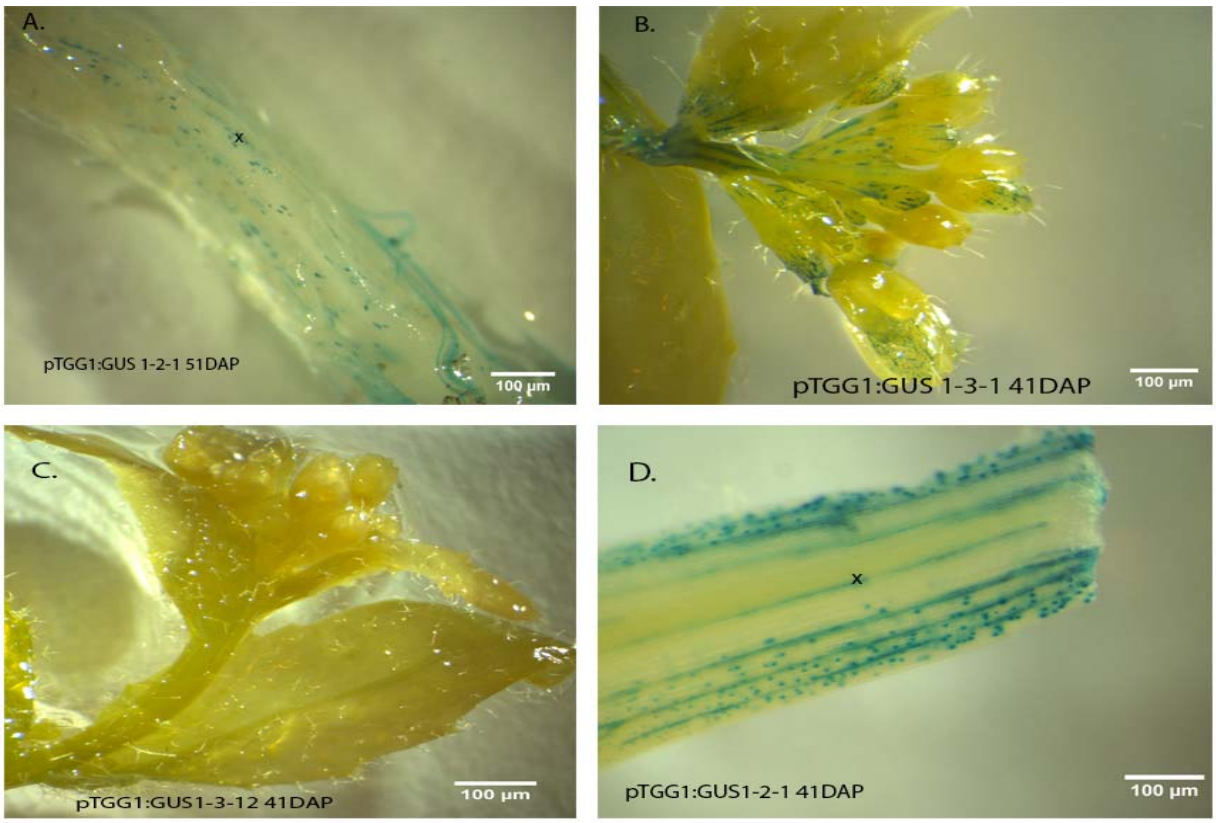

Figure 2. GUS expression of TGG1 at different stages of *Arabidopsis* plant development. A. The pTGG1: GUS (1-2-1) 51 days plant root showing rectangular shaped blue indigo. B. The pTGG1: GUS (1-3-1) 41 days old plant inflorescence with prominent blue indigo. C. The pTGG1: GUS (1-3-12) 41 days old plant inflorescence with no indication of *TGG1*. D. The pTGG1: GUS (1-2-1) 41 days plant leaf with definite blue indigo and *TGG1* expression at the base of the leaf vascular tissue.

The different GUS reporter plant lines were examined at reproductive stage. As a result, *TGG1* gene was expressed in anthers (Fig. 3A, F), gynoecium (Fig. 3B, F), and mature seeds (Fig. 3C), and sepals (Fig. 3D), and petals (Fig. 3D), and siliques (Fig. 3E), and cauline leaves of pTGG1: GUS (1-2-1) and pTGG1: GUS (1-3-1) plants. However, no indication of *TGG1* expression was observed in the pTGG1: GUS (1-3-12) GUS reporter plant line at reproductive stage.

Arabidopsis contains six different myrosinase genes (*TGG1-6*) with somewhat different function and location. However, *TGG3* and *TGG6* are pseudo genes and their specific role in plants is not known. In this study, 2days old pTGG1: GUS (1-2-1), pTGG1: GUS (1-3-1) and pTGG1: GUS (1-3-12) plants revealed a specific location in guard cells. However, *TGG1* was not only confined to guard cells of the leaf but also occurred in mesophyll, petiole, cauline leaf, and siliques of the plant. The *TGG1* gene is expressed in guard cells, not *TGG2* according to other studies [38]. To

compare the myrosinase activity of the *TGG1* and *TGG2* genes, according to western blot result *TGG1* is expressed in rosette leaves and flower, less in siliques, flower stalk and seedlings whereas *TGG2* are expressed in siliques and flower [16]. The GUS test in inflorescence showed that *TGG1* was detected in sepal, petal, gynoecium, and anther for pTGG1: GUS (1-2-1) and pTGG1: GUS (1-3-1) but no *TGG1* gene indication on inflorescence of pTGG1: GUS (1-3-12) plant. However, *in situ* hybridization with *TGG1* and *TGG2* probes detected transcripts in sepal, petal, and gynoecium but not in stamen [40]. *TGG1* expression was randomly distributed on a young leaf of the plant but concentrated at the periphery of the leaf with increasing age.

Moreover, *TGG1* gene expression was observed at a root of 28days old pTGG1: GUS (1-2-1) and pTGG1: GUS (1-3-1) plants and the same observation were detected in 41days and 51days old plants. Those plants were grown on natural soil, not *in vitro* in a growth chamber. Normally, plant root myrosinase as *TGG4* was expressed at the tip of the main root and lateral root but in that case, *TGG1* expression was found in the main root with a rectangular shape, not at root tip. Previous data analysis and several scientific papers revealed that *TGG1* was only expressed above-ground, not below-ground. Abiotic factors at different developmental stages could play a vital role for the variation of the myrosinase enzyme system [42].

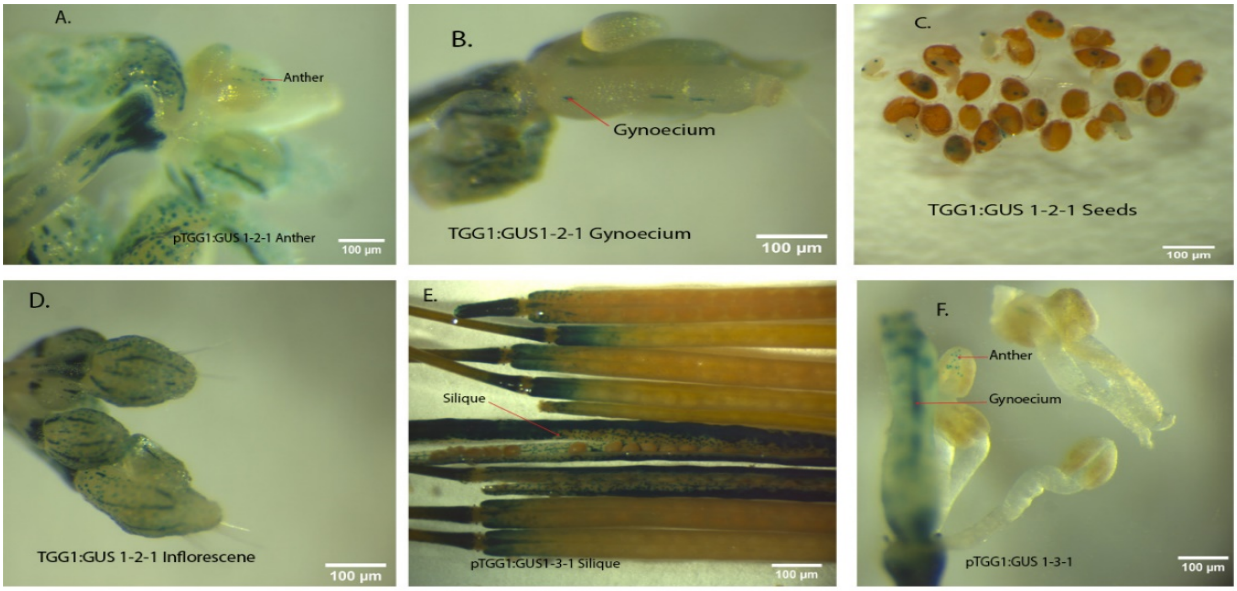

Figure 3. Tissue-specific gene expression was observed in inflorescence and silique and mature seeds. 3(A, F), *TGG1* was active in pTGG1: GUS (1-2-1) and pTGG1: GUS (1-3-1) plants, respectively, in anthers. 3(B, F), dissected gynoecium of pTGG1: GUS (1-2-1) and pTGG1: GUS (1-3-1) plant showed a positive response to GUS activity. 3C, the dissected embryo and seed coated mature seed with endosperm exhibited GUS expression. 3D, Different flower parts of a TGG promoter: GUS reporter plant showed blue indigo 3E, dissected siliques with young seed showed GUS expression.

The young seeds collected from 41 days old plant showed no GUS staining for any reporter line. *TGG1* expression was detected in imbibed mature seeds of the pTGG1: GUS (1-2-1) and pTGG1: GUS (1-3-1) plant. The GUS expression was observed in cotyledons of dissected embryo and seed coat including endosperm at cotyledon region (Fig. 3C). However, no gene expression were found in pTGG1: GUS (1-3-12), pTGG4: GUS (4-4-5) and pTGG4: GUS (4-7-5) plant mature seeds.

In young seeds there was no sign of myrosin cells but mature seeds had a distinct indication of myrosin cells. Only siliques from 41days old plants were analysed for the young seed myrosin cell experiment. At what stage of seed development that myrosin cells are produced remains to be established.

At flower developmental stage, the silique contained a high proportion of myrosin cells, as well as the sepal, petal, anther, and gynoecium, also contained a number of myrosin cells. In this experiment, it was clearly indicated that when the plant was wounded more myrosin cells were produced than a non-wounded plant. When siliques were cut with a sharp knife and tested with GUS staining solution it produced more blue indigo than fresh siliques (Fig. 3E). Accordingly, when the plant was wounded more myrosin cells were formed adjacent to the wounded site.

GUS analysis revealed that *TGG1* was active in mature seeds. GUS staining was observed both in coated seeds and seeds without seed coat. In situ hybridization of mature seeds revealed that some *TGG1* transcripts were found in Arabidopsis seed [40]. So, *TGG1* is expressed at a later stage of the mature seed but not in young seed. In contrast, *TGG4* was not expressed at any seed stage but expression was found in 2days old plant root and at all later time points. *TGG4* and *TGG5* have root specific expression pattern according to RT-PCR analysis [37].

Another *Brassicaceae*, *B. napus* have >20 myrosinase gene distributed into three subfamilies, MA, MB and MC [15]. High level of myrosinase activity was reported in the mature seed of *B. napus* [23]. The different subfamilies are found at different stages of seed embryo development according to *in situ* hybridization analyses [41]. *MA* and *MC* genes are expressed in seed axis whereas *MB* genes are expressed in cotyledon of *B. napus* seed according to *in situ* hybridization [41]. Using the antibodies (K505 and 3D7) the 72k Da myrosinase was identified in leaf, stem, pedicel, flower and silique of *B. napus* mature plants [41]. In case of the root of *B. napus*, MB transcripts were not regularly observed [41]. Whereas in *S. alba* very low or no

myrosinase activity was observed at mature seed stage and *MA* and *MB* expression during embryo development had no consistency [21]. *MA* expression was found in the axis and *MB* in cotyledon [21].

Localization of myrosinase of *B. napus* plant was observed in parenchyma cells and vascular cells. At an adult stages, immunohistochemistry showed that myrosinages were localized in parenchyma cell of mesophyll, vascular tissue as well as at guard cell [19]. In siliques and petals of *B. napus* the myrosin cell was detected in mesophyll and adjacent to vascular tissue and in the root, the myrosin cells were located at cortical cells [43]. A similar result was observed for *S. alba* plants by *in situ* hybridization.

Myrosinase distribution has also been studied in *Raphanus sativus, Brassica comprestris*, and *Wasabia japonica* plants. Myrosinase was localized at epidermis and vascular cambium of those plant species using tissue printing techniques [45]. In *B. juncea* plants myrosinase was found in cotyledons of imbibed seeds by immunohistochemical analysis with an antibody technique [46].

Less glucosinolate content was not affected during plant germination and senescence process [39]. Furthermore, *TGG4* and *TGG5* both gene has an additive effect on root myrosinase activity [47].

Stress developmental expression

The effect of plant growth regulating substances and signaling mechanism managing as a result of UCBM5113 colonization on *Arabidopsis* transgenic plant root was tested. Transgenic plant seedlings were grown vertically on 0.5x MSA medium. Ten days after inoculation with UCMB5113 a GUS test was performed. The GUS expression was detected in leaf and root of the TGG promoter: GUS reporter plant line.

For the stress development expression experiment five *Arabidopsis* TGG promoter: GUS reporter lines underwent four different treatments. Plants treated with water served as a control. Plants were treated with *Bacillus* UCMB5113 that promote plant growth of roots and above ground tissues [44]. Plants were also treated with a detrimental microorganism (*A. brassicicola*) that produces leaf spot disease on *Brassicaceae* plants. Finally, plants were treated with both beneficial (UCMB5113) and detrimental (*Alternaria*) microorganisms to study the effect on TGG expression.

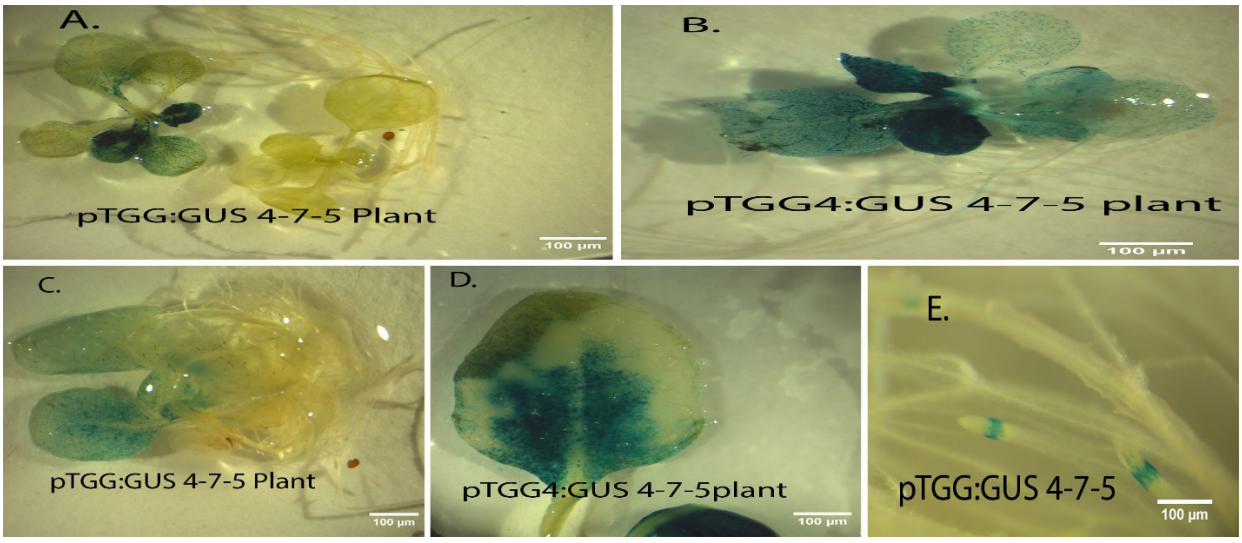

Figure 4. Arabidopsis myrosinase gene expression upon UCMB5113 treatment and subsequent challenge with *Alternaria* pathogen. A. 14 days old pTGG4: GUS (4-7-5) plant treated with UCMB5113 showed leaf and root myrosinase activity. B, C. 14 days, and 19 days old pTGG4: GUS (4-7-5) plant, respectively, challenged with pathogens. D. 21days old pTGG4: GUS (4-7-5) plant treated with UCMB5113 and challenged with pathogen showed distinct blue indigo in leaves. E. Control pTGG4: GUS (4-7-5) plant showed root specific TGG4 activity.

Gus expression was observed after 48hours later plant challenged with pathogen. As a result, *TGG1* gene expression was observed in leaves of pTGG1: GUS (1-2-1), pTGG1: GUS (1-3-1) and pTGG1: GUS (1-3-12) plants and *TGG4* was expressed at root tip of pTGG4: GUS (4-4-5) and pTGG4: GUS (4-7-5) plants. A similar result was also observed for 12 days old stressed plants.

Stress analysis of plants occurred between days 10 to 21. No distinguishable feature was obtained from 10days, 11days or 12days old plants. A distinct difference was observed for 14days old pTGG4: GUS (4-7-5) plant treated with *Alternaria* (Fig. 4A, B). Normally *TGG4* was expressed in the root but *Alternaria* treated 14days old plant showed GUS staining in leaves but the combined *Alternaria* and UCMB5113 treatment plant showed normal expression. The same observation was observed for 19days old pTGG4: GUS (4-7-5) plants treated with detrimental fungus (Fig. 4C) and 21days old pTGG4: GUS (4-7-5) plants treated with both *Alternaria* and UCMB5113 (Fig. 4D).

Table 2: GUS staining result of different TGG promoter: GUS reporter line plant with different treatment and time points. TGG1 and TGG4 expression is indicated as +/- (positive/negative) and * marks are used to denote any additional findings. The different samples were; control (H_2O), *Bacillus* UCMB5113 treatment (5113) or *Alternaria* challenge (Al).

Plant	**pTGG1:GUS 1-2-1**				**pTGG1:GUS 1-3-1**				**pTGG1:GUS 1-3-12**				**pTGG4:GUS 4-4-5**				**pTGG4:GUS 4-7-5**			
Treatment / **Days**	**H_2O**	**5113**	**Al**	**AL+5113**	**H_2O**	**5113**	**Al**	**Al+5113**	**H_2O**	**5113**	**Al**	**AL+5113**	**H_2O**	**5113**	**Al**	**AL+5113**	**H_2O**	**5113**	**Al**	**AL+5113**
10	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
11	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*+	+
19	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	*+	+	*+	+
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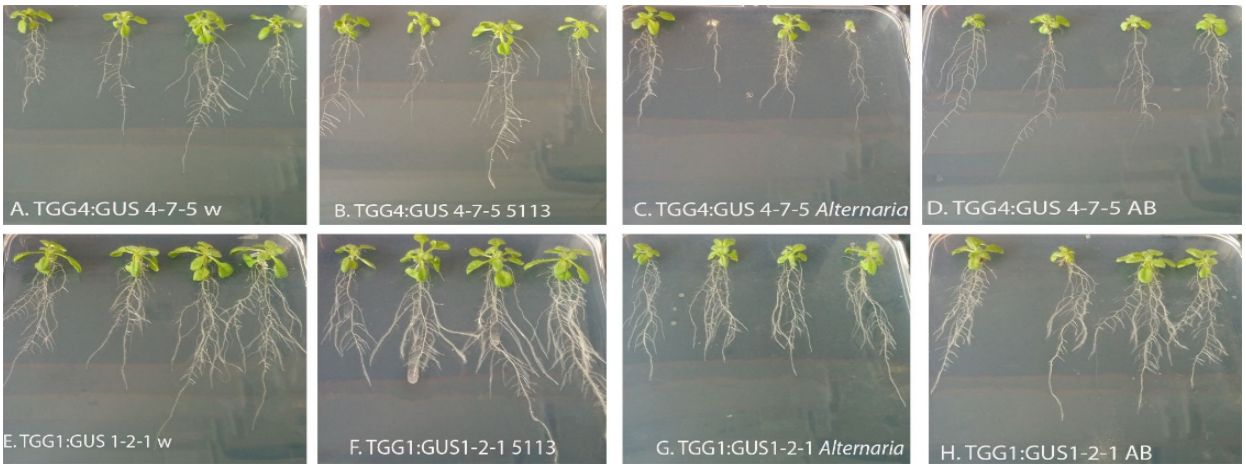

Figure 5. Effect of H_2O, UCMB5113, *Alternaria* and *Alternaria*+5113 on 19days old *Arabidopsis* Col-O plants. (A, B, C, D) pTGG4: GUS (4-7-5) plants treated with water, UCMB5113, *Alternaria* and (*Alternaria*

+*Bacillus*), respectively. (E, F, G, H) pTGG1: GUS (1-2-1) plants were treated with water, UCMB5113, *Alternaria* and (*Alternaria* +*Bacillus*), respectively.

Morphological observation was made with open eye observation and found no difference in 10days old plants among the different treatments. Beneficial bacteria treated 12days old plants showed a positive effect on plant roots. The bacteria treated plant was produced more lateral root than control plant.

The most prominent difference among treatments were observed in 19days old plants. The lateral root were observed in UCMB5113 treated plants compared to control and some black spots were seen on leaves of plants treated with *Alternaria*. However, mixed result was observed for plants treated with both beneficial and detrimental fungus. Furthermore, the effect differed among the different TGG promoter: GUS reporter plant lines

Histochemical localization of GUS enzyme

To analyse the cellular localization of GUS, histochemical analysis was performed of the *Arabidopsis* lines. Intensive staining was found in vascular bundle parenchyma cells of the leaf and epidermal parenchyma cells or guard cells of the stomata (Fig. 6-A, C). Moreover, in a case of roots, profound staining appeared in epidermal parenchyma cells (Fig. 6D) and GUS staining was observed in the vascular tissue of the petiole (Fig. 6B).

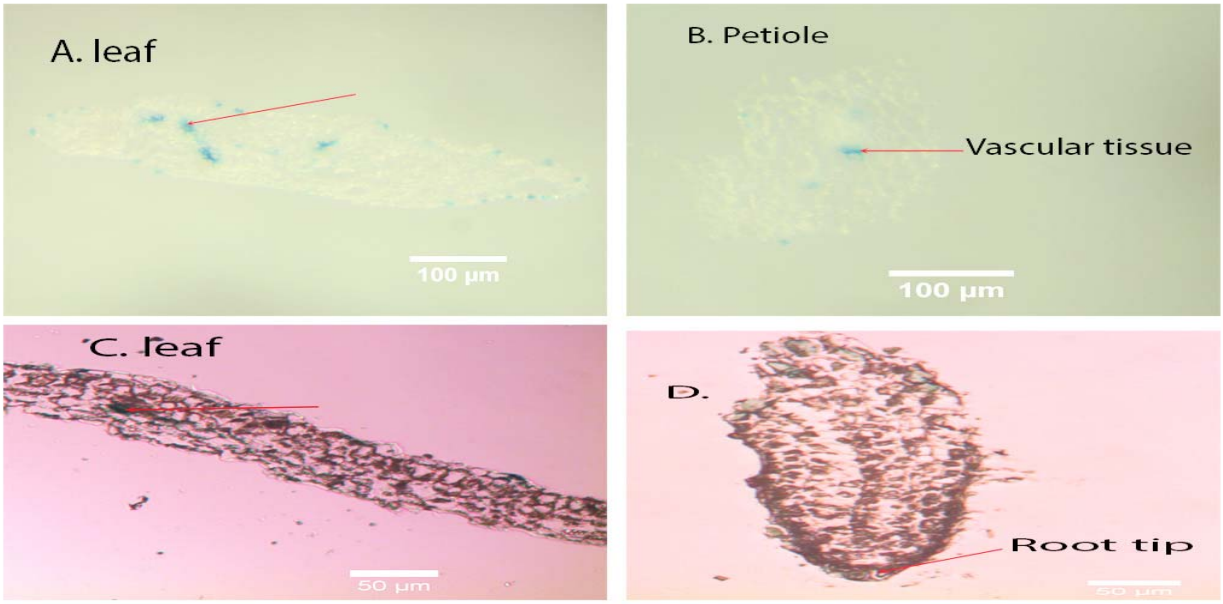

Figure 6. Histochemical localization of TGG promoter: GUS reporter line plants. A, C. Transverse section of leaf showed a clear indication of gene expression in the vascular tissue and the epidermal cells. B. The transverse section of petiole clearly indicates gene expression of the vascular tissue. D. Longitudinal section of root showing extensive staining in the epidermal cells and downward facing root tip.

The histochemical analysis of *Arabidopsis* plants revealed that myrosin cells localized in scattered parenchyma guard cells at an early stage of seedling development. With the gradual development of the plant, the myrosin cells were detected in scattered parenchyma cells and in vascular tissue. The myrosin cells were concentrated more at the rib of the leaf. In stem tissue, no myrosin cells were found.

Myrosinase coupled enzyme assay

The myrosinase enzyme activity was determined by the amount of glucose liberated under a defined time period using a coupled enzyme assay. The GSL was broken down into glucose and other substances in the presence of myrosinase. The activity was $1.56x10^{-3}$mol/(min x mg). Thus, it can be said that, *Arabidopsis* plant has myrosinase activity. To calculate the amount of enzyme activity the substrate sinigrin (allyl-glucosinolate) was added to the sample and absorbance measured after addition of the glucose test reagent.

The potential complex formation properties of myrosinase

To determine the potential complex formation properties of myrosinase, leaves of *Arabidopsis* plants that plant grown on soil were collected and a protein extraction was made from leaves of plant. The protein extract was then fractionated by size exclusion chromatography using Superpose 6 columns and FPLC. The fractions were analyzed by catalytic activity (Fig. 7). The sample fraction size was 0.5 ml and the flow rate was 0.5 ml min^{-1}. Fraction aliquots (20 µl) were used for myrosinase assay. The absorbance was taken at 280nm. The experiment was done two times. The elution profile showed activity around fractions, respectively, (Fig. 7) where the first peak may represent complexes and the second free enzyme.

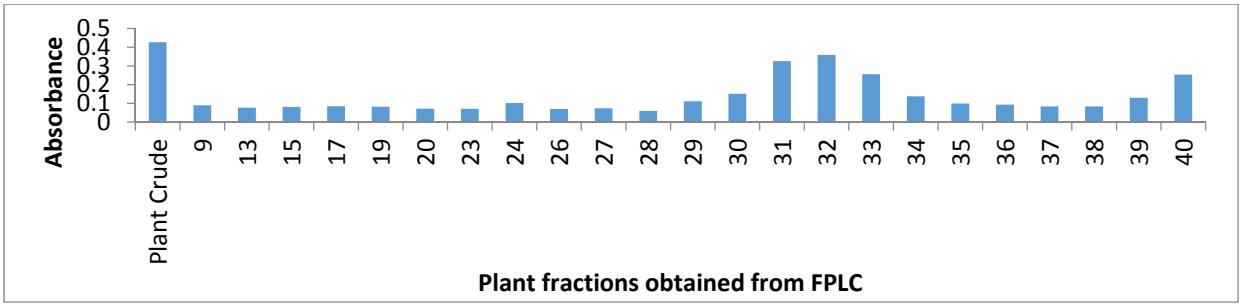

Figure 7. Column chart showing the fractionation of *Arabidopsis* leaf extract and myrosinase activity and implicit complex formation properties.

For analysis of complex formation properties of myrosinase, plant leaf extract was fractionated using FPLC and the fractions analysed spectrophotometrically for protein and myrosinase activity. No clear data was obtained and further analysis is required to analyse the protein content using SDS-page and antibodies. It could also be of interest to analyze root and seed protein fractions from Arabidopsis for myrosinase complex formation ability.

Quantification of GUS enzyme activity

Quantification of GUS enzyme expression using a fluorometric GUS assay was performed with selected *Arabidopsis* samples. No activity was found in pTGG4: GUS (4-4-5) plant as compared with 21 days old WT plant. However, pTGG4: GUS (4-7-5) plants treated with UCMB5113 and challenged with pathogen showed a response. The pTGG1: GUS (1-2-1), pTGG1: GUS (1-3-1) and pTGG1: GUS (1-3-12) plants treated with UCMB5113 and stressed with *Alternaria* displayed a distinct value compared to the control.

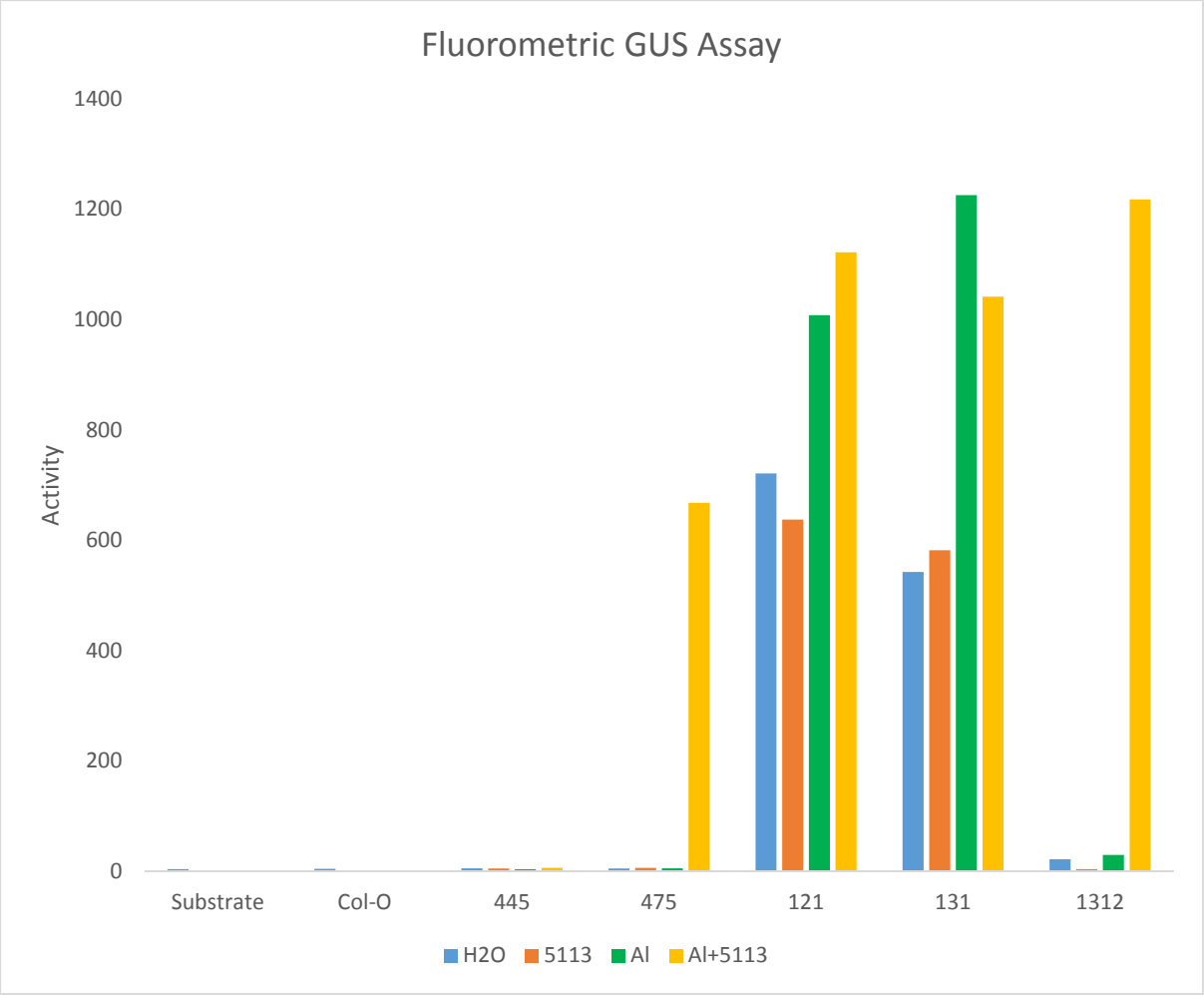

Figure 8. Fluorometric analysis of GUS expression of 21 days old plant with H_2O (blue colour), UCMB5113 (orange colour), *Alternaria* (green colour) and *Alternaria* with UCMB5113 (yellow colour) treatment derived from *Arabidopsis* Col-0. Substrate and Col-0 wild-type plants served as control for expression in pTGG4: GUS (4-4-5) as 445, pTGG4: GUS (4-7-5) as 475, pTGG1: GUS (1-2-1) as 121, pTGG1: GUS (1-3-1) as 131 and pTGG1: GUS (1-3-12) as 1312 plants.

When GUS activity was quantified using a fluorometric GUS assay, a significant difference was obtained among the samples and compared with the controls. The highest value was observed for pTGG1: GUS (1-3-1) plants treated with *Alternaria* and the lowest value was for pTGG4: GUS (4-4-5) plants treated with *Alternaria*. The plant TGG4: GUS (4-7-5) treated with both beneficial bacteria and detrimental fungus showed distinct differences compared with plants treated with water as control.

Conclusions

Based on the results, some concluding remarks can be stated as follows

- Among Two different TGG promoter GUS constructs, *TGG1* expressed in guard cells of the leaf, in mesophyll, petiole, cauline leaf, and siliques of the plant. *TGG1* gene also expressed in anthers, gynoecium, sepals, and petals of *Arabidopsis* inflorescence and mature seeds.
- *TGG4* expressed only root tip in developmental stage of *Arabidopsis* plant.
- However, *TGG1* was also expressed in root of *Arabidopsis* plant when plant grown on soil after 28days later.
- At stressed condition, *TGG4* expressed on stressed plant root as well as plant leaves.
- The histochemical analysis of *Arabidopsis* plants revealed that myrosin cells localized in scattered parenchyma cells and vascular tissue.
- A coupled enzyme assay showed that *Arabidopsis* plant has myrosinase activity under a defined time period.

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