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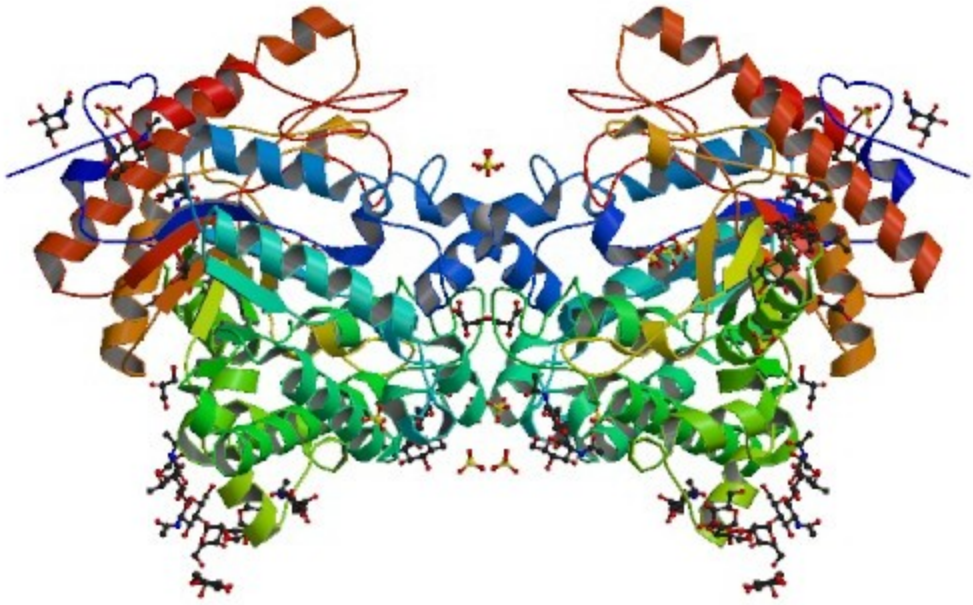

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STUDIER AV POTENTIELLA EFFEKTORER AV MYROSINAS FUNKTION
STUDIES OF POTENTIAL EFFECTORS OF MYROSINASE FUNCTION

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Masterprogrammet i växtbiologi

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Key words: Myrosinase, glucosinolate, ascorbic acid, *Arabidopsis thaliana*, Isothiocyanate

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Cover page illustration shows a mustard myrosinase **http://www.rcsb.org/pdb/explore.do?structureId=1E6S**

ABSTRACT

Glucosinolates are sulphur and nitrogen containing secondary metabolites which are present in the order Brassicales. These compounds are hydrolysed by enzymes called myrosinases upon tissue damage and serve to protect plants against insects and pathogens. Protein extracts from leaves of fully developed *Arabidopsis thaliana* Col-0 plants and the ascorbic acid deficient mutants *vtc1-1* and *vtc4-1* were utilised to characterize effects on basal myrosinase expression. The catalytic activity of myrosinases against the glucosinolate sinigrin was lower in *vtc4-1* compared to Col-0 and *vtc1-1*. The content of myrosinase and myrosinase related proteins in Col-0, *vtc1-1* and *vtc4-1* was analysed by Western blotting and immunoprecipitation using monoclonal and polyclonal antibodies. Differences were found regarding myrosinase and myrosinase related proteins in the evaluated genotypes. However, further analysis is required to clearly identify all the proteins present upon Western blot and IP analysis. His-tagged recombinant Arabidopsis myrosinases TGG1 and TGG4 were over-expressed in *Pichia pastoris,* extracted and purified through immobilised metal affinity chromatography. The effect of the sinigrin product allyl isothiocyanate (AITC) for enzyme inactivation ("suicide inactivation") of TGG1 and TGG4 was measured. Decrease of myrosinase activity was observed both for TGG1 and TGG4 in the presence of 1 mM AITC. The catalytic activity of TGG1 was completely inhibited by 5 mM and 10 mM AITC. On the other hand, 5 mM and 10 mM AITC caused a reduction of activity in TGG4 but more than 60 % activity was still remaining. Accordingly it seems that glucosinolate products can affect myrosinase activity in plant tissues if not finding other nucleophiles to react with which may represent an endogenous regulatory mechanism.

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1. INTRODUCTION

Glucosinolates ("mustard oil glucosides") are a group of plant secondary metabolites present in the order Brassicales (fomer Capparales); mainly in the Brassicaceae family. These sulphur- and nitrogen-containing metabolites are hydrolysed into different products by enzymes called myrosinases upon tissue damage (Kissen et al., 2009). In *Arabidopsis thaliana* six myrosinase genes have been identified which are called TGG (Thio-Glucose Glucosidase) genes. There is a basal constitutive expression of myrosinases, but they also seem to be stress-inducible (Rask et al., 2000). Different kinds of stimuli have been reported to affect myrosinase levels, such as jasmonate treatment (Jost et al., 2005), or Diamond back moth (*Plutella Xylostella*) feeding (Pontoppidan et al., 2005).

Glucosinolates are stored in vacuoles of giant S-cells which are located outside phloem bundles in floral stalk and leaves, and account for 40% of total sulphur in floral stalk tissue (Koroleva et al., 2010). S-cells seem to undergo programmed cell death at early stages after tissue differentiation (Koroleva et al., 2010). Myrosinases, on the other hand, are often localized to vacuoles of special idioblasts called myrosin cells whose distribution may vary between different species, organs of the plant, and according to developmental stage (Andréasson et al., 2001). Based on promoter reporter gene analysis of Arabidopsis, other cells have also been described to contain myrosinases, such as guard cells (Barth and Jander, 2006).

The “Mustard oil bomb” theory was first proposed by Luthy and Matile (1984). This theory explained the stability of the glucosinolate-myrosinase system; which was based on spatial separation of substrate and enzyme under normal conditions. The degradation of glucosinolates by myrosinases is therefore carried out only after tissue damage (“wounding”) when the mechanical damage disrupt the spatial sequestration of enzyme and substrate allowing catalysis to occur.

Even though no other proteins are required for primary glucosinolate hydrolysis except for myrosinases, there are two groups of proteins called myrosinase binding proteins (MBP) and

myrosinase associated proteins (MyAP) which form complexes with myrosinases in oilseed rape (*Brassica napus*), but the roles of these complexes have not been fully understood (Rask et al., 2000).

Studies in *B. napus* led to the identification of MBP in seeds, seedlings, pistils and sepals. Along with MBP's other proteins have been identified based on immunoreaction to MBP antibody, although those proteins (MBPRP) are unable to form complexes with myrosinases (Kissen et al., 2009). Two MBPs were identified in floral organs of *A. thaliana,* but their function is yet unknown (Tackechi et al., 1999). These proteins were not recognized by the MBP antibody (raised to *B. napus* MBPs) and the presence of functional MBP in *A. thaliana* was questioned (Andréasson et al., 2001). However, several proteomic studies (Carter et al. 2004; Casasoli et al. 2007; Chen et al. 2006; Huttlin et al. 2007) have detected putative MBP's in *A. thaliana.* MBP's were detected in myrosin cells of 7 day old seedlings of *B. napus* (Geshi et al., 1998) and proteomic studies in *A. thaliana* have detected MBP in vacuoles (Carter et al., 2004; Chen et al., 2006).

Myrosinase associated proteins (MyAP) were detected in seeds of *B. napus* as a glycoprotein in myrosinase complexes (Falk et al., 1995). In *A. thaliana* some genes have been identified based on sequence similarity with MyAP of *B. napus,* although only Epithiospecifier modifier 1 (*ESM1*) has been characterized. ESM1 expression was detected in rosette leaves of *A. thaliana* but its subcellular localization has not been clearly established (Zhang et al., 2006). It appears that ESM1 interacts with the epithiospecifier protein (ESP), albeit it has not yet been proven (Kissen et al., 2009).

Epithiospecifier proteins (ESPs) and thiocyanate forming protein (TFP) are not directly involved in glucosinolate hydrolysis, but they seem able to transform the primary product of hydrolysis (the aglycone) or a derivative of that into epithionitriles or thiocyanates (Figure 1)(Kissen et al., 2009; Lambrix et al., 2001). In addition many other products can be formed such as nitriles, thiocyanates and oxazolidine-2-thiones. Epithionitriles and thiocyanates appear to be less toxic than isothiocyanates and appear to have an important role as signals of certain pest parasitoids (Wittstock, personal comunication). However, not all Brassicaceae species have detectable activity of ESP or TFP, and within *A. thaliana* some ecotypes presents ESP activity

and some do not (Kissen et al., 2009). The common Columbia ecotype lacks functional ESP while for example Landsberg *erecta* contains ESP (Zhang et al., 2006).

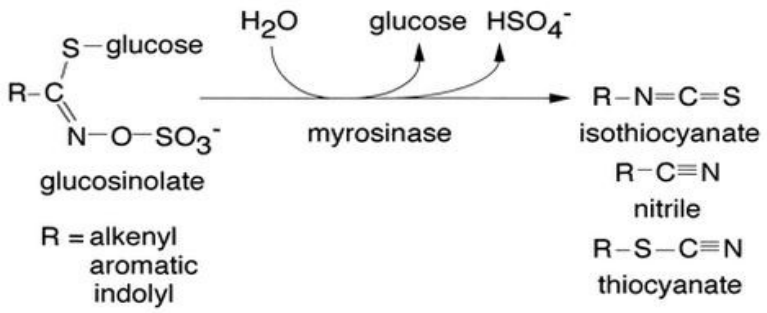

Figure 1. Diagram showing the main products of glucosinolate degradation. ESP and TFP are required for nitrile and thiocyanate formation, respectively. Taken from Hara et al., 2000.

The hydrolysis products (Figure 1) act as defence molecules since they have toxic effects on insects and some pathogens due to their electrophilic nature causing alkylation of nucleophiles present e.g. in membrane proteins and DNA. However, some specialist insects have developed mechanisms to overcome this plant defence. Ratzka and colleagues (2002) described that *P. xylostella* has gut sulphatases, which cleave the sulphur residue of glucosinolates (Figure 1) and prevent their hydrolysis by myrosinase not being recognized as substrate any longer. *Pieris rapae* posses an endogenous nitrile specifier protein (NSP) capable of converting isothyocianates to nitriles, which are less toxic (Wittstock et al., 2004).

Isothiocyanates are the most common product after hydrolysis, although, epithionitriles or nitriles are produced in the presence of either ESP or NSP, respectively (Bones and Rossiter, 2006). Allyl Isothiocyanates (AITC) is the most abundant and toxic compound formed by glucosinolates hydrolysis in *B. juncea.* AITC is the major degradation product from the allylglucosinolate sinigrin which is highly enriched in mustard plants and have insecticide potential and negative effects on *Cyclocephala spp* populations (Noble et al., 2002). Further, Brown and Morra (1997) reported anti-fungal and anti-bacterial activity of isothiocyanates. Biofumigation properties of Brassica crops have been known for long and useful in organic pharming (Gimsing and Kirkegaard, 2009).

Temperature, pH conditions and the presence of cofactors may alter the result of the hydrolysis of glucosinolates (Kissen et al., 2009). Catalytic activity of myrosinases is

modulated by ascorbic acid where low concentrations activate the enzyme, whereas high concentrations are inhibitory (Burmeister et al., 2000). It is worth nothing that the oxidation product of ascorbic acid, dehydroascorbic acid, does not activate myrosinases (Shikita et al., 1999). Even though myrosinases are capable of hydrolysing glucosinolates in the absence of ascorbic acid, the reaction is generally much slower. In the presence of ascorbic acid a radish myrosinase was stimulated ten-fold compared to the control without ascorbic acid (Shikita et al., 1999).

Andersson and colleagues (2009) described the effects of ascorbic acid on recombinant TGG1, TGG4 and TGG5 proteins overexpressed in *Pichia pastoris*, which reached maximal myrosinase activity at a concentration around 0.7 mM. However, the relative activation of different myrosinases varied with the ascorbate concentration used. It appears that ascorbic acid acts as a cofactor in the release of the glucose residue from myrosinase (Burmeister et al., 2000), i.e. the second step of the reaction. This was based on structure modelling analysis, and has not been confirmed by biochemical or kinetic studies.

The Arabidopsis mutants *vtc1-1* and *vtc4-1* have point mutations in genes encoding enzymes with important roles in an ascorbic acid biosynthetic pathway. The *vtc1-1* plants contain 30% of the ascorbic acid content of wild type (Mukherjee et al., 2010). The *VTC1* gene encodes a GDP-mannose pyrophosphorylase, which is involved in the predominant pathway of ascorbic acid synthesis (Figure 2). The *VTC4* gene encodes a L-Gal-1-phosphate phosphatase required

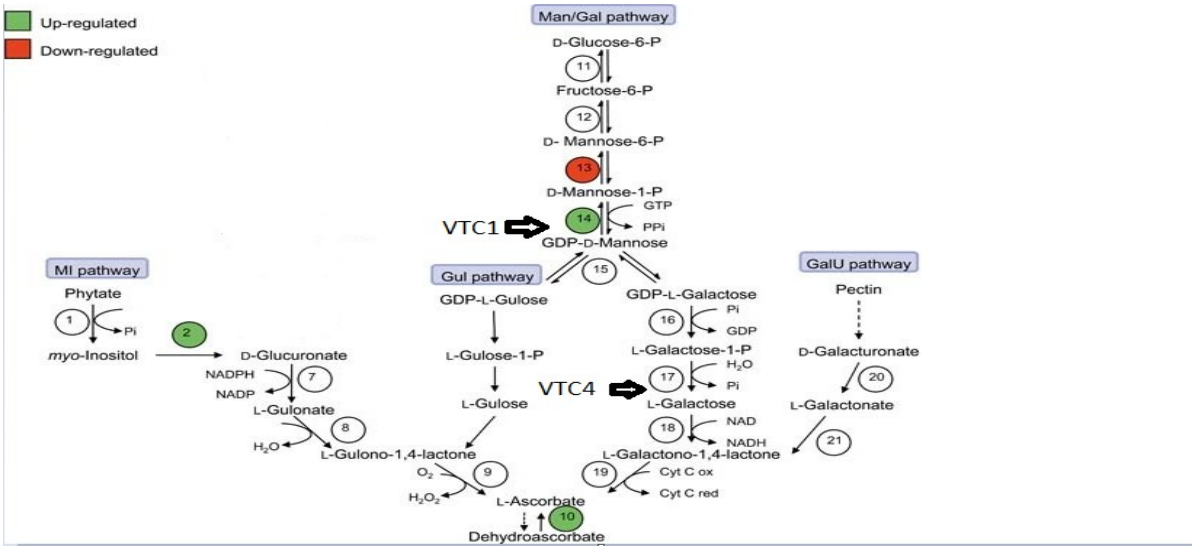

Figure 2. Ascorbic acid biosynthesis pathways. Taken from Goggin et al. (2010)

for ascorbic acid and myoinositol synthesis (Figure 2) (Torabinejad et al., 2009). The *vtc4-1* mutant presents a reduction of 50% in ascorbic acid content compared with wild type (Muckjerjee et al., 2010).Ascorbic acid mutants seem more susceptible to herbivore attack since the lower level of ascorbic acid affects the breakdown of glucosinolates (Goggin, Avila and Lorence, 2010). On the other hand, *vtc1-1* and *vtc4-1* plants present resistance to the bacterial pathogen *Pseudomonas syringae* along with a high level of salicylic acid (Muckjerjee et al., 2010).

2. AIM OF THE WORK

In order to investigate a role of ascorbic acid for myrosinase activity and product formation in *Arabidopsis thaliana,* myrosinase activity was measured in the Col-0 ecotype and compared to the ascorbic acid deficient mutants *vtc1-1* and *vtc4-1.* Along with myrosinase activity measurement, myrosinase complexes of the three genotypes were characterized through Western blot analysis and immunoprecipitation.

The effect of allyl isothiocyanate on recombinant protein performance was measured through myrosinase activity analysis to determine if there is any enzyme inhibition by a common product of the glucosinolate-myrosinase system.

3. MATERIALS AND METHODS

3.1 PLANT GROWTH CONDITIONS

Seeds of ascorbic acid deficient mutants *vtc1-1* and *vtc4-1* (obtained from NASC) along with Col-0 wild type were put out on soil-vermiculite mix (3:1) in separate pots without previous sterilization. In order to optimize growth conditions seedlings were transplanted into single pots 15 days after germination. Temperature (20°C) and light conditions (16 h/ 8h, L/D; ~200 µmol m^{-2} s^{-1}) were kept constant using controlled environment provided in a growth chamber.

3.2 PROTEIN EXTRACTION FROM LEAVES

Leaves of Col-0, *vtc1-1* and *vtc4-1* were harvested from 35 day-old plants. Subsequently, 1 gr of leaves of each genotype were ground with 2 mL extraction buffer (250 mM Tris-HCl, pH7, 2.5 mM EDTA, 1 mM PMSF) using a pre-chilled mortar and pestle. The lysates were transferred into 1.5 mL Eppendorf tubes to be homogenized by vortexing. Then, the extracts were centrifuged at 12,500 rpm at 4°C for 10 minutes. The supernatant was recovered and kept on ice. Myrosinase activity and protein estimation was performed with supernatants of each genotype.

3.3 SODIUM-DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

4-20% Tris-Glycine precast gels (CBS-Scientific) were used for protein separation. Samples were mixed with 4x LDS sample buffer and 0.1 M DTT. Subsequently, samples were heated at 95°C to denature the proteins prior to loading into the gel. The gel was assembled in the chamber, which was filled with Tris-Tricine SDS-Running buffer (CBS-Scientific). The gel was run at room temperature at 110 mA for 60 minutes. A protein molecular mass standard (Fermentas Page ruler SM0671 or SM0661) was also loaded into the gel. The gel was either incubated overnight in Coomassie Brilliant blue dye in order to visualize the proteins present on the gel or used for western blotting.

3.4 WESTERN BLOT ANALYSIS

In order to detect myrosinases from total protein extract and analyze any differences among Col-0, *vtc1-1* and *vtc4-1,* an immunoblot analysis was performed. The gel containing the proteins was placed along with a PVDF membrane and three pieces of Whatman paper into a cassette in order to transfer the proteins onto the membrane (Hybond C, Amersham). The cassette was filled with Transfer buffer (25mM Tris, pH8.5, 192mM glycine, 10% methanol) and placed into the chamber for 90 minutes at 30 mA. Then, the membrane was immersed in blocking solution (PBS, 1% milk powder) for 60 minutes at room temperature. Subsequently, the membrane was incubated with either 1:100 dilution of a mouse monoclonal antibody (3D7) raised to oilseed rape myrosinases or 1:500 dilution of a rabbit polyclonal antibody (antiTGG4) raised to recombinant TGG4 overnight at 4°C in order to detect myrosinases. After incubation with primary antibody, the membrane was placed in PBS + 0.1% Tween 20 for 30 minutes. Subsequently, the membrane was exposed for 90 minutes to either a goat anti-mouse HRP secondary antibody (for monoclonal primary antibody) or swine anti-rabbit HRP secondary antibody (previous incubation with polyclonal primary antibody). Chemiluminescence reagents were used to detect the presence of myrosinases, which were visualized by placing the membrane in the imaging system (Fujifilm LAS-3000).

3.5 TGG4 ANTIBODY PURIFICATION AND COUPLING TO DYNABEADS

Serum (2 ml) from a rabbit ("Kanin 218/02 TGG4A") was loaded into a packed column of protein A (Amersham) in order to purify antiTGG4 polyclonal antibodies. The antibodies were eluted by adding 20 ml of 1 M glycine (pH 3) and were collected as 1 ml fractions that were neutralized by the addition of 100 µl of 1M Tris buffer (pH 9). Protein concentration was measured in a microplate reader (Fluostar Omega) at 280 nm. The best fractions were pooled and placed in a dialysis cassette (Slide-A-Lyzer 10K) (Pierce) to remove excess of salts and nitrogen containing compounds. In parallel, Dynabeads MyOne™ Carboxylic Acid (Invitrogen) were activated according to the protocol provided by Invitrogen. The coupling chemistry is based on carboxylic acids of proteins that react with amine derivatives created by the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC). The protein A purified

antibodies (400 µg) were added to the activated dynabeads for coupling. The sample was incubated in a roller overnight at 4°C. The unbound antibody was removed after putting the samples in a magnetic stand and the beads were washed before use and then resuspended in 50mM Na-phosphate buffer, pH 7.
In order to remove Rubisco from the samples and prevent interference of subunits on SDS-page rubisco antibodies (Agrisera ®) were coupled to magnetic beads as describe above.

3.6 IMMUNOPRECIPITATION

To pull out myrosinases and associated proteins from whole leaf protein extracts of Col-0, *vtc1-1* and *vtc4-1,* 10 µl of lysate of each genotype were placed in separate Eppendorf tubes along with 150 µl of resuspended dynabeads coated with antiTGG4 antibody. Samples were incubated in a shaker for 90 minutes at 4°C. Then, samples were placed in a magnetic stand and the supernatant was removed. The immunoprecipitation (IP) products were washed with PBS+ 0.1% Tween, PBS + 500 mM NaCl and water and resuspended in 20 µl of loading dye to perform SDS-PAGE as previously described.

3.7 MYROSINASE ASSAY

Glucose is released as a sub-product together with the aglycone of sinigrin (allyl side chain glucosinolate) during breakdown by myrosinases (Fig. 1). In order to determine differences in myrosinase activity among Col-0, *vtc1-1* and *vtc4-1*, myrosinase assay was performed. Supernatants (10 µl) of protein extracts were dissolved in 50 mM Na-citrate (pH 4.5) to a final volume of 25 µl. Then, 62 µl of 12.5 mM sinigrin (Sigma) was added. The reaction was incubated in a thermo-mixer for 60 minutes at 37°C and 350 rpm. The reaction was stopped by placing the samples at 95°C to inactivate myrosinase. The samples were put on ice to let them cool before adding 250 µl of glucose GLUC-PAP reagent (Randox Co.). The samples were incubated at 37°C for 15 minutes. Then, the samples were transferred into a 96 well microplate and the amount of glucose was quantified at 550 nm using a microplate reader (Fluostar Omega). The coupled enzyme assay is based on that glucose is oxidized liberating hydrogen peroxide that further reacts to produce a coloured product that is finally measured.

The aglycone can form many different products so there is no easy way to study these final products. The results were compared to a glucose standard curve (GLUC-PAP Cal., Randox Co.) run in parallel with the samples.

3.8 BRADFORD PROTEIN ASSAY

Bradford assay was run to determine protein concentration in the extract supernatants of Col-0, *vtc1-1* and *vtc4-1* and, in this way, be able to calculate specific myrosinase activity per µg of protein for each genotype. Clear crude extract (2 µl) was loaded into a 96-well microplate and mixed with 8 µl of water and 200 µl of dye reagent. A protein standard curve was also prepared in order to compare the protein concentration of the samples to known concentrations. After 15 minutes incubation the samples were analysed by a microplate reader (Fluostar Omega) at 595 nm.

3.9 Pichia pastoris GROWTH AND INDUCTION

Overnight cultures (5 ml) of cells of *Pichia pastoris* clones expressing the TGG1 or TGG4 gene were placed in 200 ml BMGY medium for biomass accumulation in a shaker at 28°C and 130 rpm. When the OD value of the culture was around 2, the culture was collected in 50 ml Falcon tubes and centrifuged at 5000 rpm for 5 minutes. Supernatants were discarded and the pellets were resuspended and placed in 500 ml BMMY medium kept at 28°C and 130 rpm in Fernbach flasks to allow good aeration for four days to induce expression of recombinant proteins. Methanol (50 ml of 10% solution) was added on a daily basis during four days to maintain a constant concentration of methanol in the medium. After four days of induction the cultures were centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and the pellets were pooled.

3.10 RECOMBINANT PROTEIN EXTRACTION FROM *Pichia pastoris* cells.

An equal volume of start buffer (20mM Tris-HCl, pH 7.5, 40 mM Imidazole, pH 7.5, 500 mM NaCl, 1mM PMSF) was added to the pooled pellet. The mixture was homogenized by pipetting

up and down several times before transfer into 2 ml tubes containing 0.5 mm glass beads. The *P. pastoris* cells were broken by putting the 2 ml tubes in the bead beater for 1 minute at maximum speed followed by 1 minute cooling in an ice bath. In order to optimize the protein extraction this step was repeated three times. The supernatants were pooled and centrifuged at 15,000 rpm at 4°C until no pellet was observed. Then, the crude extract was filtrated through a 0.2 μm filter.

3.11 IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY (IMAC)

Since recombinant proteins TGG1 and TGG4 have a his-tag in the C-terminus (not to disturb the N-terminal leader sequence for the ER), IMAC is a suitable and efficient technique to purify them from the cell crude extract. His-TALON gravity columns (Clontech) were equilibrated by adding 7 ml of equilibration buffer (50mM sodium phosphate, pH 7.4, 300 mM NaCl). Subsequently, the crude extract was placed into the column. Unbound proteins were removed by adding 8 ml of wash buffer (50mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 7.4). Recombinant proteins were eluted from the column by adding 8 ml of elution buffer (50mM sodium phosphate, 300 mM NaCl, 150 mM imidazole, pH 7.4). One ml fractions were collected and analyzed for myrosinase activity and protein (Bradford) and also run on SDS-PAGE. The most active fractions were pooled and stored at 4°C before further use.

3.12 SUICIDE INACTIVATION OF MYROSINASE

In order to better suit these experiments the volumes of the myrosinase assay was changed. Recombinant TGG1 or TGG4 proteins were dissolved in 50 mM sodium citrate up to a volume of 79 μl. A 1 ul aliquot of allyl isothiocyanate (AITC) dissolved in 100% ethanol (final concentration 1%) was added to each sample to determine the effect of different concentrations of AITC on myrosinase activity. The samples were pre-incubated for 1 hour at 37°C and 350 rpm. Then, 20 μl of 10 mM sinigrin (Sigma) were added to the samples, which were incubated for 2 hours (30 min for TGG4 only). The reaction was stopped by placing the samples at 95°C for 5 minutes. Myrosinase activity was determined through quantification of glucose as described previously in the myrosinase assay.

4. RESULTS

4.1 Col-0, *vtc1-1* AND *vtc4-1* ANALYSIS

Phenology of plants

The seeds from Col-0, *vtc1-1* and *vtc4-1* showed an uneven germination where *vtc4-1* was the most delayed in germination. Compared to Col-0 and *vtc1-1* seedlings of *vtc4-1* presented slow growth and slightly unhealthy appearance with yellowish leaves during the first developmental stages. However, after floral stalk development, the appearance of the three genotypes was normal. The criteria of which stage to select for harvesting leaves for subsequent experiments was based on chronological age, and Col-0, *vtc1-1* and *vtc4-1*leaves were harvested after 35 days of planting at which time the appearance was similar between the genotypes.

Myrosinase activity of plants

Differences in myrosinase activity were found when Col-0 WT, *vtc1-1* and *vtc4-1* lysates were evaluated through myrosinase assay and protein estimation. In terms of specific activity, Col-0 and the *vtc1-1* mutant were similar with a value of 30 nmol min^{-1} mg $protein^{-1}$ (0.5 nkat mg $protein^{-1}$), whereas the *vtc4-1* mutant showed a lower activity, with a value of 19 nmol min^{-1} mg $protein^{-1}$. v*tc4-1* specific activity represents 66% of the activity of both Col-0 and *vtc1-1*(Figure 3.)

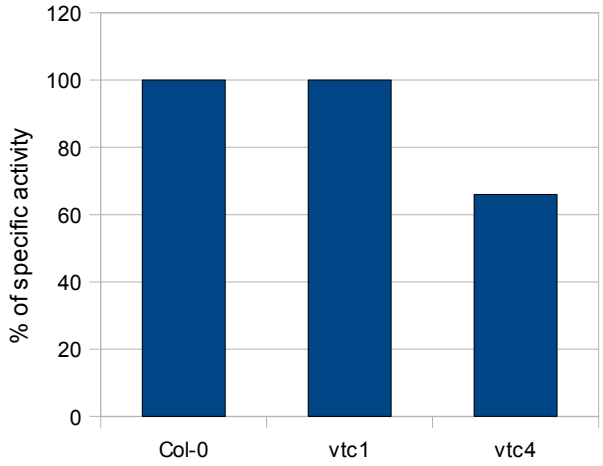

Figure 3. Myrosinase specific activity of Col-0 *vtc-1 and vtc-4* at pH 4.5. Specific activity (nmol glucose min^{-1} mg $protein^{-1}$) data was plotted as percentage, taking the activity of Col-0 as 100%.

Myrosinase protein expression of plants

Polyclonal (antiTGG4) and monoclonal (3D7) antibodies were used to probe myrosinases and detect differences among Col-0, *vtc1-1* and *vtc4-1*. Slight differences were found when using the 3D7 antibody (Fig. 4 and 5 lanes A,B,C). Analysis of *vtc4-1* (lane A), *vtc1-1* (lane B) and Col-0 (lane C) displayed three bands with a molecular mass of 59 kDa, 26.5 kDa and 14 kDa. In addition, Col-0 (lane C) also showed a 44 kDa band.

However, when myrosinases were probed with the antiTGG4 antibody, *vtc1-1* (Fig. 4 lane F; Fig 5 lane E) displayed one band only with a molecular mass of 78.5 kDa, whereas Col-0 (Fig.4 and fig. 5 lane G) and *vtc4-1* (Fig 4lane E; Fig 5 lane F) showed two bands with molecular masses of 83 kDa and 66 kDa. Additionally, Col-0 presented a band with a molecular mass of 35 kDa (Fig. 4). In a subsequent experiment (Fig. 5) Col-0 displayed an additional band just below the 35 kDa band. When the positive control was probed by the antiTGG4 antibody (Fig. 4 and 5 lane D), it showed a smear with e.g. a 68 kDa band. When 3D7 was used on the positive control (Fig. 4 and 5 left side lane A) no band was visible, indicating lack of an appropriate epitope in the recombinant protein.

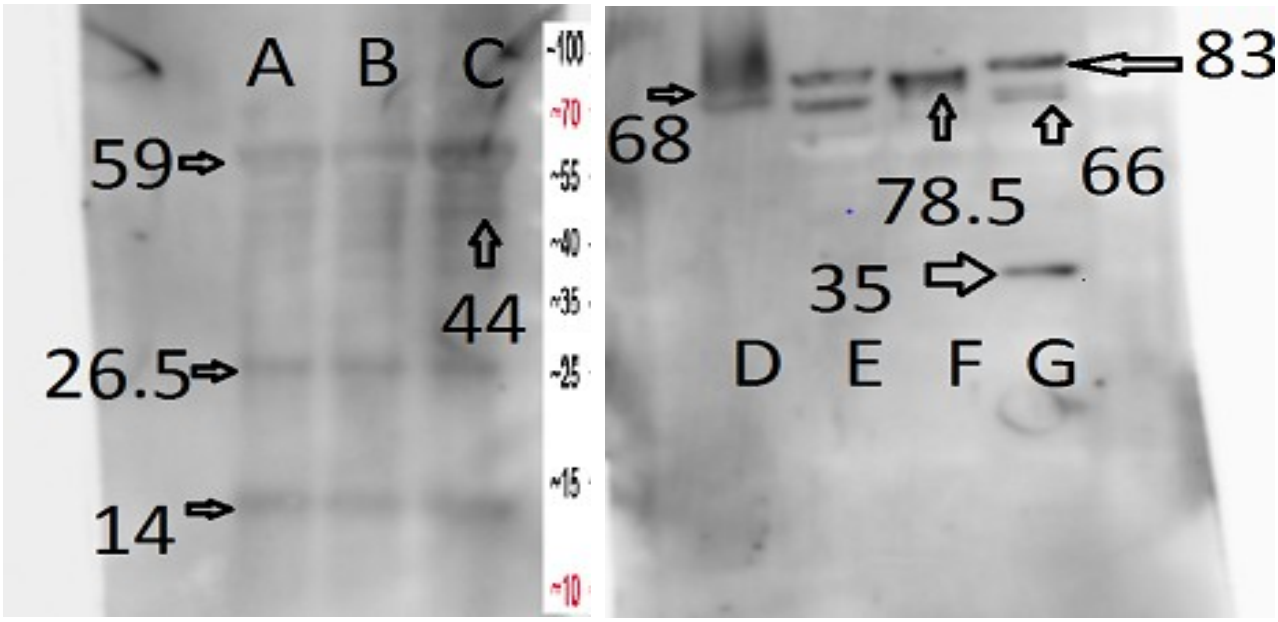

Figure 4. Western blotting to probe myrosinases from leaf lysates of Col-0 (lane C and G), *vtc1-1* (lane B and F) and *vtc4-1* (lane A and E). 3D7 Monoclonal antibody (lanes A, B and C) and antiTGG4 polyclonal antibody (lanes D,E, F and G) were used for detection. Lanes A, B, C, E, F and G contain 30 µg of protein, whereas lane D contains

15 ng of TGG4 recombinant protein as positive control. The sizes of the protein mass standard is indicated in kDa besides the gels.

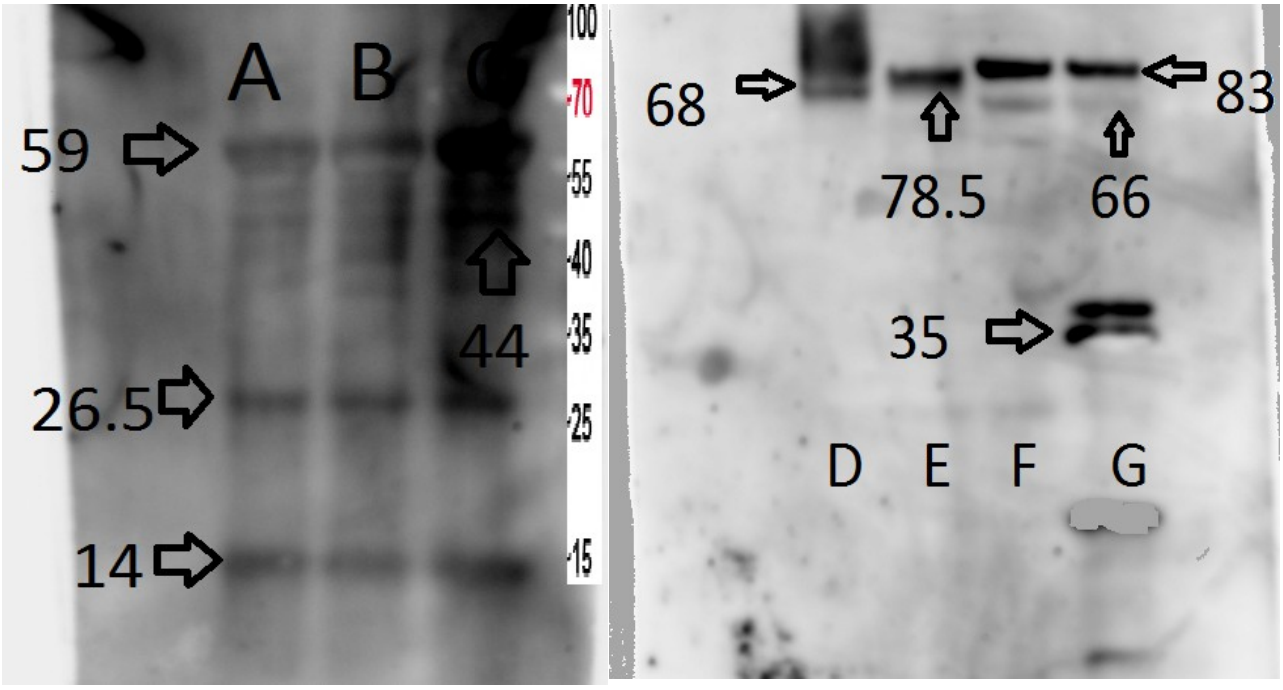

Figure 5. Western blotting to probe myrosinases from leaf lysates of Col-0 (lane C and G), *vtc1-1* (lane B and E) and *vtc4-1* (lane A and F). 3D7 Monoclonal antibody (lanes A, B and C) and antiTGG4 polyclonal antibody (lanes D,E, F and G) were used for detection. Lanes A, B, C, E, F and G contain 30 µg of protein, whereas lane D contains 15 ng of TGG4 recombinant protein as positive control. The sizes of the protein mass standard is indicated in kDa besides the gel.

Purification of antibodies

Anti-TGG4 polyclonal antibodies were enriched (i.e. the immunoglobulin fraction purified) from rabbit serum to be used for immunoprecipitation. After purification through a Protein A column and dialysis to remove nitrogen-containing low molecular weight compounds, the protein concentration of the enriched antibody fraction was estimated and the best fractions (Fractions 4, 5 and 6; Figure 6) were pooled.

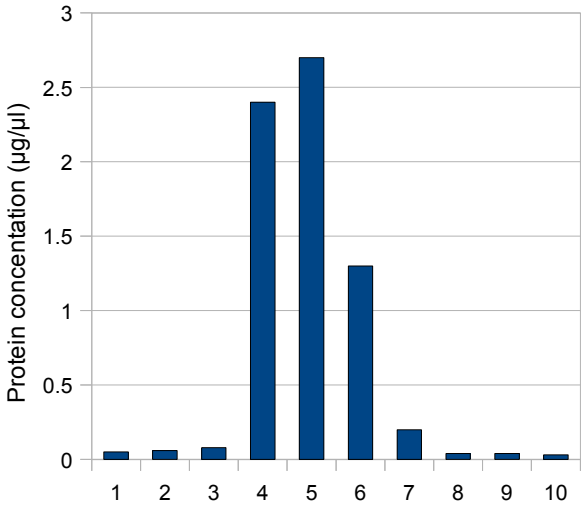

Figure 6. Protein concentration of TGG4 polyclonal antibody' fractions after Protein A column. Concentration was estimated by microplate reader at 280 nm.

The protein concentration was ~ 5 mg/ml in the pooled fractions. The antibody was then coupled to activated dynabeads (Invitrogen) with a 45% efficiency. Coated and washed dynabeads were used for the immunoprecpitation and contained approximately 225 ug of antibody/ ml beads. Likewise, Purified Rubisco antibody (Agrisera ®) was dissolved in 15 mM MES. Then the antibody was coupled to activated dynabeads (Invitrogen) with a 85 % efficiency. Coated and washed dynabeads were used prior to Immunoprecipitation (Figure 8) and contained 153 µg of antibody/ml of beads .

Immunoprecipitaion of myrosinases

In order to pull out myrosinases and associated proteins for complex formation analysis of Col-0, *vtc1-1* and *vtc4-1* crude lysates, immunoprecipitation analysis was performed. The results were analysed by SDS-PAGE (Figure 7). One band with an approximative molecular mass of 56 kDa was detected in Col-0 (Fig. 7, Lane D), *vtc1-1* (Fig. 7, Lane E) and *vtc4-1* (Fig. 7, Lane F). A blurred band with a molecular mass of 42 kDa was detected in Col-0 (Fig. 7 Lane D) only. A previous experiment with Col-0 only (Figure 8) presented the same pattern of bands except for one extra band with a molecular mass of 70 kDa. In order to remove rubisco from the samples and prevent interference of the large subunit (55kDa) of rubisco (Agrisera®) in the experiment, previous 1 hour incubation of Col-0 with rubisco antibody was performed (Figure 8 lane C). The supernatant of the incubation was used for IP with TGG4 antibody. No

differences were observed between the supernatant of incubation with rubisco antibody (Figure 8 lane C) and crude extract of Col-0 (Figure 8 lane D). for that reason the incubation with rubisco antibody was removed from the IP analysis.

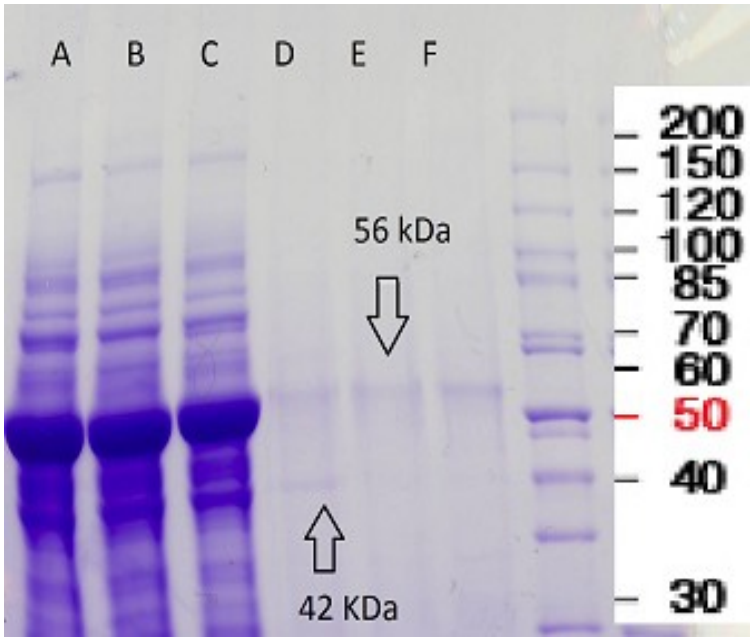

Figure 7. SDS-PAGE analysis of immunoprecipitation products using TGG4 antibody to detect myrosinases and associated proteins in crude lysates of Col-0 *vtc1-1* and *vtc4-1.* Lanes A, B and C contain crude lysate of Col-0 *vtc1-1* and *vtc4-1*, respectively. Lanes D, E and F contain the IP product of Col-0 *vtc1-1* and *vtc4-1.* The sizes of the protein mass standard is indicated in kDa.

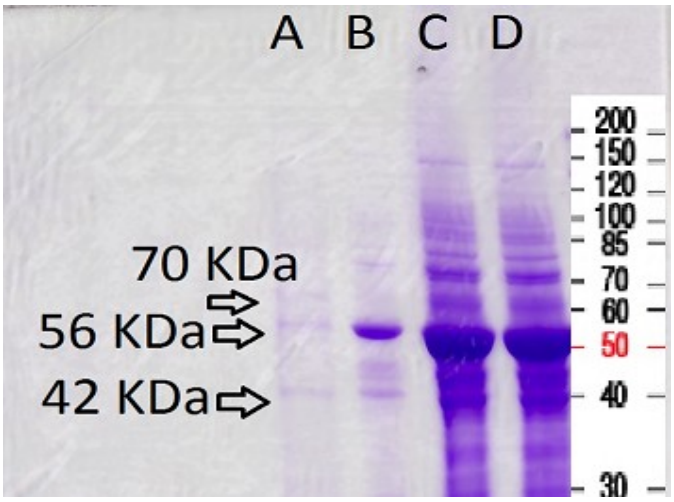

Figure 8. SDS-PAGE analysis of immunoprecipitation products using antiTGG4 antibody to detect myrosinases and associated proteins in crude lysates of Col-0. Lane A contains immunoprecipitation products of Col-0, lane B contains unbound proteins (supernatant). Lane C contains supernatant after incubation with rubisco antibody and lane D contain crude extract of Col-0. The sizes of the protein mass standard is indicated in kDa.

4.2 TGG1 AND TGG4 SUICIDE INACTIVATION

TGG1 and TGG4 recombinant proteins were extracted from *P. pastoris* clones and pulled out of the crude extracts through Immobilized metal affinity chromatography (IMAC). Bacterial contamination occurred sometimes in the *P. pastoris* cultures, which may reduce the synthesis of the recombinant proteins. Additionally, TGG1 recovery in the fractions after IMAC was poor, even though the crude extract showed high myrosinase activity. Maybe there were compounds in the crude extract that somehow blocked interaction with the cobalt resin. Prior DNA sequencing showed the sequence of the *P. pastoris* clone to be correct but use of an anti-his-tag antibody might be used to verify the presence of a his-tag in the recombinant protein.
One ml fractions were collected and myrosinase activity and protein concentration was assayed. Bacterial contamination in the cultures was the main constraint for optimal growth of *Pichia* cells. TGG1 recovery in the fractions was poor, even though the crude extract showed high myrosinase activity (data not shown). TGG4 recovery was slightly better and pure TGG4 displayed higher specific activity than TGG1 (Figure 9). The amount of TGG1 and TGG4 obtained from 0.5 L of culture was on average 1.5 ng/µl and 6 ng/ µl respectively. In order to corroborate the purity of TGG1 and TGG4, SDS-PAGE analysis was performed. No contaminants were detected on the gel (Figure 10). Apparent molecular masses of 62 kDa and 50 kDa were estimated for TGG1 (Figure 10 lanes A, B, C) and TGG4 (Figure 10 lanes D, E, F).

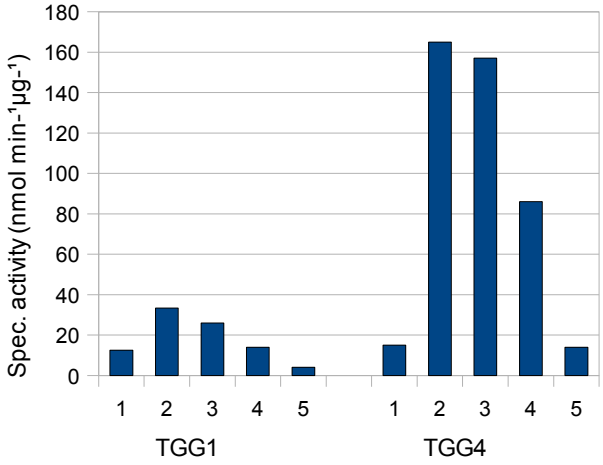

Figure 9. Specific myrosinase activity of fractions of recombinant proteins TGG1 and TGG4 after purification through IMAC.

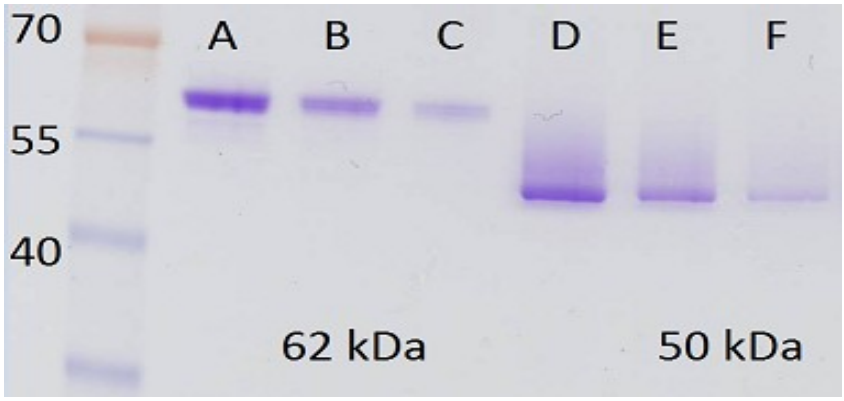

Figure 10. SDS-PAGE analysis of recombinant proteins TGG1 and TGG4. Lanes A, B and C contain TGG1, while lanes D, E and F contain TGG4.

To elucidate the effect of allyl isothiocyanates (AITC) on myrosinase activity, three concentrations (1mM, 5 mM and 10 mM) of AITC were tested on TGG1 and TGG4 recombinant proteins at pH 4.5 or pH 6. The results of suicide inactivation are summarized as follows:

Table1. Effect of different concentrations of AITC on myrosinase specific activity (nmol glucose/min*µg protein) of TGG1 and TGG4 recombinant proteins at pH 4.5 or 6. To calculate relative activity, TGG1 and TGG4 control incubations were taken as 100%.

PROTEIN	pH	AITC (mM)	Spec. activity (nmol $min^{-1}µg^{-1}$)	Relative activity (%)
TGG1	4.5	0	33	100
TGG1	4.5	1	4.12	12
TGG1	6	0	15.7	100
TGG1	6	1	10.7	68
TGG4	4.5	0	160	100
TGG4	4.5	1	130	81
TGG4	4.5	5	116	72
TGG4	4.5	10	108	67
TGG4	6	0	143	100
TGG4	6	1	121	84
TGG4	6	5	100	70
TGG4	6	10	92	64

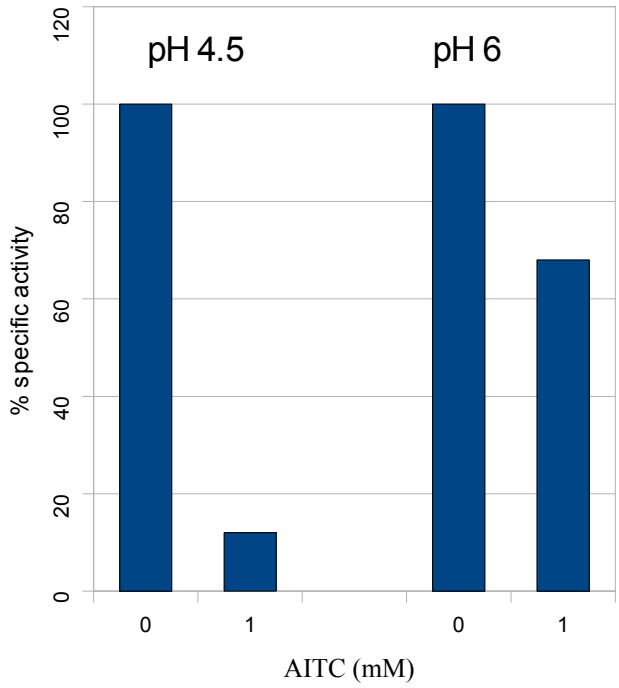

Figure 11. Myrosinase specific activity of TGG1 recombinant protein at pH 4.5 or 6 with or without 1 mM AITC. Specific activity of control incubations without AITC were taken as 100%. Each reaction contained 28 ng of TGG1 protein.

TGG1 presented higher activity when incubated at pH 4.5. Myrosinase activity of TGG1 at pH 4.5 was two-fold higher compared to that of TGG1 at pH 6 in control treatments (Table 1, Figure 11). After addition of 1 mM AITC, there was a drastic reduction in myrosinase activity of TGG1 at pH 4.5, down to 12% of the activity of the control treatment. TGG1 at pH 6 presented a reduction in the activity after addition of 1mM AITC, but the reduction was less and the remaining activity represented 68% of the control (Table 1, Figure 11). No activity was obtained for TGG1 after addition of 5 mM and 10 mM AITC at either pH 4.5 or 6.

TGG4 showed somewhat higher activity at pH 4.5. The performance of TGG4 at pH 6 and pH 4.5 was rather similar, but it is worth noting that myrosinase activity of TGG4 in control treatments was fivefold higher than that of TGG1 (Table 1, Figures 11 and 12). Even though TGG4 myrosinase activity was affected by the addition of AITC at different concentrations (Table 1, Figure 11), there was activity at both pH values tested when adding 5 mM and 10 mM AITC. There was a slight drop in activity after AITC addition, although the pattern of

activity at pH 4.5 and 6 have the same tendency and the inhibition was not as severe compared to that observed for TGG1 (Table 1, Figures 11 and 12).

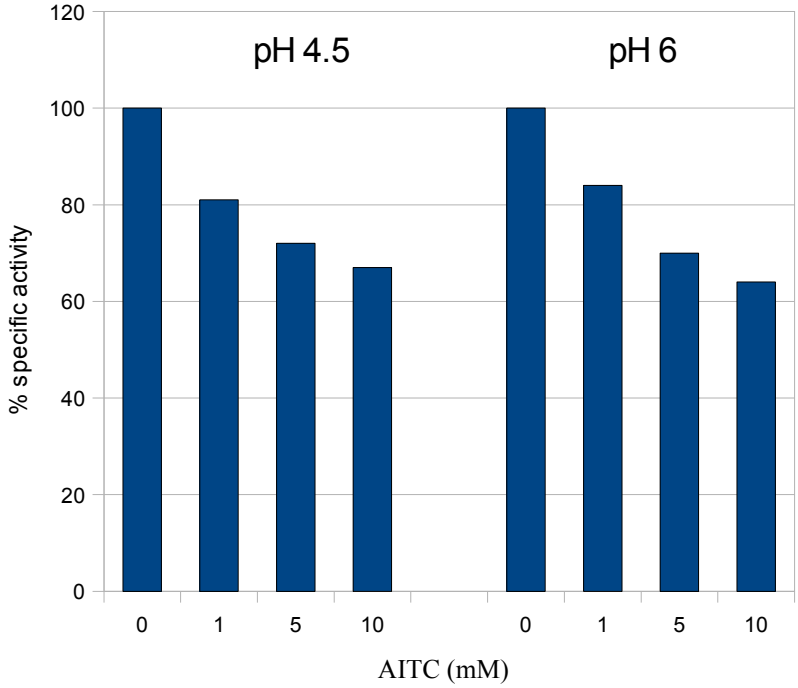

Figure 12. Myrosinase specific activity of TGG4 recombinant protein at pH 4.5 and 6 after addition of different concentrations (0, 1, 5 and 10 mM) of AITC. Specific activity of control treatments were taken as 100%. Each reaction contained 30 nanogram TGG4.

Lower concentrations of AITC were tested on TGG1 (Figure 13). Concentrations up to 100 µM did not inhibit TGG1, but 1 mM AITC did cause a down-regulation in activity (Figure 11). However, the glucose conversion of control treatment (Table 2) presented a 50% reduction compared to that of the previous experiment with TGG1 (Table 1). The inhibition when adding 1 mM AITC was about 24% compared to control treatment (Figure 12), albeit the decrease of activity was not as sharp as in the previous experiment with TGG1 (Figure 11). TGG1 was two-fold more active in pH 4.5 than in pH 6 as was seen previously in TGG1 and TGG4 (Table 1).

Table2. Effect of different concentrations of AITC on myrosinase specific activity (nmol glucose/min*µg protein) of TGG1 recombinant protein at pH 4.5 or 6. To calculate % specific activity TGG1 and TGG4 control incubations were taken as 100% .

PROTEIN	pH	AITC (µM)	Spec. activity (nmol $min^{-1}µg^{-1}$)	Relative activity (%)
TGG1	4.5	0	17.3	100
TGG1	4.5	1	17.2	99.8
TGG1	4.5	10	17.2	99.8
TGG1	4.5	100	17.2	99.4
TGG1	4.5	1000	11.9	68
TGG1	6	0	9.08	100
TGG1	6	1	9.07	99.6
TGG1	6	10	9.06	99.5
TGG1	6	100	9.06	99.6
TGG1	6	1000	5.36	59

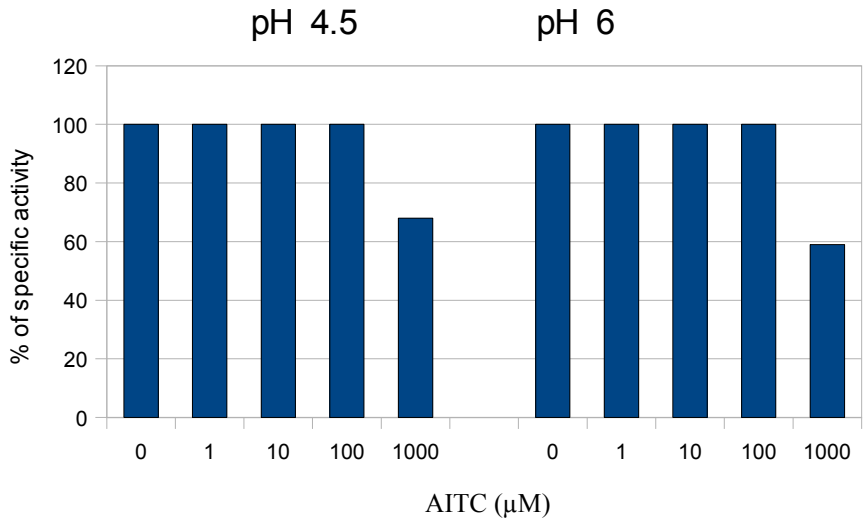

Figure 13. Myrosinase specific activity of TGG1 recombinant protein at pH 4.5 and 6 after addition of different concentrations (0, 1µM, 10 µM, 100 µM and 1 mM) of AITC. Specific activity of control incubations were taken as 100%. Each reaction contained 28 ng of TGG1

5. DISCUSSION

5.1 Col-0, *vtc1-1* AND *vtc4-1* ANALYSIS

The antiTGG4 antibody detected differences in myrosinase composition of leaves derived from Col-0 and the ascorbic acid deficient mutant *vtc1-1* (Figure 4). Western blot analysis showed three bands with approx. molecular masses of 83 kDa, 66 kDa and 35 kDa in Col-0, whereas only one band with a molecular mass of 78. 5 kDa was detected in *vtc1-1*.

The molecular mass of the band of *vtc1-1* did not match the size of any band of Col-0 or *vtc4-1.* The difference in molecular mass might be due to the fact that VTC1 gene encode an enzyme that also provides GDP-mannose for protein glycosylation (Hashimoto et al.,1997). Mannose along with fucose and N-acetylglucosamine residues constitute the extensive carbohydrate side chain attached as N-linked "sugar trees" to consensus sequences Asn-Xaa-Ser/Thr present in myrosinase naked peptide after translation in the endoplasmic reticulum (Rask et al., 2000). Myrosinases have a variable number of glycosylation sites, which seem to be important for molecular stability and solubility (Burmeister et al., 1997). Myrosinases from *S. alba* seeds present more glycosylation sites than enzymes that are expressed in leaves and roots, presumably due to the dehydrated conditions of the seeds (Figure 13). This post-transductional modification might increase the molecular mass of the proteins with 20% or more, but it is highly variable between the different isoforms of myrosinases (Rask et al., 2000).

Molecular mass differences are not only due to the size of the sugar tree, but also the fact that protein charges may occur, that are not masked appropriately by the SDS molecules causing erroneous migration in the gel (James, 1990). Another problem is that the sugar trees are extensively modified in the Golgi apparatus and this is not a homogeneous process causing blurring of the bands. Finally heterologous expression of a glycoprotein will not result in the same glycosylation pattern as the endogenous protein due to differences among different organisms, which should be kept in mind when interpreting data (Demain and Vaishnav, 2009). Since the GDP-mannose synthesizing gene is partially defective in *vtc1-1* the molecular

mass of glycoproteins including myrosinases are most probably altered in addition to effects on ascorbic acid.

```
Salba      ------------------------DEEITCQENLPFTCGNTDALNSSSFSSDFIFGVAS 35
TGG1       -------MKLLMLAFVFLLALATCKGDEFVCEENEPFTCNQTKLFNSGNFEKGFIFGVAS 53
TGG4       MAIPKAHYSLAVLVLLFVVVSSSQKVCNPECKAKEPFHCDNTHAFNRTGFPRNFTFGAAT 60
                                    :  *: : ** *.:*. :*  .*  .* **.*:

Salba      SAYQIEGTIGRGLNIWDGFTHRYPNKSGPDHGNGDTTCDSFSYWQKDIDVLDELNATGYR 95
TGG1       SAYQVEGGRGRGLNVWDSFTHRFPEKGGADLGNGDTTCDSYTLWQKDIDVMDELNSTGYR 113
TGG4       SAYQIEGAAHRALNGWDYFTHRYPEK-VPDRSSGDLACDSYDLYKDDVKLLKRMNVQAYR 119
           ****:**   *.** ** ****:*:*  .* ..** :***:  ::.*:.::..:*  .**

Salba      FSIAWSRIIPRGKRSRGVNEKGIDYYHGLISGLIKKGITPFVTLFHWDLPQTLQDEYEGF 155
TGG1       FSIAWSRLLPKGKRSRGVNPGAIKYYNGLIDGLVAKNMTPFVTLFHWDLPQTLQDEYNGF 173
TGG4       LSIAWSRVLPKGRLTGGVDENGITYYNNLINELKANGIEPYVTIFHWDVPQTLEDEYGGF 179
           :******::*:*: : **:  .* **:.**. *  :.: *:**:****:****:*** **

Salba      LDPQIIDDFKDYADLCFEEFGDSVKYWLTINQLYSVPTRGYGSALDAPGRCSPTVDPSCY 215
TGG1       LNKTIVDDFKDYADLCFELFGDRVKNWITINQLYTVPTRGYALGTDAPGRCSPKIDVRCP 233
TGG4       LSTRIVEDYTNYAELLFQRFGDRVKFWITLNQPFSLATKGYGDGSYPPGRCTG----CEL 235
           *.  *::*:.:**:* *: *** ** *:*:** :::.*:**. .  .****:

Salba      AGNSSTEPYIVAHHQLLAHAKVVDLYRKNYT-HQGGKIGPTMITRWFLPYNDTDRHSIAA 274
TGG1       GGNSSTEPYIVAHNQLLAHAAAVDVYRTKYKDDQKGMIGPVMITRWFLPFDHSQ-ESKDA 292
TGG4       GGDSGVEPYTVAHNQLLAHAKTVSLYRKRYQKFQGGKIGTTLIGRWFAPLNEFSELDKAA 295
           .*:*..*** ***:****** .*.:**..*   * * **..:* *** * :. .  .  *

Salba      TERMKEFFLGWFMGPLTNGTYPQIMIDTVGERLPSFSPEESNLVKGSYDFLGLNYYFTQY 334
TGG1       TERAKIFFHGWFMGPLTEGKYPDIMREYVGDRLPEFSETEAALVKGSYDFLGLNYYVTQY 352
TGG4       AKRAFDFFVGWFLDPLVYGKYPTIMREMVGDRLPEFTPEQSALVKGSLDFLGLNYYVTQY 355
           ::*   ** ***:.**. *.** ** : **:***.*:  :: ***** ********.***

Salba      AQPSPNPVNSTNHTAMMDAGAKLTYINASGHYIGPLFEKDKADSTDNIYYYPKGIYSVMD 394
TGG1       AQNNQTIVPSDVHTALMDSRTTLTSKNATGHAPGPPFN---AAS----YYYPKGIYYVMD 405
TGG4       ATDAP---PPTQLNAITDARVTLGFYRN-GVPIGVVAP--------SFVYYPPGFRQILN 403
           *        .   .*: *: ..*   .  *   *              *** *:  :::

Salba      YFKNKYYNPLIYVTENGISTP--GDENRNQSMLDYTRIDYLCSHLCFLNKVIKEKDVNVK 452
TGG1       YFKTTYGDPLIYVTENGFSTP--GDEDFEKATADYKRIDYLCSHLCFLSKVIKEKNVNVK 463
TGG4       YIKDNYKNPLTYITENGVADLDLGNVTLATALADNGRIQNHCSHLSCLKCAMKDG-CNVA 462
           *:* .* :** *:****.:    *:     :  *  **:  ****. *. .:*:   **

Salba      GYLAWALGDNYEFNKGFTVRFGLSYIDWNNVT-DRDLKKSGQWYQSFISP---------- 501
TGG1       GYFAWSLGDNYEFCNGFTVRFGLSYVDFANITGDRDLKASGKWFQKFINVTDEDSTNQDL 523
TGG4       GYFAWSLMDNYEFGNGYTLRFGMNWVNFTNPA-DRKEKASGKWFSKFLAK---------- 511
           **:**:* ***** :*:*:***:.:::: * : **. * **:*:..*:

Salba      ------------------
TGG1       LRSSVSSKNRDRKSLADA 541
TGG4       ------------------
```

Figure 14. Sequence alignment of myrosinases of *S. alba* seeds and A. thaliana leaves (TGG1) and roots (TGG4). N-glycosylation prediction was based on NetNGlyc 1.0 server. Prediction sites are underlined and bold while proven sites are displayed in red.

Differences in myrosinase composition (Figure 4) did not affect specific myrosinase activity, since Col-0 and *vtc1-1* had the same performance (Figure 3). TGG1 and TGG2 transcripts were detected by RT-PCR in leaves and above-ground tissues (Andersson et al., 2009). The *tgg1tgg2* double knock-out mutant does not display myrosinase activity in above-ground tissues, which demonstrates that those genes are essential for glucosinolate breakdown in leaves (Barth and Jander, 2006). However, a *tgg1* mutant showed around 5% of myrosinase activity in above-ground tissues compared to Col-0 and a *tgg2* mutant, showing that TGG1 contributes the majority of detectable activity (Barth and Jander, 2006). GUS lines driven by TGG1 and TGG2 promoters allowed differentiation of their spatial expression pattern (Barth and Jander, 2006). TGG1:GUS lines showed expression in guard cells and in phloem associated cells of above-ground tissues, whereas TGG2 promoter:GUS showed expression in phloem associated cells only (Barth and Jander, 2006). At least the *TGG1* gene must be present in *vtc1-1*as its catalytic activity resembles Col-0 activity and, as previously mentioned, TGG1 gene accounts for the majority of myrosinase activity in above-ground tissues of *A. thaliana.*

The vtc4 mutant presented a myrosinase composition similar as that of Col-0 in western blot analysis except for one band (35kDa)(Figure 4). However, the similarity between Col-0 and *vtc4-1* regarding the bands on the membrane did not correlate with the performance of *vtc4-1* concerning myrosinase activity, which showed a drop of 32 % compared with Col-0 (Figure 3). The *vtc4* seeds were highly sensitive to surface sterilization and showed slow germination. Apparently the mutation affects general properties of the plant. Besides, insects seem to feed more on *vtc4-1* plants than on *vtc1-1* and col-0 plants; which was seen after an unintentional fly and aphid infestation in the growth chamber, indicating that these plants are compromised in wound responses.

The *VTC4* gene codes for a IMP bifunctional enzyme (Figure 2) that is involved in synthesis of ascorbic acid via myoinositol (Torabinejad et al., 2009). VTC4 homologs in tomato presented the highest transcription level in tissues undergoing cell division (Gillapsy et al., 1995). Myoinositol is required for multiple processes including auxin perception, phosphorus storage, stress tolerance and oligosaccharide synthesis. It is also incorporated into compounds

such as myoinositol phosphatases, which are required for signal transduction (Torabinejad et al., 2009). The reduced levels of both ascorbic acid and myoinositol may affect the performance of *vtc4-1* at different levels of primary metabolism and directly or indirectly affect the insect defence responses in addition they may also change the host plant nutrient value for the insect as well as feeding stimulants.

Slight differences among wild type, *vtc1-1* and *vtc4-1* plants were found when myrosinases were probed with 3D7 antibody(Figure 4 and 5). Three bands in the region of 59 kDa, 26.5 kDa and 14 kDa were visualized in Col-0, *vtc1-1* and *vtc4-1*. One blurred band of 44 kDa was observed in Col-0 only. Nevertheless, the size of the bands differed to that of the bands detected with the antiTGG4 antibody. Previous findings (Andréasson et al., 2001) demonstrated the detection of a 70 kDa band (myrosinase) when probing Col-0 leaves with the 3D7 monoclonal antibody, although the epitope that is bound by the 3D7 is unknown. In the same way, TGG1 and TGG2 were detected by 3D7 antibody after immunohistochemical analysis (Andréasson et al., 2001). Taipalensuu and colleagues (1996) stated that 3D7 is unable to recognize MBP or MyAP in *B. napus* seeds, by which it is unlikely that the probed bands to be either MBP or MyAP. Inmunoblot experiments using 34:14 (MBP specific) and B1H5 (MyAP specific) antibodies would allow to see whether the recognizes bands are myrosinase related proteins and if such bands were identified by 3D7.

Unexpectedly Arabidopsis show a much higher complexity concerning MBP and MyAP genes than *B. napus*. At least17 genes with sequence homology to MBP of *B. napus* have been detected by sequence analysis studies of *A. thaliana* (Rask et al., 2000, unpublished), but only MBP1 and MBP2 have been characterized. These genes have detectable expression in flowers of *A. thaliana* only (Arabidopsis.org-1), but no experimental evidence has been reported regarding complex formation between MBP and myrosinases in *A. thaliana* (Kissen et al., 2009). On the other hand, MBP from *B. napus* have been shown to form complexes with myrosinases. MBP's role in complex formation was demonstrated in MBP anti-sense plants of *B. Napus* (Erikson et al., 2002), which were unable to form complexes.

Only one *A. thaliana* MyAP has been characterized, but no report of expression pattern was

published (Rask et al., 2000). It seems that ESM1 (MyAP) expression is restricted to *A. thaliana* rosette leaves and the molecular mass of the naked peptide is around 44 kDa (Arabidopsis.org-2). MyAP's were first detected in *B. napus* seeds as a glycoprotein in myrosinase complexes, although presumably they are not involved in complex formation (Erikson et al., 2002). MyAP's are structurally unrelated to MBP's and seem to interact with ESP (Zhang et al., 2006) affecting the ratio of conversion of isothiocyanates to nitriles, although the latter remains purely speculative.

When proteins were stained after immunoprecipitation analysis (Figure 7), Col-0 showed two bands (56 kDa and 42 kDa) with molecular masses similar to those bands present in western blot analysis with the 3D7 antibody (Figure 4 and 5). The 56 kDa band was also detected in *vtc1-1* and *vtc4-1,* but not the 42 kDa band, which correlate with the Western blot outcomings (Figure 4 and 5). None of the bands detected by the antiTGG4 antibody (Figure 4 and 5) were visualized on SDS-PAGE after immunoprecipitation analysis (Figure 7). Nevertheless, a previous immunoprecipitation experiment with Col-0 only (Figure 8) allowed to visualize a band with approx. size of 70 kDa along with two bands (56 kDa and 42 kDa) with similar molecular masses to the bands detected by the 3D7 antibody in Col-0 (Figure 4 and 5). The immunoprecipitation protocol used in the previous experiment (Figure 8) just differed in the washing step, which was less rigorous and PBS and 0.1% Tween 20 was used only. To further characterize the complexes between myrosinases and other proteins in Col-0, *vtc1-1* and *vtc4-1* it is advisable to utilise 21 day-old plants for protein extraction. Barth and Jander (2006) described an increase of myrosinase activity of Col-0 from 14 day-old seedlings to 21 day-old mature plants. Subsequently, myrosinase activity decreased as the plants aged.

5.2 TGG1 AND TGG4 SUICIDE INACTIVATION

Isothiocyanates are the result of the breakdown of glucosinolates by myrosinases as a response to wounding of the plant, tissue damage or mastication of fresh plants (Bones and Rossiter, 2006). The mechanism of toxicity of AITC seems to be related with electrophilic binding to biological thiols, alcohols and amines (Pechacek et al., 1997). AITC stability relies on different parameters including pH. Alkaline conditions along with high temperatures accelerate the decomposition of AITC , while acidic pH provides stability (Chen and Ho,

1998).

AITC did cause down-regulation in the activity of both recombinant proteins, although , total inactivation was observed only for TGG1 when the concentration of AITC was 5 mM or higher (Figure 11). TGG4 showed the highest activity in the absence of AITC. The decrease in activity of TGG4 turned out to be slight and not totally inhibitory (Figure 12). TGG1 and TGG4 were more active at pH 4.5. This finding differs from a previous report (Andersson et al., 2009) where TGG1 and TGG4 showed higher myrosinase activity at pH 6 than at pH 4.5.

When concentrations lower than 1 mM AITC were tested on TGG1 (Figure 13) no inhibitory effect was observed. However, when 1 mM AITC was added there was a decrease of myrosinase activity, as was observed previously (Figure 11). This reduction was rather similar to that in the previous experiment at pH 6. On the other hand, the decrease of activity in pH 4,5 with 1 mM AITC was highly variable (Figure 11 and 13).

It is worth noting that TGG4 was highly active and needed 30 minutes to reach maximal activity within the range of the glucose standard curve, whereas TGG1 needed two hours of incubation to show maximal activity in control treatments. Low concentration of TGG1 and TGG4 from *P. pastoris* cultures allowed use of only 28 and 30 nanograms of TGG1 and TGG4, respectively; rather than 50 nanograms as the experiment had been designed for. However, this provides only small difference in the molar ratios of enzyme to chemicals tested.

It appears that AITC is able to bind to the myrosinase and affect its catalytic activity probably forming a stable complex with myrosinases,. However, the reactivity varies between the myrosinases tested and may say something about the relative reactivity of the active site residues and provide further information concerning the kinetic mechanism and properties of different myrosinases. Isothiocyanates have been known for a long time to be reactive and harmful but also considered for that reason as a therapeutic for example to combat cancer (Wu et al., 2009). Product inactivation has been described in other contexts as well. Lantum and colleagues (2002) described the inactivation of Gluthathione Transferase Zeta (GTZ) by maleylacetone (MA) and fumarylacetone (FA). *In vitro* experiments showed that MA (substrate) and FA (product) are independently able to inactivate GTZ and such an

inactivation is time and concentration dependent. However, when GTZ was preincubated with glutathione, that is needed for the catalytic reaction but it is not consumed, the inactivating effects of FA and MA were blocked. Presumably other components of the myrosinase-glucosinolate system such as MBP or MyAP may play a central role in protecting myrosinases from inactivation by AITC *in planta*, as they form complexes with myrosinases although not required for glucosinolate hydrolysis. Maybe suicide inactivation is a mechanism to protect plant cells if for some reason myrosinase becomes active in the absence of pests and it should be noted that some glucosinolates seem to be under normal turnover during development in the absence of any stressor (Rask et al., 2000).

Even though AITC plays a central role in defence against hervibores in Brassicaceae, it might fulfil another role in inducing apoptosis in glucosinolate accumulating cells (S-cells), as isothiocyanates can induce programmed cell death(PCD) of human pre-cancerous cells (Koroleva et al., 2010). S-cells undergo apoptosis during differentiation and subsequent accumulation of glucosinolates, but the plasma membrane apparently remains at least for twon weeks after PCD (Koroleva et al., 2010). S-cells have similarities with lacticifer cells, which are present in *Hevea brasiliensis* and also undergo PCD. Lactifier cells contain a range of secondary metabolites involved in defense against insects.

5.3 CONCLUSIONS

Differences among the Columbia ecotype and the ascorbic acid deficient *vtc1-1* and *vtc4-1* mutants were observed when myrosinase activity was assayed. In the same way, western blot analysis with 3D7 and antiTGG4 antibodies revealed differences of either myrosinases or myrosinase-related proteins in the evaluated genotypes. However, the proteins were not identified. Immunoprecipitation analysis displayed differences among the genotypes, but the bands on SDS-PAGE after Coomassie staining were weak. In order to obtain stronger signal on SDS-PAGE and further characterize the mentioned genotypes, perhaps it is advisable to utilise younger plants than 35-day-old material, which may have higher content of myrosinases and related proteins.

TGG1 and TGG4 recombinant proteins were extracted from *P. pastoris* cells to test the effect of AITC on myrosinase performance. A reduction of myrosinase activity was observed in both TGG1 and TGG4 when adding 1 mM AITC but TGG1 was more affected than TGG4 and also showed a stronger pH effect.

To further characterize the myrosinase-AITC interaction adduct formation must be tested. MALDI-TOF mass spectrometry might be used for identification of the amino acid residues in TGG1 and TGG4 that are alkylated by AITC. This technique was successfully used by Fisher and colleagues (2009) to determine chemical- protein adduct formation *in vitro.* Further experiments are required to identify whether MBP and MyAP play a role in blocking myrosinase inactivation by AITC. Suicide inactivation experiments with previous incubation of myrosinase and MyAP or MBP might be useful to elucidate whether these proteins protect myrosinases from AITC.

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