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Study of a bacterial enzymatic assay for rapid screening of GRETCHEN HAGEN 3 (GH3) family

DIPLOMA THESIS

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V Olomouci dne .....

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Abstrakt

Kyselina indol-3-yloctová (IAA) je v rostlinách jednou z nejdůležitějších biologicky aktivních látek, a proto je její koncentrační hladina přísně regulována. U rostlin se můžeme setkat s různými mechanismy regulace hladiny IAA, z nichž jednou z nejdůležitějších je konjugace s aminokyselinami. Tyto reakce katalyzují enzymy z druhé podrodiny enzymové rodiny Gretchen Hagen 3 (GH3). V rámci této diplomové práce byla nedávno vyvinutá metoda pro charakterizaci enzymů z rodiny GH3 využita pro studium aktivity jednoho z jejích členů. Tato metoda je založená na heterologní produkci enzymu z housenky rolního v buňkách *Escherichia coli*. Hostitelské buňky jsou transformovány vektorem s vneseným genem vybraného GH3 enzymu. Exprese tohoto genu je dosaženo pomocí isopropyl-ß-D-thiogalaktopyranosidu (IPTG) při 20 °C. Přítomnost histidinové značky umožňuje sledování produkce rekombinantního proteinu po indukci. Enzymatická reakce probíhá přímo v transformovaných bakteriích. Produkty enzymové reakce jsou stanoveny přímým nástřikem supernatantu bakteriální kultury pomocí kapalinové chromatografie spojené s tandemovou hmotnostní spektrometrií (LC-MS/MS). Oproti dříve používaným *in vitro* přístupům není v tomto případě nutná izolace rekombinantního proteinu. V této práci byla metoda použita pro charakterizaci enzymu GH3.2 z housenky rolního.

Klíčová slova	IAA, Gretchen Hagen 3, GH3.2, rekombinantní enzym, kapalinová chromatografie, tandemová hmotnostní spektrometrie
Počet stran	60
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Abstract

Indole-3-acetic acid (IAA) is one of the most important molecules with biological activity in plants, therefore, its level must be tightly controlled. In plants, various mechanisms occur for regulation of IAA concentration, including conjugation of IAA to amino acids as one of the major inactivation pathways. These conjugation reactions are catalysed by acyl acid amido synthetases of the Group II of the Gretchen Hagen 3 (GH3) family. In this diploma thesis, a recently developed method for characterization of GH3 enzymes has been used to study the activity of one member of this family. This method is based on the heterologous production of a recombinant enzyme from *Arabidopsis thaliana* in *Escherichia coli* cells. Bacterial cells are transformed with a protein expression vector in which the *Arabidopsis GH3* coding sequence was cloned with an N-terminal 6× His-tag. Recombinant enzyme expression is achieved by incubating these engineered bacterial cultures with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20 °C. The presence of the His-tag allows monitoring of recombinant protein production after the induction. The enzymatic reaction is carried out directly in bacteria that produce the recombinant protein. The enzymatic products are analysed by liquid chromatography coupled to tandem spectrometry (LC–MS/MS) using direct injection of a small supernatant fraction from the bacterial culture. Compared to existing *in vitro* approaches, no purification of the recombinant protein is required, enabling a faster screening of the enzymatic activity. Herein, this method was used to characterize the activity of *Arabidopsis* GH3.2 enzyme with IAA.

Keywords	phytohormones, Gretchen Hagen 3, GH3.2, recombinant enzyme, liquid chromatography, tandem mass spectrometry
Number of pages	60
Number of appendices	0
Language	English

CONTENT

OBJECTIVES OF THE WORK

The aim of this work was to investigate the activity of recombinant *Arabidopsis* GH3.2 (AtGH3.2) enzyme with IAA heterologously expressed in *Escherichia coli* by performing the enzymatic assay directly in bacterial cultures.

Within the theoretical part, my research focused on the following issues:
- introduction to bacterial tests and cloning,
- introduction to enzymology,
- metabolism of selected groups of phytohormones and the role of enzymes from the GH3 family,
- methods for targeted profiling of phytohormones.

Within the experimental part, experiments focused on:
- cloning and transformation of *E. coli*,
- induction of recombinant protein,
- optimization of conditions for enzymatic assay,
- sampling and subsequent analysis of enzymatic products by mass spectrometry,
- data analysis.

1 INTRODUCTION

Phytohormones are low molecular weight substances with biological activity occurring in plants. They are connected with long-term regulation of plant growth as well as immediate reaction to current stimuli. One of the most important plant hormones is indole-3-acetic acid (IAA), which, among other things, affects the growth and development of the plant. Concentration of IAA in plant tissues is regulated by its metabolism and transport. It may be degraded by oxidation, or it may be conjugated to glucose or amino acids.

Gretchen Hagen 3 (GH3) family of acyl acid amido synthetases typically conjugate amino acids to IAA. The substrates for the GH3 enzymes were identified based on *in vitro* activity measurements and supported by *in planta* analyses. The *in vitro* approaches are usually based on heterologous expression of plant genes in bacterial or yeast cultures. However, this procedure requires the purification of the recombinant protein that can sometimes result in either poor yield or inactivation of the protein of interest and in not optimal protein solubility and stability.

This work deals with a bacterial assay for rapid characterization of an enzyme belonging to the GH3 family. The method has been suggested as an alternative approach to the existing *in vitro* experiments. The enzymatic reaction takes place directly in the bacterial culture expressing the recombinant enzyme. Formed conjugates can be determined by liquid chromatograph combined with tandem mass spectrometer with electrospray ionization using direct injection of a small supernatant fraction from the bacterial culture.

2 THEORETICAL PART

2.1 Basics of enzymology

Enzymology is science discipline that studies proteins with catalytic activity. First information about biocatalyst appeared as early as the 18th century. Later in the 19th century, some scientists focused more on the processes connected with digestion of food. From the beginning, they thought that fermentation was connected only with living cells, but this was refuted in 1897 by E. Buchner, who used yeast extract for fermentation. In 1840, J. von Liebig predicted that biocatalysts are proteins, as confirmed by J. B. Summer by the crystallization of urease in 1926 (Poulsen and Buchholz, 2002).

The word 'enzyme' was used for the first time by F. W. Kühne in 1876. From the beginning, scientists thought that specificity of enzymatic activity can be explained by lock and key theory postulated by E. H. Fisher in 1894. According to this theory, only complementary substrate (key) can fit perfectly into the active site (keyhole of the lock), so the catalytic reaction can take place (Poulsen and Buchholz, 2002). If different substrate with another shape is bound, no reaction occurs. Not all experimental results could be explained by lock and key theory, so in 1958, it was improved by D. E. Koshland (Koshland Jr., 1958). He came up with an induce-fit model, in which shape of the active site is influenced by the substrate. The final shape is formed by interactions between side chains of enzyme amino acids around the active site and the substrate. Only a substrate of a certain size with certain groups can cause forming of proper final shape of the active site with a certain catalytic activity (Bosshard, 2001; Johnson, 2008).

2.1.1 Cofactors

In many cases, presence of some other molecule is necessary for catalysed reaction. This molecule is called cofactor and the enzyme is in this case called apoenzyme. Cofactors can be organic compounds or often also metals in different oxidation state. Organic cofactors can be divided in two groups. Coenzymes are small organic molecules that can transfer some chemical groups (phosphate, hydride ion, etc.). They are not bonded tightly and can dissociate from enzyme. Mostly known coenzymes are NAD^+, $NADP^+$ and ATP. Conversely, a prosthetic group also transfers chemical groups between molecules, but is covalently bonded to the apoenzyme and is not able to dissociate. Their regeneration is ensured by reaction with a second substrate. For example, flavin mononucleotide, biotin or lipoic acid belong to prosthetic groups (Berg *et al.*, 2002).

2.1.2 Classes of enzymes

Enzymes are divided into seven classes according to the reaction they catalyse. The first class includes oxidoreductases. These enzymes catalyse redox reactions, in which they transfer an electron from a donor molecule to an acceptor molecule. In this class, enzymes usually utilize NAD^+ or $NADP^+$ cofactors or FAD prosthetic group. The second class includes transferases that mediate transfer of functional group. In this case, for example, biotin prosthetic group is used for carboxylic group transfer. Similarly, ATP as a coenzyme often occurs as a phosphate donor in kinases (Berg *et al.*, 2002). In the third class, we can find hydrolases that catalyse the cleavage of bonds using water. A special case of hydrolases are restriction endonucleases. These enzymes cleave DNA at specific recognition sites creating blunt or sticky ends. This is widely used in molecular biology for molecular cloning (Williams, 2003). Another class of enzymes is lyases. They also cleave bonds, but by applying another mechanism than oxidation or hydrolysis. The fifth class is isomerases that convert isomers. Ligases catalyse the formation of a new chemical bond between two molecules, generally with hydrolysis of a chemical group on one of the molecule (Berg *et al.*, 2002). In the last class, we can find translocases. Enzymes of this newly formed class serve to transport molecules, often across a membrane (Tao *et al.*, 2020).

2.1.3 Reaction mechanisms

Several steps for enzymatic catalysis were described. In fact, enzymatic catalysis is alternative pathway for reaction, where activation energy is lowered compared to uncatalysed reaction. In the first step, enzyme is bonded to the substrate creating an enzyme-substrate complex. After that, enzyme-substrate complex is in second reaction turned to enzyme and product (Berg *et al.*, 2002).

Several reaction mechanisms of enzyme catalysis are known. First type is acid-base catalysis. In this case, there are H^+ acceptors or donors present in active site, which stabilize charges associated with a transition state. This mechanism is also often used to stabilize leaving groups or to activate nucleophile or electrophile (Kalninysh, 1991).

In covalent catalysis, there is a covalent bond forming between the substrate and residues in the active site. The formation of this covalent bond reduces the energy of transition states and can occur either *via* an electrophilic or nucleophilic reaction. An electrophile, that is generally a positively charged or an electron-deficient chemical species, is attacked to the functional group during the electrophilic covalent catalysis.

On the other hand, nucleophilic catalysis involves attack of nucleophiles, that are negatively charged or electron-rich chemical species, to the functional group. While during nucleophilic covalent catalysis H^+ is attacked by nucleophile and a covalent bond is formed, during basic catalysis nucleophile provides partial H^+ abstraction that lowers the energy of the transition state (Berg *et al.*, 2002; Toth and Richard, 2007).

Another reaction mechanism depends on metal ions that are needed for stabilization or shielding of negative charges. Furthermore, metal ions can also provide proper substrate orientation for the reaction and mediate redox reaction by reversible change of oxidation state of the ion (McCall *et al.*, 2000).

Ionic bonds between the residues in active site and the substrate stabilize the charged transition state in electrostatic catalysis. When the substrate is bonded, water is usually displaced from the active site, leading to strengthening the electrostatic interactions. In some enzymes, charge distribution also guides polar substrates to the binding site (Berg *et al.*, 2002; Fried and Boxer, 2017).

The fifth reaction mechanism relates to proximity and orientation. Interactions between enzyme and substrate cause its optimal geometry and appropriate positions of chemical groups. Thanks to this, effective molarity of the substrate is increased and so the reaction speed is increased as well (Catalano *et al.*, 2016; Speltz and Zalatan, 2020).

The last mechanism uses stabilization of transition state. These enzymes have actually higher affinity to the transition state compared with substrate (Warshel *et al.*, 2006). Interaction with substrate causes conformational changes, which force the substrate to acquire the conformation of the transition state resulting in decreased activation energy.

2.1.4 Kinetics

As other catalysts, enzymes do not alter the equilibrium of the reaction compared to uncatalysed reaction. Reaction speed generally depends on concentration of reagents. If concentration of substrate is low compared to the amount of the enzyme, reaction speed depends on the substrate concentration, but when all enzyme molecules are saturated, reaction speed cannot be increased by adding more substrate (Frey and Hegeman, 2007).

Enzyme reactions can be divided according to the number of substrates in reaction. As the given name suggests, “single-substrate enzymatic reactions” are those in which only one substrate occurs. We can find this type of reactions for example in case of isomerases and some lyases. For kinetics of these enzymes, a model was postulated by L. Michaelis and M. Menten in 1913. It works with simple reaction scheme,

where substrate reacts reversibly with the enzyme forming complex enzyme-substrate. Therefore, the enzyme-substrate complex can be brought back to original enzyme and substrate or irreversibly processed to the final product with the release of the regenerated original enzyme. For enzymes to which Michaelis-Menten kinetics can be applied initial reaction grade exhibits hyperbolic growth while increasing substrate concentration (Frey and Hegeman, 2007; Choi *et al.*, 2017).

When more substrates participate in the reaction, mechanism is much more complicated. Most common reactions are those with two substrates (*e.g.*, transferases and oxidoreductases). For these enzymes, several main mechanisms are known. Ternary-complex mechanism is characterized by forming a ternary complex of enzyme with both substrates. This complex can be formed with random order. However, in an ordered mechanism, substrates are bonded to the enzyme in a precisely defined sequence. The reaction products are released in the same way. In the case of "Ping-Pong" mechanism, one substrate is bonded first and it is converted into the first product, which is released from the modified form of the enzyme. The second substrate reacts with this modified enzyme, forming the second product and the original enzyme is regenerated. Reaction rate of these reactions depend on concentration of both substrates (Frey and Hegeman, 2007; Ulusu, 2015).

There are also enzymes, which do not behave according to the Michaelis-Menten model, they are called allosteric enzymes. Unlike hyperbolic dependence of the reaction rate on the substrate concentration, in this case it is sigmoidal and their kinetics can be described using Hill-Langmuir equation (Berg *et al.*, 2002). This is common in multimeric enzymes, where binding substrate or regulator molecule to the one active site may affect other active sites (Srinivasan *et al.*, 2014).

2.1.5 Regulation of enzyme activity

Different levels of regulation modulate the enzymatic activity. Regulation by altering the gene expression prevents wasting of resources and energy, so enzymes are synthetised only when needed, but response is quite slow. When fast response is necessary, control of enzyme activity can be mediated by action of inhibitors or activators. Inhibition can occur reversibly and irreversibly. In case of irreversible inhibitors, covalent bond is formed and enzyme is permanently inactivated (Berg *et al.*, 2002; Strelow, 2016). In case of reversible inhibition, inhibitors bind to the enzyme temporarily and the inhibitory effect can be depleted by dialysis or other methods. Mechanisms for enzyme activity attenuation

can involve also competitive inhibitors that being structurally analogue to the substrate, but inactive, do not allow the enzyme to react with the active substrate. Instead, non-competitive inhibitors bind to allosteric sites of enzyme, thus not affecting the binding of the substrate. However, the catalytic efficiency is reduced because of presence of inhibitor. Uncompetitive inhibitors cannot directly bind to enzyme, but only through the formation of an inactive complex enzyme-substrate-inhibitor. The last option is mixed inhibition. In this case, inhibitor can bind to both, enzyme and complex enzyme-substrate (Berg *et al.*, 2002).

2.2 Molecular Cloning

Molecular cloning methodology is needed to work with enzyme-encoding genes. Thanks to these methods, genetic information from one organism can be extracted, modified and after that transferred to another species, which serves as a host system for replication and expression of our genes of interest. In the past, the only way how to alter genetic information was random mutagenesis and selection of individuals with advantageous characteristics. Later, new methods for targeted alteration of genetic information were discovered (Lodish *et al.*, 2016).

Generally, cloning strategy consists of several steps. At the beginning, the coding sequence of the gene of interest is isolated from the studied organism, amplified using PCR and inserted into the proper vector by ligation or recombination. Host cells are than transfected with these vectors and cultures are prepared. Because not all cells are transformed properly, they must be selected using antibiotics or other methods. Successful transformation can be validated by colony PCR and/or DNA sequencing. Expression of target gene in the host system depends on several factors, such as promoter and termination sequence and strength of ribosome binding. Also, the number of gene copies in plasmid or the host chromosome can affect the heterologous expression of the recombinant protein (Fakruddin *et al.*, 2012). Furthermore, codon bias exists among the amino acids codons within species, thus causing possible translational problems (Tuller *et al.*, 2010).

2.2.1 Selection of host organism and cloning vector

Very often, bacterial cells are used as host system, because they can be cultivated fast on simple and quite cheap media (Gopal and Kumar, 2013). But there are also disadvantages

of prokaryotic host organisms. Prokaryote transcripts are not spliced after transcription and there are also no post-translation modifications. So, if the target gene contains introns, or the protein needs to undergo to eukaryotic post-translational modifications after translation, the recombinant protein cannot be properly synthetised. Problems with accuracy of the protein folding, assembling of multimeric proteins, rare codon bias can occur as well. (Fakruddin *et al.,* 2012). In these cases, it is better to use eukaryotic host cells such as yeast (Gomes *et al.*, 2018), insect (Kollewe and Vilcinskas, 2013) or mammalian cell cultures (O'Flaherty *et al.*, 2020). As a prokaryotic expression system, *Escherichia coli* is mostly used because of short life cycle, well-known genetics and easy cultivation (Gopal and Kumar, 2013). In the case of yeast, usage of *Saccharomyces cerevisiae* or *Pichia pastoris* and others is common (Gomes *et al.*, 2018).

There are several types of vectors usable as carriers of recombinant DNA. Plasmids are double-stranded small circular molecules of DNA (Lodish *et al.,* 2016). They occur mainly in the cytoplasm of prokaryotes, but also in archaea and even some eukaryotes. Non-essential genes occur on them and their replication is independent of the nucleus. If plasmid is used as a vector, it must contain multiple cloning site for insertion of exogenous DNA with restriction sites, selection marker, strong regulatable promoter (Fakruddin *et al.*, 2012) and origin of replication (Lodish *et al.*, 2016). As selection markers, genes that provide resistance against antibiotics are used (Rosano and Ceccarelli, 2014).

As a promotor for target gene, promoter from *E. coli lac* operon can be used. It is activated by lactose or its synthetic analogue isopropyl-beta-D-thiogalactopyranoside (IPTG). Constitutive promoters are not suitable, because permanent expression causes high energy demands on the host cells and can lead to the loss of the plasmid or host cell death (Ringquist *et al.*, 1992).

2.2.2 Template DNA

There are several ways how to get target gene from the studied organism. DNA from organism can be extracted and target gene is multiplied using PCR with gene specific primers designed according to the gene sequence (Gibbs, 1990). Alternatively to DNA, RNA can be used as mRNA do not contain introns. RNA is extracted from biological material and treated with DNase (Dotti and Bonin, 2011). Complementary DNA (cDNA) is than synthetised using reverse transcriptase. Oligo(dT) primer is often used, because it

binds to the poly(A) tail of eukaryotic mRNAs (Adomas *et al.*, 2010). Obtained DNA is amplified again using PCR (Jia and Jeon, 2016).

In another approach, genomic library is created and screened. Genomic DNA is cleaved using restriction enzymes and fragments are inserted into vectors and host cells are transformed. After that, cells containing target gene are identified and isolated (Marsischky and LaBaer, 2004; Lodish *et al.*, 2016). There is also possibility to synthetize the PCR template *de novo* (Kosuri and Church, 2014; Jia and Jeon, 2016).

2.2.3 PCR

This method is based on multiplication of DNA molecule using DNA polymerase. It was invented by K. Mullis in 1983. Reaction takes place in thermocycler, because temperature must vary in different stages of the reaction (Lorenz, 2012). At the beginning, double strand DNA molecule must be denatured by exposure to about 95 °C. After that, temperature is decreased to 55 °C so primers can pair with complementary sequences of single-stranded DNA molecules. In the next step, temperature is set to approximately 75 °C when polymerization reaction takes place. The polymerase recognize the primer and cDNA strand is synthesized in the direction 5′-to-3′ (Gibbs, 1990). Because of high temperature, it is necessary to use DNA polymerase from thermostable organisms like *Thermus aquaticus*, which activity is highest at temperatures between 75 and 80 °C (Lawyer *et al.*, 1993). These three steps (denaturation, annealing and elongation) are repeated many times and with each repetition, number of copies of DNA molecule is doubled. After the last cycle, final elongation step can take place. It is used to ensure that the second strand will be synthesized for all remaining single-stranded molecules (Lorenz, 2012).

To verify the PCR reaction products agarose gel electrophoresis is often used. By comparison with a DNA ladder, the size of PCR product is determined. DNA molecules are in gel visualised using ethidium bromide or other fluorescent intercalating dyes such as SYBR Green or GelRed (Miller *et al.*, 1999; Huang *et al.*, 2010; Lee *et al.*, 2012).

For the correct course of the PCR, except for DNA polymerase, template DNA and primers, is necessary to add enough amount of each deoxynucleoside triphosphates. Suitable chemical environment to reach optimal activity and stability of polymerase is secured by appropriate buffer solution. Presence of divalent cations of Mg is necessary for the correct activity of *Taq* DNA polymerase (Lorenz, 2012).

2.2.4 Digestion, ligation and competent cells transformation

Chosen vector must be prepared for insertion of recombinant DNA. Commonly used is cleavage using restriction endonucleases. The same enzyme is used also to cleave inserting DNA. Restriction endonucleases are group of enzymes that catalyse cleavage of double strand DNA molecules on a specific short palindromic sequence (Lodish *et al.*, 2016). Restriction enzyme creating sticky ends are more advantageous, because of higher efficiency of ligation.

After cleavage, alkaline phosphatase is often used. It dephosphorylates ends of DNA molecule, so the ends cannot be ligated spontaneously creating the empty vector again. Ligation is catalysed by DNA ligase, which links two DNA molecules together by covalent bond. Ligase join DNA ends randomly, so also molecules of insert DNA or vector linked to itself may occur (Lodish *et al.*, 2016). Because of this, cleaving with two different restriction enzymes, one on each side of inserting sequence is useful. This provides insertion of recombinant DNA in only one orientation in recombinant vector and moreover no ligation of multiple molecules of recombinant DNA or vectors occur.

Ligated vector must be transferred to the living host cells. Cells, that are able to take up foreign DNA, are called competent cells. Naturally, cells are turned into competent in high cell density or when starving. Competence can be also induced artificially. This can be done with bacterial cells by electroporation (Tu *et al.*, 2016) or addition of $CaCl_2$ into the cell suspension and exposure to heat shock (Li *et al.*, 2010; Chang *et al.*, 2017). Electroporation is using electric pulses, which cause destabilization of the membrane. Permeability is increased and foreign DNA can enter the cell (Dower *et al.*, 1988). Heat shock method is using divalent cation, which reduces interactions between proteins and lipopolysaccharides. Membrane fluidity is increased which simplify the transformation (Froger and Hall, 2007). Divalent cations are also shielding the negative charge on the cell surface so the negatively charged DNA can adhere to the surface (Panja *et al.*, 2006; Rahimzadeh *et al.*, 2016). There are natural channels called Bayer's bridges, that can serve potentially for uptake of DNA (Dreiseikelmann, 1994; Sperandeo *et al.*, 2007).

2.2.5 Screening of positive colonies

After introducing recombinant DNA to host cells, selection is necessary, because transformation can be ineffective. Presence of vector is usually checked by cultivation of transformed cells on the media containing antibiotics (Peubez *et al.*, 2010). Only cells containing vector with the gene for resistance against this antibiotic can grow on this

medium. Successful incorporation of target DNA into the vector can be checked using multiple methods such as colony PCR. Colonies are sampled and primers corresponding to target gene and other necessary components are added. PCR takes places only in samples containing desired genes (Bergkessel and Guthrie, 2013). Correctness of insert can be then checked using DNA sequencing.

2.3 Metabolism of plant hormones mediated by GH3

Phytohormones are necessary for plant adaptation to external conditions and for control of the growth. They are important biologically active molecules involved in the regulation of almost all physiological processes in plants. They are involved in fast reactions on stress conditions as well as in long term growth regulations (Davies, 2010). For effective regulation, in addition to biosynthesis, their rapid conversion to inactive metabolites is necessary (Westfall *et al.*, 2016). These metabolic pathways can lead to degradation as well as to creation of stock forms (with lower or no biological activity) that can be converted back to fully active molecules. In some cases, also more active conjugate of phytohormones can be formed (Meesters *et al.*, 2014).

Gretchen Hagen 3 (GH3) is a family of phytohormone-modifying enzymes occurring in plants. Although not all the members of this family have been characterized yet, involvement to metabolic pathways of auxins, salicylic acid, jasmonic acid and also benzoates have been found (Chen *et al.*, 2010; Westfall *et al.*, 2010). The first enzyme of this family was discovered in 1984 and up to this day, 19 members of GH3 family have been identified in *Arabidopsis thaliana* (Westfall *et al.*, 2010). Enzymes from GH3 family can be divided according to phylogenetic analysis into three subfamilies (Staswick *et al.*, 2005; Gan *et al.*, 2019). The first group catalyse conjugation of jasmonates, the second group encode for enzymes that conjugate auxins and salicylic acid and the last one provides conjugation of benzoates (Chen *et al.*, 2010). These enzymes function as acyl acid amido synthetases. For example, in the case of GH3.8 enzyme, mechanisms can be described simply as follows: In the beginning, ATP is bonded and after that, it is followed by IAA. Adenylated IAA is formed and pyrophosphate is released, then amino acid (e.g. Asp) binds and reaction with adenylated IAA takes place. In the last step, conjugated IAA (e.g. IAA-aspartate) and AMP are released (Chen *et al.*, 2010).

2.3.1 Auxin metabolism

Biosynthesis of the main auxin, indole-3-acetic acid (IAA) starts from tryptophan. There are four different ways how can it be metabolised to IAA, but also biosynthetic pathway independent on tryptophane was discovered although it has only minor importance and cannot replace tryptophan-dependent biosynthesis (Normanly *et al.*, 1993; Zhao, 2010; Zhao, 2014; Malka and Cheng, 2017). IAA can be further metabolised in multiple ways (Fig. 1). For degradation, it is prevalently oxidised to 2-oxindole-3-acetic acid (oxIAA), which has only little biological activity (Pěnčík *et al.*, 2013; Zhang and Peer, 2017). Other option is conjugation with glucose, which is mainly catalysed by UDP-glycosyltranferase (UGT84B1) (Jackson *et al.*, 2001; Aoi *et al.*, 2020; Casanova-Sáez *et al.*, 2021). Also, oxIAA can be conjugated with glucose forming oxindole-3-acetyl-1-glucosyl ester (Ljung, 2013). This reaction is in *Arabidopsis* catalysed by UDP-glycosytransferase 74D1 (Kai *et al.*, 2007; Tanaka *et al.*, 2014).

A very important inactivation step is the formation of conjugates with amino acids. These are created in reactions catalysed by enzymes from group II of the GH3 family (Westfall *et al.*, 2010; Porco *et al.*, 2016). IAA can be conjugated with multiple amino acids, but not all of them have the same function. Some of them can serve as storage

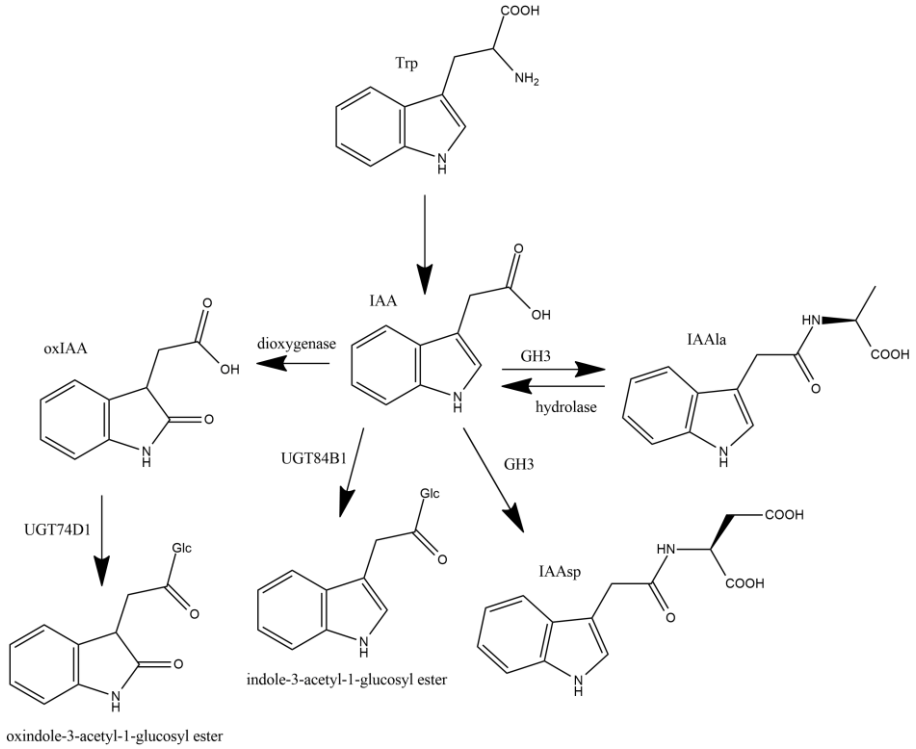

Fig. 1 Scheme of selected reaction of IAA metabolism (tryptophan, Trp; Indole-3-acetic acid, IAA; Indole-3-acetyl-L-aspartic acid, IAAsp; Indole-3-acetyl-L-alanine, IAAla; 2-oxindole-3-acetic acid, oxIAA; UDP-glycosyltranferase 74D1, UGT74D1; UDP-glycosyltranferase 84B1, UGT84B1; GRETCHEN HAGEN 3, GH3) (according to Malka a Cheng, 2017).

forms, which can be hydrolysed back to the active form by IAA-amido hydrolases (LeClere *et al.*, 2002; Normanly, 2010). This mechanism was discovered in conjugates with Ala and Leu (Staswick *et al.*, 2005; Westfall *et al.*, 2010). Conversely, conjugation of IAA to Asp and Glu is considered irreversible and therefore leads IAA to the degradation (Östin *et al.*, 1998; Woodward and Bartel, 2005; Kramer and Ackelsberg, 2015).

In *Arabidopsis*, members of subfamily II of GH3 family such as GH3.2, GH3.3, GH3.4, GH3.5, GH3.6 and GH3.17 catalyse the conjugation of IAA with amino acids (Staswick *et al.*, 2005; Sugawara *et al.*, 2015). For most of these enzymes, Asp was found to be the best substrate *in vitro*. Also, Glu is strongly reactive with all of them, GH3.17 and GH3.6 even prefer it to Asp (Staswick *et al.*, 2005; Brunoni *et al.*, 2019). All these enzymes form IAA conjugates *in vitro* also with Ala, Gly, Val and Leu. Only some of them can use other amino acids such as Phe, Trp, Tyr, Met, Ile and Gln (Staswick *et al.*, 2005). Not all of these possible conjugates accumulate in plants, because of the activity of hydrolases or because of the lack of these free amino acids (Kowalczyk and Sandberg, 2001). There are not big differences in the substrate specificity among the acyl acid-amido synthetases in *Arabidopsis*. From this, we can deduce, that presence of multiple similar enzymes, are results of their tissue specificity more than substrate specificity (Staswick *et al.*, 2005).

2.4 Analyses of phytohormones

For studying of phytohormones, it is necessary to have appropriate methods for determining of their concentration in plant tissues. The main problem is, that phytohormones occur in plant in very low concentrations and also large differences in chemical properties of multiple phytohormones (Šimura *et al.*, 2018). Due to presence of many other interfering molecules that can complicate the analysis, purification procedures must be used (Davies, 2010; Tarkowská *et al.*, 2014). To prevent degradation of the analytes, all extraction and purification steps must be performed at low temperature to reduce the activity of hormone-degrading enzymes (Nováková and Vlčková, 2009; Davies, 2010).

2.4.1 Extraction

Initially, biological material must be homogenized. For large amounts, knife homogenisers or a mortar and pestle is often used. A bead mill with beading balls added can be also used to homogenise the samples using high frequency shaking. Moreover,

small amounts of tissue can be homogenised directly in microtubes (Novák *et al.*, 2014). Importantly, it is necessary to keep the sample in low temperature even during gridding step. From homogenised sample, target molecules are extracted using suitable solvent (Davies, 2010). Due to different physical-chemical properties of phytohormones, it is difficult to find optimal extraction solvent. The ideal extraction solvent has a high solubility for the target molecules and a low solubility for the interfering molecules. Inhibition effect to degradation enzymatic reaction is also required (Salem *et al.*, 2020). As extraction solvent, aqueous solutions of methanol in different concentrations can be used as well as ethyl acetate, acetonitrile or (modified) Bieleski's solution (Bieleski, 1964) (Hoyerová *et al.*, 2006). In higher concentrations of organic solvents, phytohormone degradation processes are inhibited, however, interfering molecules such as chlorophylls are also obtained (Šimura *et al.*, 2018). Extraction efficiency can be increased using sonication and mixing the extraction mixture in rotator. Finally, centrifugation at 4 °C is often used to separate the extract from the tissue.

2.4.2 Purification

Compared to other molecules, phytohormones are present in very low concentrations. The presence of large amount of other compounds can cause problems during analysis, because of interference with target molecules. Therefore, multiple isolation methods, such as solid-phase extraction or immunoaffinity extraction, can be used to isolate selected phytohormones from complex biological matrices (Novák *et al.*, 2017).

2.4.2.1 Purification using recombinant protein

In recent years, methods based on recombinant proteins were also investigated. One of them was developed for purification of IAA. It uses specific binding capacity with IAA of AUXIN RESISTANT/INDOLE-3-ACETIC ACID INDUCIBLE proteins (Su *et al.*, 2017). This recombinant protein, expressed in *E. coli*, is via the glutathione S-transferase tag solidified in a column pre-filled with resin. After loading of the sample, IAA is bonded to the binding sites of recombinant protein and then released by alkaline elution buffer.

2.4.3 Determination of phytohormones

After extraction and purification steps, the concentration of plant hormones must be determined using sensitive and selective analytical methods. Nowadays, gas or liquid chromatography with mass spectrometry is mostly used (Novák *et al.*, 2017). Most

phytohormones are not volatile and must therefore be derivatized before analysis by gas chromatography (GC). However, the derivatization step can lead to analyte loss and make their determination difficult (Fu *et al.*, 2011; Rawlinson *et al.*, 2015; Vallarino and Osorio, 2016). In comparison, liquid chromatography (LC) does not require modification of the target molecule prior to analysis in most approaches (Wang *et al.*, 2020).

Nowadays, chromatography techniques are usually combined with mass spectrometry (MS). Other detectors, such as UV/VIS or fluorescent detectors, can also be used to determine auxins, cytokinins, jasmonic acid, salicylic acid, ethylene or abscisic acid (Nováková *et al.*, 2005; Rozhon *et al.*, 2005; Diopan *et al.*, 2009; Rawlinson *et al.*, 2015). However, these optical detectors are less used due to low sensitivity and insufficient selectivity (identification is based only on retention time) (Fu *et al.*, 2011).

Using MS-based method, the ratio between mass and charge is detected, so target molecules must be easily ionised. This is done by chemical or electron ionization in the case of GC-MS (Fu *et al.*, 2011; Tarkowská *et al.*, 2014) and by atmospheric pressure chemical ionization or electrospray ionization (ESI) for LC-MS (Wang *et al.*, 2020).

The main detection technique used in the analysis of phytohormones are quadrupole analyzers operating in several modes. The first detection is based on single ion monitoring, where only one type of ion is selected. Therefore, this mode can be used with an instrument equipped with a single-quadrupole analyzer (Nehela *et al.*, 2016). The other detection mode is multiple reaction monitoring (MRM). In this scan mode, precursor ion is separated in the first quadrupole and fragmented in collision cell. In the third quadrupole, product ion is selected and then recorded by detector (Riet *et al.*, 2016; Šimura *et al.*, 2018). The MRM mode provides higher selectivity and specificity (Wang *et al.*, 2020). There are also another analyzers except quadrupoles, such as ion trap, orbitrap or time-of-flight analyzers that can be used for quantification of phytohormones alone or in combination with a quadrupole (Wang *et al.*, 2020). In combination of quadrupole, collision cell and orbitrap used, parallel reaction monitoring approach can be applied. Precursor ion is selected in a quadrupole, then fragmented in a collision cell, and all product ions are scanned in the orbitrap. This approach provides higher selectivity and specificity compared to MRM approach (Kisiala *et al.*, 2019).

In general, liquid chromatography with mass spectrometry is the most common method for quantification of phytohormones (Novák *et al.*, 2014). This analytical

technique was used for determination of auxins (Pěnčík *et al.*, 2009; Novák *et al.*, 2012; Pěnčík *et al.*, 2018), cytokinins (Tarkowski *et al.*, 2010; Svačinová *et al.*, 2012; Kisiala *et al.*, 2019; Xin *et al.*, 2021), abscisic acid (Xiong *et al.*, 2014; Perin *et al.*, 2018), jasmonic acid (Liu *et al.*, 2010), salicylic acid (Zhang *et al.*, 2013) or gibberellins (Li *et al.*, 2017). Also simultaneous quantification of different plant hormones in one LC-MS run is commonly used (Segarra *et al.*, 2006; Forcat *et al.*, 2008; Cao *et al.*, 2016; Schäfer *et al.*, 2016; Wang *et al.*, 2017; Šimura *et al.*, 2018).

Except determination of phytohormone levels directly in plant tissues, it is also possible to determine concentration of phytohormones and their metabolites in bacterial cultures expressing gene connected with phytohormone metabolism. In that case, no purification steps may be required and a small fraction of supernatant from the culture can be directly injected, and the activity of recombinant proteins can be screened using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Brunoni *et al.*, 2019).

There are also several other methods for determining phytohormone concentrations (Bai *et al.*, 2010). Enzyme linked immunosorbent assays (ELISA) was used for quantification of several phytohormones from different groups (Patel and Thaker, 2007; Zhang *et al.*, 2009; Sokolova *et al.*, 2011). The basic principle of ELISA is similar to immunoextraction. Antibodies specifically bind with carrier-conjugated phytohormones (haptens) and concentration is determined using the activity of enzyme, by which the antibody or antigen is labelled (Fu *et al.*, 2011). The advantage of this method is high specificity and low detection limit, but cross-reactions with phytohormone analogues may occur, leading to reduced specificity (Fu *et al.*, 2011; Porfírio *et al.*, 2016).

Low-cost electrochemical methods can also be used for determination of phytohormones, but their application is highly affected by pH and matrix of the sample (Fu *et al.*, 2011). Currently, these methods are used mainly in construction of biosensors, which offer a completely different approach on quantification of phytohormones, providing real-time information about concentration of analyte (Novák *et al.*, 2017). Electrochemical biosensors measure current or resistance during enzymatic redox reactions, but fluorescent reporters or immunoanalytical methods can also be used for construction of biosensors (Pařízková *et al.*, 2017). Many approaches using biosensors to determine phytohormones has been developed (Hernández *et al.*, 1994; Wu *et al.*, 2003; Li *et al.*, 2005; Mancuso *et al.*, 2005; Li *et al.*, 2008; Wells *et al.*, 2013; Sun *et al.*, 2017).

3 EXPERIMENTAL PART

3.1 Material and equipment

3.1.1 Chemicals and material

Methanol (99.9 %), di-sodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate, sodium dodecyl sulfate and agarose for analytical nucleic acid electrophoresis were used (Merck, Darmstadt, Germany). Ethanol (96 %) and agar powder for bacteriology were supplied by VWR International (Radnor, Pennsylvania, USA). Acetic acid (99.8 %), sodium chloride (99.5 %), potassium chloride (99.0 %), magnesium chloride (97.0 %), 2-mercaptoethanol for molecular biology (99 %), acrylamide solution (40 %) suitable for electrophoresis, ATP, dNTPs, IAA, kanamycin, Monoclonal Anti-polyHistidine−Peroxidase antibody and all used amino acids: L-Glutamic acid (99.5 %), L-Aspartic acid (98 %), L-Tyrosine (99.0 %), L-Leucine (98 %), Glycine (99 %), L-Phenylalanine (98.5-101.0 %), L-Aspartic acid (98 %), L-Isoleucine (99.5 %), L-Methionine (99.5 %), L-Tryptophan (98 %), L-Glutamine (99 %), L-Alanine (99.5 %), L-Valine (99.5 %) were used (Sigma-Aldrich, Steinheim, Gemany). Saccharose were obtained from Lach-Ner (Neratovice, Czech Republic). LB Broth High salt, Murashige & Skoog medium including vitamins and MES monohydrate was purchased from Duchefa Biochemie (Haarlem, Netherlands). Tris base was supplied by PENTA company (Prague, Czech Republic). Gellan gum was used (Alfa Aesar company, Haverhill, Massachusetts, USA). Tween® 20 was purchased from Carl Roth company (Karlsruhe, Germany). From the company Bio-Rad Laboratories (Hercules, California, USA) was obtained TEMED, ammonium persulfate and 4× Laemmli Sample Buffer. Amersham™ ECL™ prime western blotting detection reagent was supplied by GE Healthcare (Chicago, Illinois, USA). GoTaq® G2 Flexi DNA Polymerase was bought from Promega company (Madison, Wisconsin, USA). From the company Thermo Scientific (Waltham, Massachusetts, USA) was obtained RevertAid H Minus First Strand cDNA Synthesis Kit, Phusion™ High-Fidelity DNA Polymerase, 10× FastDigest Buffer, 10× FastDigest Green Buffer, FastDigest BamHI, FastDigest NotI, T4 DNA Ligase, 10× T4 DNA Ligase Buffer, FastAP Thermosensitive Alkaline Phosphatase, Exonuclease I, TriTrack DNA Loading Dye (6×), GeneRuler 1 kb Plus DNA Ladder and PageRuler™ Prestained Protein

Ladder (10 to 180 kDa). All used primers were synthesized by Generi Biotech (Hradec Králové, Czech Republic).

Deionized water was prepared using Millipore Milli-Q™ reference ultrapure water purification system supplied by Merck (Darmstadt, Germany). All used chemicals meet purity grade p.a., and chemicals used for liquid chromatography meet purity criteria „HPLC gradient grade".

Used internal standards labeled with stable isotopes ^{13}C were synthesized in company OlchemIm, s.r.o. (Olomouc, Czech republic) ([$^{13}C_6$]oxIAA, [$^{13}C_6$]IAAsp and [$^{13}C_6$]IAGlu) and Sigma Aldrich (Steinheim, Germany) ([$^{13}C_6$]IAA). From these, a mixture was prepared with final concentration $1 \cdot 10^{-6}$ mol·l^{-1}of each standard.

RNA from plant materials was extracted using Spectrum ™ Plant Total RNA Kit from Sigma-Aldrich company (Steinheim, Gemany). For DNase treatment, DNA-free™ Kit supplied by Thermo Scientific (Waltham, Massachusetts, USA) was used. The same company was also a manufacturer of nitrocellulose membrane used for SDS-PAGE. NucleoSpin® Gel and PCR Clean-up kit for purification of PCR products were purchased from Macherey-Nagel (Bethlehem, Pennsylvania, USA). QIAprep® Miniprep kit for extraction of plasmids was obtained from QIAGEN company (Hilden, Germany).

3.1.2 Instruments

Weighing of samples and chemicals was done using analytical balances XP26 Excellence Plus Micro Balance and classic light balances PL602-L supplied by Metler Toledo (Columbus, Ohio, USA). Adjustment of pH of used buffers was done using pH meter UB-10 UltraBASIC with High performance pH/ATC Glass-body electrode from Denver Instrument (Bohemia, New York, USA). Centrifugation steps were carried out in centrifuge Mega Star 600R from VWR International (Radnor, Pennsylvania, USA), MiniSpin Plus manufactured by Eppendorf (Hamburg, Germany) and Avanti™ 30 Centrifuge (Beckman, Chelmsford, United Kingdom). For vortexing and spinning down of the samples, Combi-spin PCV-2400 centrifuge/vortexer from Grant Instruments (Cambridge, United Kingdom) was used. For sterilisation, autoclave LaM-ECZ manufactured by SANOclave (Geislingen an der Steige, Germany) was used. Laminar flow cabinet LVG-4AG-F8 from ESCO company (Portland, Oregon, USA) was used for work under sterile conditions. Incubating Orbital Shaker, Model 3500I from VWR International (Radnor, Pennsylvania, USA), Innova S44i from Eppendorf (Hamburg, Germany) was used for incubation of bacterial cultures. Optical density of cultures was

measured using UV-1600PC Spectrophotometer from VWR International (Radnor, Pennsylvania, USA). NanoDrop™ One Microvolume UV-Vis Spectrophotometer manufactured by Thermo Scientific (Waltham, Massachusetts, USA) was used for determination of concentration and purity of nucleic acids. The same company also supplied thermocycler Doppio, Digital Heatblock and Mini Gel II electrophoresis system with accessories. Equipment for SDS-PAGE and Western blotting (Mini-PROTEAN® Tetra Electrophoresis System) was purchased from Bio-Rad Laboratories (Hercules, California, USA) as well as Gel Doc™ EZ Imager. Imaging of chemiluminescent signal of labelled antibody after SDS-PAGE and blotting was done using Luminiscent image analyzer LAS-4000 manufactured by Fujifilm (Tokyo, Japan). The cell cultures were lysed using SONOPULS HD 2070 ultrasonic homogenizer manufactured by Bandelin (Berlin, Germany). Ice was prepared using GB 903 ice maker manufactured by Brema (Villa Cortese, Italy). In general, pipets Eppendorf® Research® Plus were used (Eppendorf, Hamburg, Germany).

Analysis of IAA and their metabolites was performed using HPLC-MS/MS system combined Agilent 1260 Infinity II with column KINETEX 1.7 μm C18, 50 x 2.1 mm – Phenomenex (Torrace, USA) with mass spectrometer Agilent 6495 Triple Quad – Agilent Technologies (Santa Clara, USA). For *in-silico* molecular cloning, PCR and gene analysis, SnapGene software (version 5.2.4) was used. Alignment of sequencing data was performed using BioEdit software (version 7.2.5).

3.1.3 Biological material

For all experiments, plant material from wild type *Arabidopsis thaliana* was used. *Arabidopsis* seeds were sterilized for 3 min in 70% EtOH and after that 10 min in mixture of bleach, 70% EtOH and sterile dH_2O in ration 5:1:4. Finally, seeds were washed three times in sterile dH_2O. Seeds were under the sterile conditions transferred with a toothpick or pipette on plates with half strength medium according to Murashige and Skooge (Murashige and Skoog, 1962). Medium was prepared from 2.15 g of Murashige and Skooge salts with vitamins, 0,25 g of MES and 10 g of saccharose. Everything was dissolved in some dH_2O, pH was adjusted to 5.7 and the rest of dH_2O was added up to 1 l. Finally, 5.7 g of Gellan gum was added and medium was sterilized by autoclaving. Medium was poured on plates and after solidification, seeds were sowed. Plates were closed and sealed up using Softpore tape and stored at 4 °C for 3 days. After that,

seeds were germinated by cultivating under long day conditions (16 h of light (100 $\mu Em^{-2}s^{-1}$) at 24 °C and 8 h in dark at 18 °C). After 7 days, plants were harvested, transferred to sterile Erlenmeyer flasks containing half strength Murashige and Skooge liquid medium supplemented with or without IAA (10 $\mu mol \cdot l^{-1}$) for 3 h under gentle shaking and incubated under the same growing conditions described above. Samples were collected and immediately frozen with liquid nitrogen and stored in −80 °C.

3.2 Methods

3.2.1 Cloning of *AtGH3.2* (AT4G37390)

3.2.1.1 RNA isolation, DNase treatment and cDNA synthesis

RNA was extracted using Spectrum ™ Plant Total RNA Kit from plants, which were stored in −80 °C. 100 mg of powdered plant tissue was weighted and 500 µl of lysis mixture was added and immediately vortexed. Samples were incubated at 56 °C for 3-5 min. Samples were centrifuged for 3 min at 14,000 *g*. Lysate supernatant was transferred into filtration column placed in a 2 ml collection tube. Tubes with columns were centrifuged for 1 min at 14,000 *g*. 500 µl of binding solution was pipetted into the lysate and mixed immediately by pipetting up and down. 700 µl of mixture was transferred into binding columns placed in 2 ml collection tube and centrifuged at 14,000 *g* for 1 min. Flow-through fraction was decanted and residual liquid was drained with clean absorbent paper. Column was returned to the collection tube. The rest of mixture was pipetted into the column and centrifugation and decanting was repeated. After that, 500 µl of Wash Solution 1 was added into the column and tube with column was centrifuged for 30 s at 14,000 *g*. Flow-through fraction was discarded and the residual liquid was drained with clean absorbent paper. For the second and third time of washing, the Wash Solution 2 was used, conditions of centrifugations were the same as in the first wash. After that, column was centrifuged at 14,000 *g* for 1 min to dry. Column was carefully transferred to a new 2 ml collection tube and 30 µl of Elution Solution was added directly onto the centre of the binding matrix of the column. After 1 min, column was centrifuged at 14,000 *g* for 1 min. Elution step was repeated once more with new collection tube. Isolated RNA was stored in –80 °C.

RNA samples were treated with DNase, using DNA-free™ Kit. To the RNA sample, 0.1 volume of 10× DNase I Buffer and 1 µl of rDNase I were added and it was mixed gently. Samples were incubated in 37 °C for 20-30 min. DNase Inactivation Reagent was resuspended and 2 µl or 0.1 volume (always used at least 2 µl) of this reagent was added to the samples. During 2 min incubation, content was mixed few times. After that, samples were centrifuged at 10,000 *g* for 1.5 min and RNA (supernatant) was transferred to a fresh tube.

For synthesis of cDNA, 0.1 ng – 5 µg of RNA template was used in nuclease free tubes placed in ice. RNA samples were supplemented with 100 pmol of Oligo $(dT)_{18}$ primer. Samples were mixed gently, briefly centrifuged and incubated 5 min in 65 °C. Samples were chilled on ice and briefly centrifuged and placed again on ice. After that, 4 µl of 5× Reaction Buffer, 0.5 µl of Thermo Scientific™ RiboLock™ RNase Inhibitor (20 U), 2 µl of dNTP mix (10 $mmol \cdot l^{-1}$ each) and 1 µl of RevertAid H Minus Reverse Transcriptase (200 U) was added. Samples were incubated for 60 min in 42 °C and reaction is terminated by heating at 70 °C for 10 min.

3.2.1.2 PCR

PCR was used to amplify target DNA. 2 µl of template cDNA were mixed with 10 µl of 5× Phusion HF Buffer, 1 µl of dNTPs (containing 10 $mmol \cdot l^{-1}$ each), 2.5 µl of each forward and reverse primers and finally 0.5 µl of Phusion DNA Polymerase. Milli-Q water was added up to 50 µl. PCR tubes with the prepared reaction mixture was spun down and transferred to the thermocycler to initiate the PCR reaction. Thermocycler program parameters are listed below (Tab. 1).

Tab. 1: Thermocycler parameters for PCR.

Temperature (°C)	Time (min:s)	Cycles (nr)
98	0:30	1
98	0:10	35
55	0:30	35
72	1:00	35
72	10:00	1
8	∞	

For the PCR reaction, *AtGH3.2* specific primers containing additional *BamHI*/*NotI* restriction sites and 6×His-tag were used:

Cloning primer Fw:

TCCCCGGATCCAATAATTTTGATTTAACTTTAAGAAGGAGATATACCA
TGCATCACCATCACCATCACGCGATGGCCGTTGATTCACCTCTTCA

Cloning primer Rv:

CTCGAGTGCGGCCGCAGCAAGCTTCTAGCTAGCTTATTACTAACGACG
TCGTTCTGGTGACCA

PCR products were checked using agarose electrophoresis and were processed using NucleoSpin® Gel and PCR Clean-up kit. To the PCR product 2 volumes of NTI binding buffer was pipetted. In order to remove small fragments, diluted 25% NTI buffer was used. Column was placed into a 2 ml collection tube, 700 µl of sample was loaded and column was centrifuged for 30 s at 11,000 *g*. Flow-through fraction was discarded, the rest of the sample was loaded and centrifugation step was repeated. 700 µl of buffer NT3 was loaded on column and again centrifuged for 30 s at 11,000 *g* and flow-through fraction was discarded. Washing step was repeated once more. Silica membrane was dried using centrifugation for 1 min at 11,000 *g*. Column was placed into a new 1.5 ml microcentrifuge tube and 30 µl of Milli-Q water was pipetted directly onto the middle of the column. After 1 min incubation at room temperature, samples were centrifuged for 1 min at 11,000 *g*.

3.2.1.3 Plasmid Isolation

Isolation of plasmid pETM11 was performer using QIAprep® Miniprep kit. Bacterial culture was pelleted and resuspended in 250 µl of buffer P1. Resuspended culture was transferred to a microcentrifuge tube and 250 µl of lysis buffer P2 was added. Culture was mixed by inverting the tube. 350 µl of buffer N3 was added and culture was mixed by inverting again. Samples were centrifuged for 10 min at 17,900 *g*. Supernatant was pipetted to the QIAprep spin column and centrifuged for 60 s at 17,900 *g*. Column was washed by adding 500 µl of wash buffer PB and centrifugation at 17,900 *g* for 60 s. Flow-through was discarded and 750 µl of wash buffer PE was added onto the column. After centrifugation at 17,900 *g* for 60 s flow-through was discarded and column was centrifuged for another 60 s to remove residual buffer. Column was placed in a clean 1.5 ml microcentrifuge tube. For elution, 50 µl of Milli-Q water was pipetted to the centre of the column. After 1 min standing, column was centrifuged for 60 s at 17,900 *g*.

3.2.1.4 Digestion, ligation and competent cells transformation

Isolated plasmid and PCR product were digested using restriction enzymes *BamHI* and *NotI*. For plasmid digestion was used 3 µl of 10× FastDigest Buffer mixed with 10 µl of DNA and 1 µl of each restriction enzyme and Milli-Q water up to 30 µl. Everything was mixed gently and spun down and incubated at 37 °C for 2 h. Digested products were checked using agarose gel electrophoresis.

Digested plasmids were ligated with target DNA using T4 DNA ligase. Reaction mixture was prepared using 2 µl of 10× T4 DNA Ligase Buffer, 5 µl of opened plasmid and 5 µl of target DNA, 1 µl of T4 DNA Ligase and 7 µl of Milli-Q water. Mixture was incubated at 4 °C overnight.

E. coli BL21 (DE3) competent cells were transformed using the generated recombinant plasmid. Ten µl of mixture after ligation was pipetted to aliquots of competent cells and tubes were kept in ice for 15 min. Transformation of competent cells was performed using heat shock at 42 °C for 45 s. Cells were chilled in ice for 1 or 2 min and 800 µl of Lysogeny broth (LB) medium was added. After 1 h of incubation at 37 °C under constant shaking at 180 rpm, 20 µl of culture was plated on solid LB medium with kanamycin. Plate was grown at 37 °C for no longer than 12 h.

3.2.1.5 Colony PCR and sequencing

Presence of plasmid containing the desired insert in bacterial colonies was checked using colony PCR. Colonies were picked and transferred to a replica plates and PCR reactions using *E. coli* cells from colonies as template were performed.

For colony PCR reaction, mixture was prepared from 4 µl of 5× GoTaq Flexi Buffer, 3 µl of $MgCl_2$ (25 $mmol \cdot l^{-1}$), 0.4 µl of dNTPs (10 $mmol \cdot l^{-1}$), 1 µl of each forward and reverse primers, 0.1 µl of GoTaq G2 Flexi DNA polymerase and 10.5 µl of Milli-Q water. Thermocycler program parameters are listed below (Tab. 2, page 31).

From colonies, that were positive in colony PCR, liquid cultures were prepared. After incubation, plasmids were isolated using QIAprep® Miniprep kit and PCR was performed using primers listed below (Tab. 3, page 31). Reaction mixture for PCR consisted of 5 µl of plasmid, 2.5 µl of primers and 2.5 µl of Milli-Q water. Purified plasmids were sent for Sanger sequencing to the company SEQme to check the correctness of the sequence (Dobříš, Czech Republic).

Tab. 2: Thermocycler parameters for colony PCR.

Temperature (°C)	Time (min:s)	Cycles (nr)
95	5:00	1
95	0:30	30
55	0:30	30
72	4:00	30
72	5	1
8	∞	

Tab. 3: Primers used in the PCR to prepare samples for sequencing. *O-0186_1* and *118_4* correspond to forward and reverse primers, respectively, that were designed on the vector. *AtGH3.2_GSP_2* and *3* correspond to gene specific forward and reverse primer sequences, respectively, that were on the *GH3.2* coding sequence.

Primer	Sequence
O-*0186_1*	TAATACGACTCACTATAGGG
AtGH3.2_GSP_2	TTACAAAAGCGACCACTTCAA
AtGH3.2_GSP_3	CGCCAACTCTGTAACGGTAGA
O-*0118_4*	GCTAGTTATTGCTCAGCGG

3.2.2 AtGH3.2 protein production

3.2.2.1 SDS-PAGE

Transformed bacteria containing plasmid with target gene were grown in LB media containing kanamycin at 37 °C on orbital shaker at 180 rpm till optical density at 600 nm (OD_{600}) 0.4–0.6. One ml of each culture was sampled. Cultures were split in two parts. One aliquot was supplemented with IPTG to the final concentration of 0.1 $mol \cdot l^{-1}$ and the second one without IPTG was used as mock sample. To induce recombinant protein expression, cultures of at least 25 ml were incubated on orbital shaker at 180 rpm at 20 °C overnight. One ml of each culture was sampled after induction. Both sets of samples were immediately centrifuged at 1,500 *g* for 5 min. Supernatant was discarded, pellet was washed with 1× PBS and centrifuged again. Supernatant was discarded and pellet was stored at −20 °C. Stock solution of PBS (10×) was prepared by dissolving 80 g of NaCl, 2 g of KCl, 14.4 g of $Na_2HPO_4 \cdot 2H_2O$ and 2.4 g of KH_2PO_4 in 1 l of sterile dH_2O, pH was adjusted to 6.8. To prepare 1× PBS, the stock solution was diluted.

The following day, samples were resuspended in 1 ml of 1× PBS, transferred to 15 ml falcon tubes and kept in ice. Cells were lysed using sonicator (amplitude 40 %, pulse 10 s, pause 10 s, 10 times). During sonication, samples were kept in ice to prevent overheating. Sonicated samples were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 17,500 *g* and 4 °C for 30 min. Supernatant was transferred to fresh tubes and kept in ice. SDS-PAGE gel was prepared as follows: 12% resolving gel and 5% stacking gel. Mixture for resolving gel was prepared from 8.6 ml of sterile dH_2O water, 6 ml of 40% acrylamide, 5 ml of Tris-HCl (1.5 $mol \cdot l^{-1}$, pH 8.8), 0.2 ml of 10% sodium dodecyl sulphate (SDS) and 0.2 ml of 10% ammonium persulfate. Glasses were assembled using 1 mm thick spacer plate with casting frame and casting stand. 8 µl of TEMED was pipetted into mixture. Resolving gel was mixed gently and poured between glasses. Approximately 1 ml of isopropanol was added on the top of resolving gel to flatten it. When resolving gel was polymerized, isopropanol was poured out. Meanwhile, mixture for stacking gel was prepared from 4.35 ml of Sterile dH_2O, 0.75 ml of 40% acrylamide, 0.75 ml of Tris-HCl (1 $mol \cdot l^{-1}$, pH 6.8), 60 µl of 10% SDS and 60 µl of 10% ammonium persulfate. Six µl of TEMED was added into the mixture, stacking gel was mixed gently and poured on the top of the solidified resolving gel. Immediately after that, comb with 10 wells was placed into the stacking gel.

Loading dye was prepared from 4× LAEMMLI Sample Buffer by adding 100 µl of β-merkaptoethanol per 900 µl of the buffer. Aliquots of extracted proteins were prepared mixing 21 µl of protein with 7 µl of Loading Dye. Samples were heated up at 95 °C for 5 min, cooled down in ice for 2 min and spun down. Running buffer was diluted from 10× stock solution, that was prepared by mixing 30 g of Tris base with 144 g of glycine and 100 ml of 10% SDS and sterile dH_2O was added up to 1 l. Gels were prepared for the run, 1× running buffer was poured between gels and comb was removed. Wells were cleaned by pipetting the running buffer up and down in each well. Samples and protein ladder were loaded. Electrophoresis were run at 80 V for about 40 min, after that voltage was increased to 120 V.

3.2.2.2 Western blot

Proteins, separated with SDS-PAGE, were transferred to membrane using tank blotting. Per 1 gel, 1 nictrocellulose membrane with 0.45 µm pore size and 4 pieces of Whatman paper were cut. Stock solution of 10× blotting buffer was prepared before the start of the experiment from 30.3 g of Tris base, 144 g of glycine and sterile dH_2O up to 1 l.

Stock solution of 10× blotting buffer was diluted to 1× blotting buffer. Sandwich for blotting was assembled in the following order: cathode, sponge soaked in blotting buffer, 2 pieces of Whatman paper soaked in blotting buffer, SDS-PAGE gel, nitrocellulose membrane, another 2 pieces of soaked Whatman paper, soaked sponge and anode. Sandwiches were mounted in the blotting case that was filled with blotting buffer. Blotting was run at 100 V for about 1 h. Buffer was stirred during blotting with magnetic stirrer and cooled using cooling bags.

Stock solution of 10× TBST was prepared from 500 ml of Tris (1 $mol \cdot l^{-1}$, pH 7.4), 300 ml of NaCl (5 $mol \cdot l^{-1}$), 195 ml of dH_2O and 5 ml of Tween20. This stock solution was diluted to prepare 1× TBST. After the end of the blotting, membrane was blocked by incubating with 5% non-fat dry milk in 1× TBST (blocking buffer). After 2 h of incubation in gentle agitation, membrane was rinsed briefly 2 times with 1× TBST. Solution of antibody (Mouse anti His-Tag mAb including horseradish peroxidase) was prepared by adding antibody into the blocking buffer in a ratio of 1:10,000. Membrane was incubated with antibody solution for 2 h in gentle agitation followed by 4 washings with 1× TBST (10 min each).

Chemiluminescent substrate for horseradish peroxidase was prepared by mixing luminol and peroxide in a ratio of 1:1. Membrane was transferred between transparent sheets of plastic film and 500 µl of chemiluminescent substrate was added. Drops of the reagent were distributed by lifting up and down of the plastic sheet. After 5 min of incubation, excess detection reagent was drained. Chemiluminescent signal was measured with a CCD camera.

3.2.3 Enzyme assay

E. coli BL21 (DE3) cell cultures transformed with pETM11 were cultivated in LB with kanamycin at 37 °C and shaking at 180 rpm for 2 or 3 h till OD_{600} 0.4–0.6. As a control, *E. coli* expressing green fluorescent protein (GFP) were used. Expression of recombinant protein was induced by adding IPTG to final concentration 0,1 $mmol \cdot l^{-1}$. Bacterial cultures with IPTG were cultivated at 20 °C overnight under constant shaking at 180 rpm. The following day, 1 ml of samples were taken as time zero. After that, cultures were split in three parts. Substrate (IAA) alone was added into one aliquot, substrate and cofactors (IAA, amino acids mixture, ATP and $MgCl_2$) were added into the second aliquot and the remaining aliquot with no substrate or cofactors were used as mock sample (at least 30 ml of each culture in each conditions). Final concentration of IAA in both cases

was 1 mmol·l^{-1} and $MgCl_2$ and ATP were added up to final concentration 3 mmol·l^{-1}. Amino acids mixture was prepared dissolving Ala, Asp, Glu, Gln, Gly, Ile, Leu, Met, Phe, Trp, Tyr and Val in KOH (5 mol·l^{-1}), pH was adjusted using acetic acid (Caschera and Noireaux, 2018). Mixture was added to the culture in final concentration 1 mmol·l^{-1}. All three parts were incubated at 20 °C for 6 h. After the end of the incubation, 1 ml from each culture was sampled. Samples were centrifuged immediately after collection at 1,500 *g* for 5 min. Supernatant was pipetted into fresh tubes and stored in −20 °C.

For cell lysate experiment, the procedure was similar as described above. After overnight cultivation with IPTG, cultures were centrifuged at 1,500 *g* for 5 min. Supernatant was removed and pellet was washed with 1× PBS. Samples were centrifuged again under the same conditions and supernatant was removed again. Pellet was resuspended in 3 ml of 1× PBS and transferred to a 15 ml falcon tube. Cells were lysed using sonicator as described above. Samples were kept in ice to prevent overheating. Cell lysates were centrifuged at 17,500 *g* for 30 min at 4 °C. For enzymatic assays, 500 µl of lysate was used for each sample. As described above, three conditions were studied (mock, IAA alone and IAA with cofactors). After supplementation of the substrate, samples were incubated at 30 °C for 6 h with gentle shaking in dark. Samples before and after treatment were stored in −20 °C until LC-MS/MS analysis.

3.2.4 LC-MS/MS analysis

Samples from enzyme assays were vortexed and centrifuged at 36,670 *g* for 10 min. Using vials with inserts, 2 µl of supernatant was pipetted and 5 µl of labelled internal standard mixture was added. Samples were then diluted up to 40 µl with 10% methanol.

For LC-MS/MS analysis, Agilent 1260 Infinity II system coupled with Agilent 6495 Triple Quad was used. The LC system was equipped with a KINETEX column (1.7 µm C18, 50 x 2.1 mm) and acidified mobile phases – 0,1 % (v/v) of acetic acid in water (A) and 0,1 % (v/v) of acetic acid in methanol (B). Chromatographic run was set for 18 min, with a flow rate 0.3 ml/min and a linear gradient elution as follows: 0 min – 10% B, 11.5 min – 60% B, 11.75 min – 100% B, 14,75 min – 100% B, 15 min – 10% B. The column temperature was 30 °C and samples were stored in an autosampler at 4 °C. Injection volume of each sample was 10 µl.

Analytes were detected using mass detector in positive ESI mode using optimized MS conditions as follows: nebulizer pressure: 25 psi, flow and temperature of sheath gas: 12 l/min and 400 °C, gas flow and temperature: 14 l/min and 130 °C, output voltage gas

fittings: 0 V, capillary voltage: 2.8 kV in positive mode. Analytes were detected using optimized MRM transitions and collision energy (Tab. 4). Chromatograms were analysed using MassHunter Workstation software (Agilent Technologies, Santa Clara, USA), quantification of analytes was performed by standard isotope dilution analysis.

Tab. 4: Optimized conditions of MS/MS method for analysis of auxin metabolites.

Analyte	MRM transition	Retention time [min]	ESI	Collision energy [eV]
IAA	176 > 130	5.6	positive	30
[$^{13}C_6$]IAA	182 > 136	5.6		24
oxIAA	192 > 146	3.9	positive	12
[$^{13}C_6$]oxIAA	198 > 152	3.9		12
IAAla	247 > 130	5.2	positive	30
IAArg	332 > 130		positive	30
IAAsn	290 > 130		positive	30
IAAsp	291 > 130	3.6	positive	30
[$^{13}C_6$]IAAsp	297 > 136	3.6		36
IACys	279 > 130	4.0	positive	30
IAGln	304 > 130		positive	30
IAGlp	297 > 130		positive	30
IAGlu	305 > 130	4.4	positive	30
[$^{13}C_6$]IAGlu	311 > 136	4.4		24
IAGly	233 > 130	3.6	positive	30
IAHis	313 > 130		positive	30
IAHyp	289 > 130		positive	30
IALeu/Ile	289 > 130	10.4	positive	30
IALys	304 > 130		positive	30
IAMet	307 > 130	8.3	positive	30
IAPhe	323 > 130	10.8	positive	30
IAPro	273 > 130		positive	30
IASer	263 > 130		positive	30
IAThr	277 > 130		positive	30
IATrp	362 > 130	10.4	positive	30
IATyr	339 > 130		positive	30
IAVal	275 > 130	8.9	positive	30

4 RESULTS

4.1 RNA extraction

After extraction of RNA from plant material, extraction and purity of obtained RNA was inspected by spectrophotometric determination of absorbance of the samples at 260 and 280 nm using micro-volume Nanodrop spectrophotometer. Nanodrop measurements allowed qualitative and also quantitative analysis of the samples. To check the integrity of the extracted RNA, an aliquoted of RNA was run on agarose with gel electrophoresis. RNA was recovered from the purification column by two sequential elution steps, so concentration and purity of the obtained RNA from the first and the second elution (E1 and E2) was compared.

The results from Nanodrop measurements show (Tab. 5) that most of the RNA is eluted from the column during the first elution. In the sample from the second elution, the RNA concentration was in both mock and IAA treated samples about one-third of the first elution. Higher concentration of total RNA was obtained from plants that were incubated in medium with no IAA supplement (Mock).

Differences in RNA concentrations are visible also in results from agarose gel (Fig. 2, page 37) electrophoresis confirming Nanodrop results. In addition, these results show presence of two main fractions of RNA corresponding to bands around 1700 bp and 1200 bp. Further can be found band around 800 bp with lower intensity. In both samples from the first elution, also very weak signals around 600 bp and 250 bp can be seen.

Tab. 5 Concentration and purity of RNA isolated from untreated seven-day *Arabidopsis* plants (Mock) and plants with the additional of IAA in the medium (IAA).

	ng/ml	A_{260}/A_{280}
Mock – elution 1	1754.5	2.05
Mock – elution 2	555.5	1.97
IAA – elution 1	1291.1	2.08
IAA – elution 2	344.0	1.95

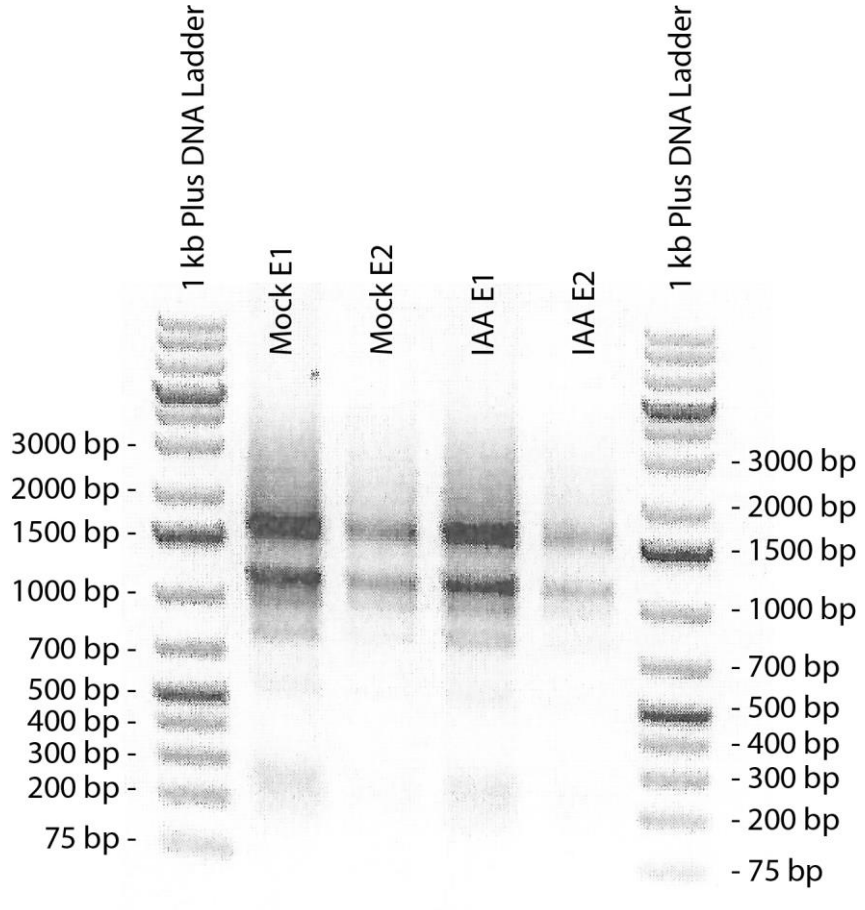

Fig. 2 Agarose gel electrophoresis of extracted RNA. Samples were prepared from plants incubated in medium with (IAA) or without (Mock) addition of IAA to the medium to the final concentration of 10 $\mu mol \cdot l^{-1}$. E1 and E2 corresponds to the first and second elution.

4.2 cDNA synthesis and PCR

From extracted RNA, cDNA was prepared and was amplified with PCR using specific primers to amplify *GH3.2* coding sequence. Accuracy of product of the PCR reaction was tested using agarose gel electrophoresis. Expected molecular weight of the PCR product was determined by *in silico* PCR using SnapGene software (Fig. 3).

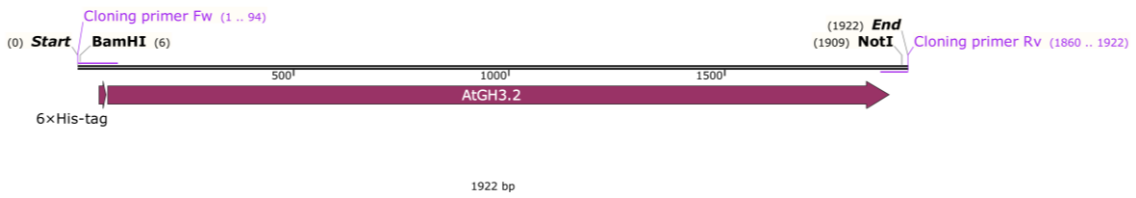

Fig. 3 Visualisation of PCR product prepared by *in silico* PCR using SnapGene software. Overhanging primers were used for insertion of *BamHI* restriction site and 6×His-tag to the 5' end and *NotI* restriction site to the 3' end.

In all four samples, two bands with size of approximately 2000 bp and 150 bp were obtained (Fig. 4). The bigger one corresponds to target gene with insertions (Fig. 3, page 37) with 1922 bp and the small one is probably formed by primer dimers. The DNA fragments corresponding to the target gene PCR products obtained from treated IAA samples resulted in thicker bands than those obtained from mock samples. *AtGH3.2* transcripts are known to accumulate more after exogenous application of IAA (Staswick *et al.*, 2005).

PCR products were further purified with PCR Clean-up kit. In order to remove undesirable small DNA fragments, usage of dilute NTI buffer solution was tested (Fig. 4). When 100% NTI buffer is used, all DNA molecules larger than approximately 50 bp are retained, but with decreasing concentration of NTI buffer, size of cut-off should be shifted towards larger fragments. Two dilutions corresponding to 12.5 % and 25 % were tested. When 12.5% NTI was used, unwanted primer dimers were removed, but also the majority of target DNA molecules were not retained on the column, so the yield of the purification step was decreased rapidly. Better results were obtained for 25% NTI. There was reduction in the intensity of the band of undesirable DNA fragments and moreover, there was no loss of target DNA, so this concentration of 25 % proved to be ideal.

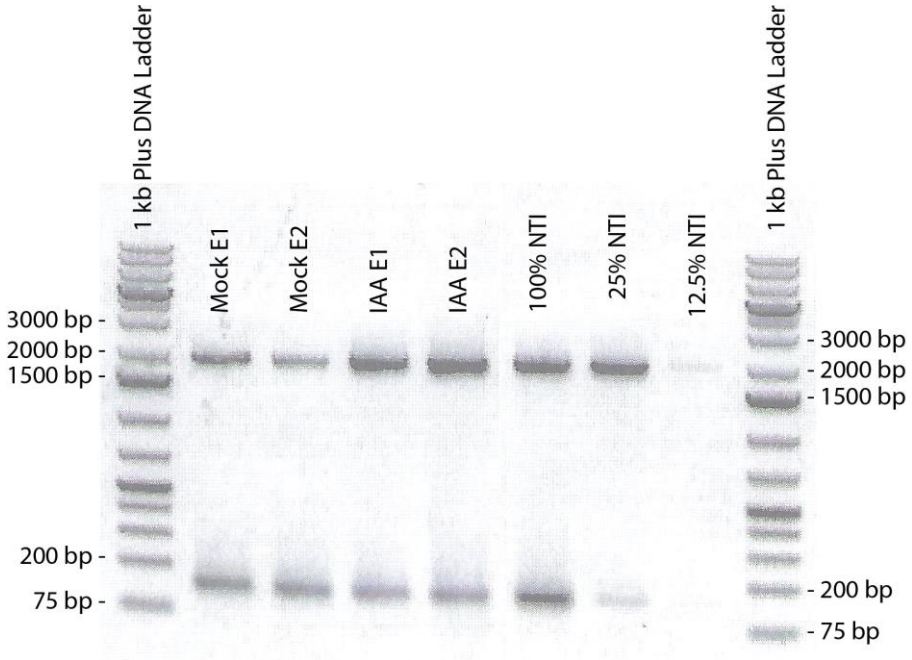

Fig. 4 Agarose gel electrophoresis of PCR products and purified PCR products using different concentration of NTI buffer in PCR Clean-up kit. Samples were prepared from plants cultivated on medium with (IAA) or without (Mock) addition of IAA to the medium to the final concentration of 10 $\mu mol \cdot l^{-1}$. E1 and E2 corresponds to the first and second elution.

4.3 Colony PCR and sequencing

To verify the presence of the target gene in the recombinant plasmid (Fig. 5), colony PCR was performed with transformed bacteria. Bacterial cultures were prepared from two colonies with a positive result. Plasmids were isolated, and the sequence of target recombinant gene was verified by Sanger sequencing (Fig. 6, pages 40-42). Obtained sequences confirmed the presence of the intact target gene without any mutations. Occasionally, mismatches occurred during individual sequencing runs. All of these were checked in sequencing chromatograms and all were considered as errors in sequencing. Most of them occurred at the beginnings or ends of the obtained sequences, where the sequencing error rate is higher.

Fig. 5 Visualisation of pETM11 vector with inserted recombinant DNA with marked cleavage sites for used restriction enzymes.

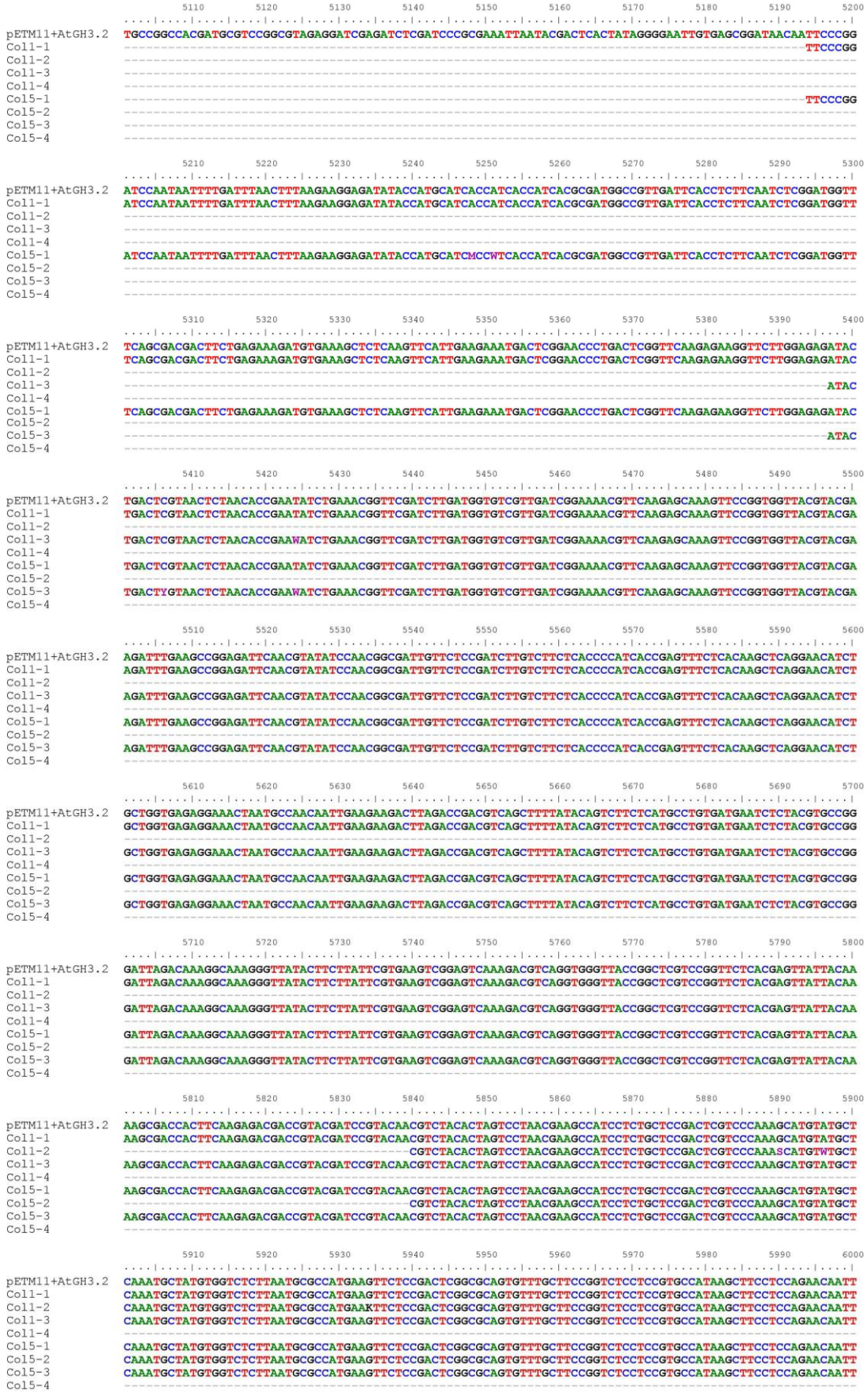

Fig. 6 Data obtained from sequencing aligned with part of the construct sequence containing the *AtGH3.2* gene.

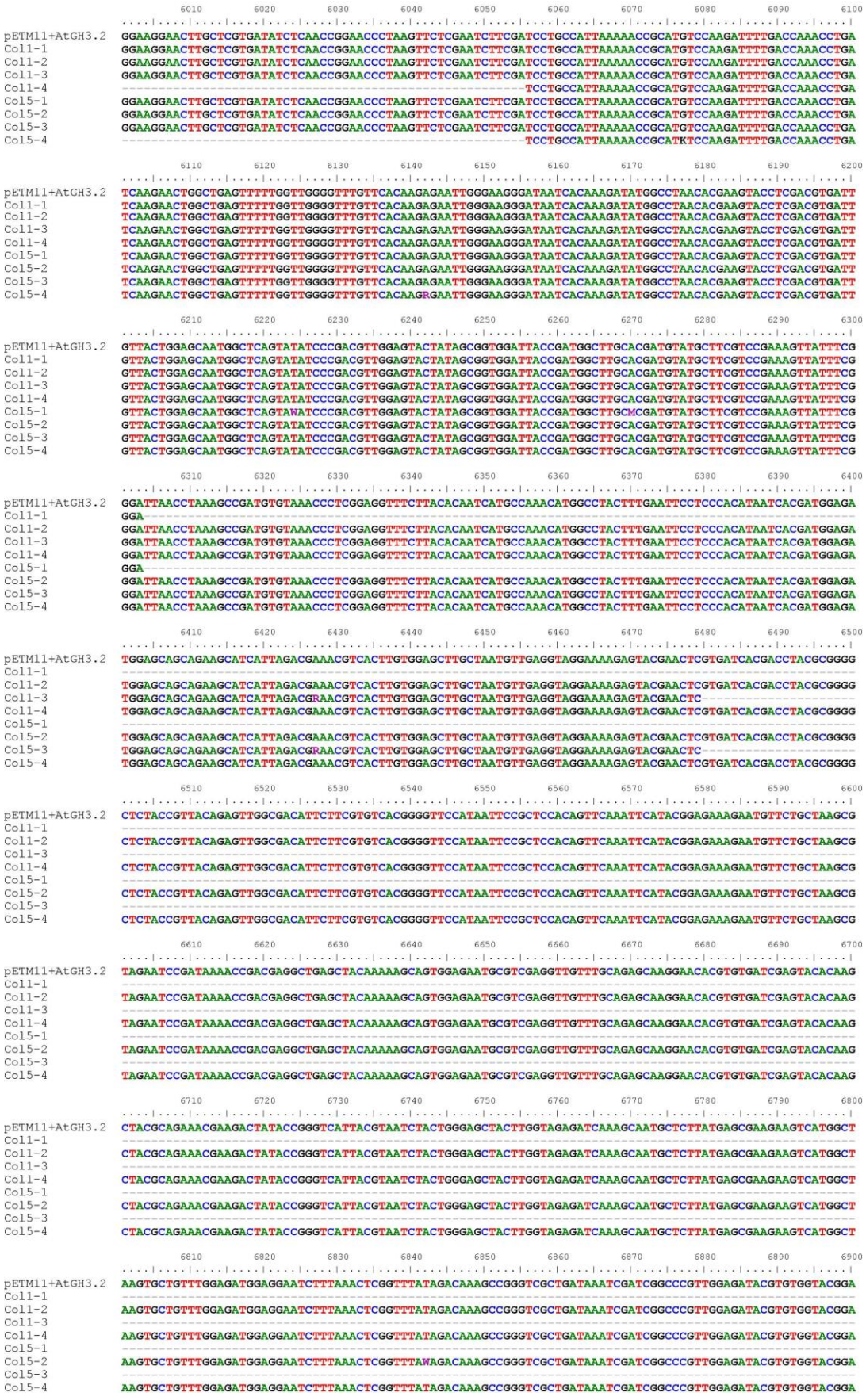

Fig. 6 Data obtained from sequencing aligned with part of the construct sequence containing the *AtGH3.2* gene *(continuation).*

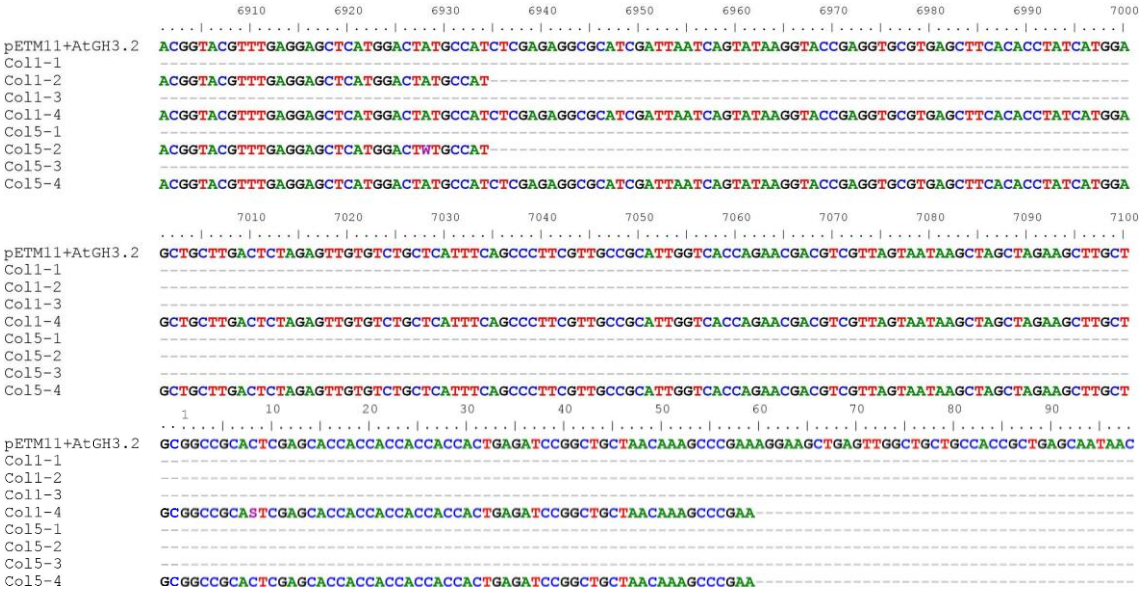

Fig. 6 Data obtained from sequencing aligned with part of the construct sequence containing the *AtGH3.2* gene *(continuation)*.

4.4 Recombinant protein production

To control the expression of the target gene from the recombinant plasmid in transformed bacterial cells, an experiment based on SDS-PAGE and following western blotting to nitrocellulose membrane was designed. Expression of target gene was induced by incubating cultures at 20 °C overnight. One aliquot of cultures was supplemented with IPTG to final concentration of 0.1 mmol·l^{-1} and the remaining one without IPTG was used as the mock sample. Since the amount of recombinant protein in the samples is unknown, both undiluted and half-diluted sample aliquots were prepared and loaded on SDS-PAGE.

Western Blot analysis (Fig. 7, page 43) shows that, no or very little expression of the target gene before recombinant protein induction is detected in samples collected before induction. There is also no expression in mock samples after incubation. However, after 6 h of incubation with IPTG, expression was clearly visible which corresponds with the role of IPTG in triggering transcription of genes under the control of *lac* operator. Position of the bands around 70 kDa corresponds to the size of the GH3 proteins with 6×HisTag.

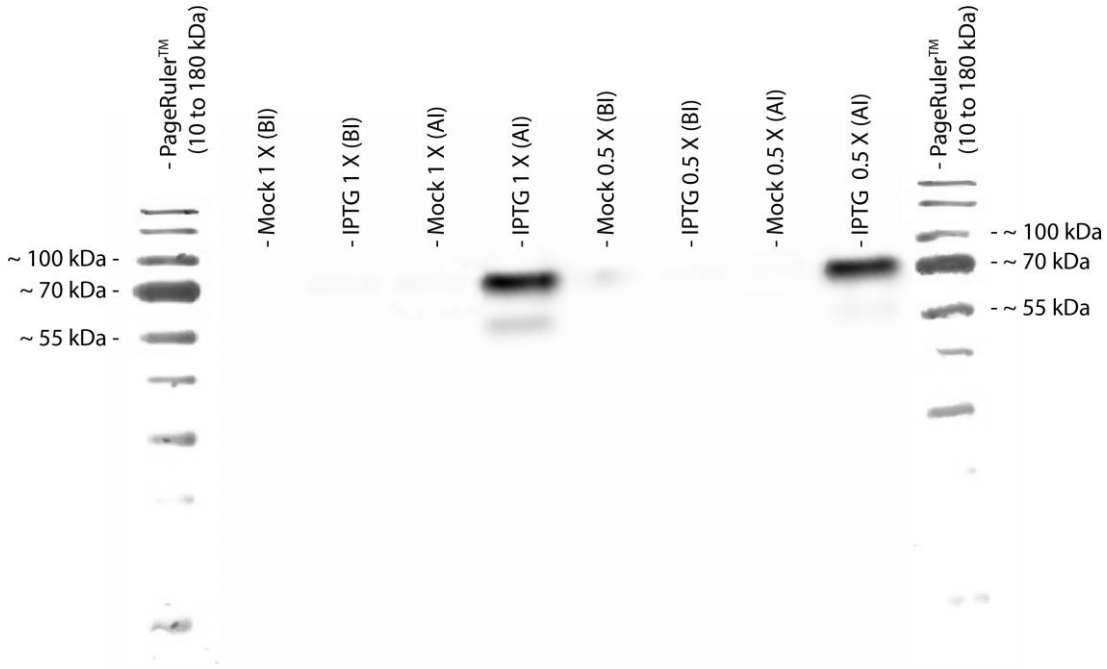

Fig. 7 SDS-PAGE of extracts from recombinant bacteria cells expressing *AtGH3.2* gene and following Western blotting to nitrocelulose membrane. BI represents samples before induction and AI samples taken after overnight incubation with or without IPTG in final concentration 0.1 mmol·l^{-1}.

4.5 Enzyme assay

An experiment was designed to determine which amino acids can be conjugated with IAA by recombinant GH3.2 (see section 3.2.3 for more experimental details). GH3.2-expressing bacterial cultures were cultivated for 6 h (Brunoni *et al.*, 2019) in presence of IAA and with or without addition of cofactor mixture (ATP, $MgCl_2$ and mix of amino acids). To test the enzymatic activity of GH3.2 in cell lysate, experiments were also done using cell lysates from GH3.2-expressing bacterial culture. Bacteria cultures expressing GFP were used as a negative control and untreated bacteria cultures or cell lysates as mock. All samples were then analysed by LC-MS/MS method (see the chapter 3.2.4).

Results from enzyme assay show levels of IAA and its metabolites in supernatant after centrifugation of recombinant cell cultures. Concentration of IAA is decreased in *AtGH3.2*-expressing cultures compared to control (GFP) (Fig. 8A, page 44), indicating depletion of IAA. This confirms, that AtGH3.2 metabolizes IAA. Similar results were obtained also for cell lysate samples with addition of cofactors (Fig. 8B, page 44). In lysate samples treated only with IAA, the concentration of IAA is not decreased compared to control, because nearly no conjugates were formed in these conditions.

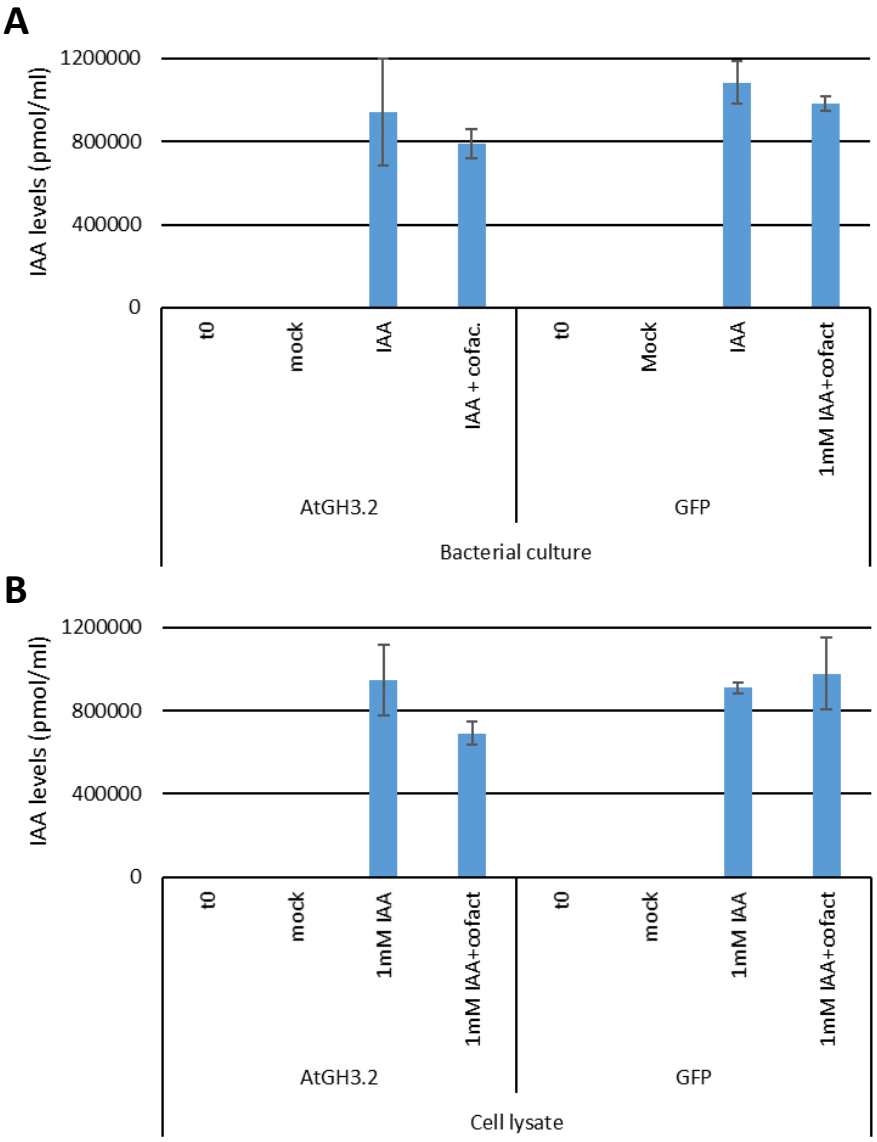

Fig. 8 Concentrations of IAA in bacterial cultures (A) and cell lysates (B). Bar graphs show mean values ± standard deviation (n=3).

Concerning the conjugates of IAA and amino acids, the highest levels were measured for IAAsp (Fig. 9, page 45). We can also see that concentration is even higher when cofactors were added, which can be caused by lack of Asp in culture without addition of cofactors. AtGH3.2 also conjugated efficiently IAA with Glu and Trp, but the levels of these conjugates were much lower than IAAsp. Concentration of conjugates in these samples with addition of cofactors is even lower compared to the samples with supplemented with IAA alone. Activity of AtGH3.2 enzyme with Asp was observed also in cell lysate samples (Fig. 10, page 46). As well as in the case of IAAsp, conjugation reaction forming IAGlu and IATrp take place also in cell lysate, but only after addition of cofactors.

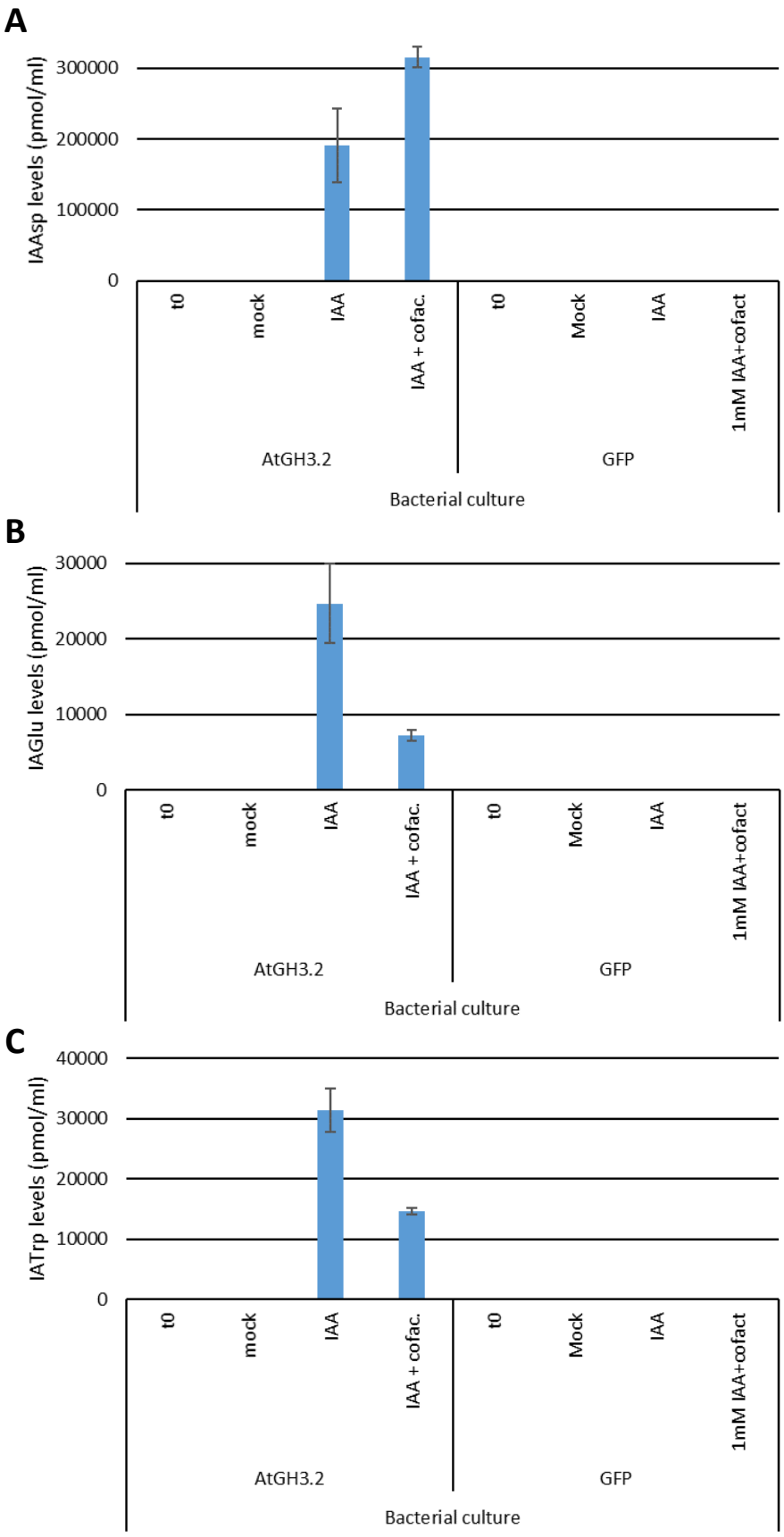

Fig. 7 Concentrations of IAAsp (A), IAGlu (B) and IATrp (C) in bacterial cultures. Bar graphs show mean values ± standard deviation (n=3).

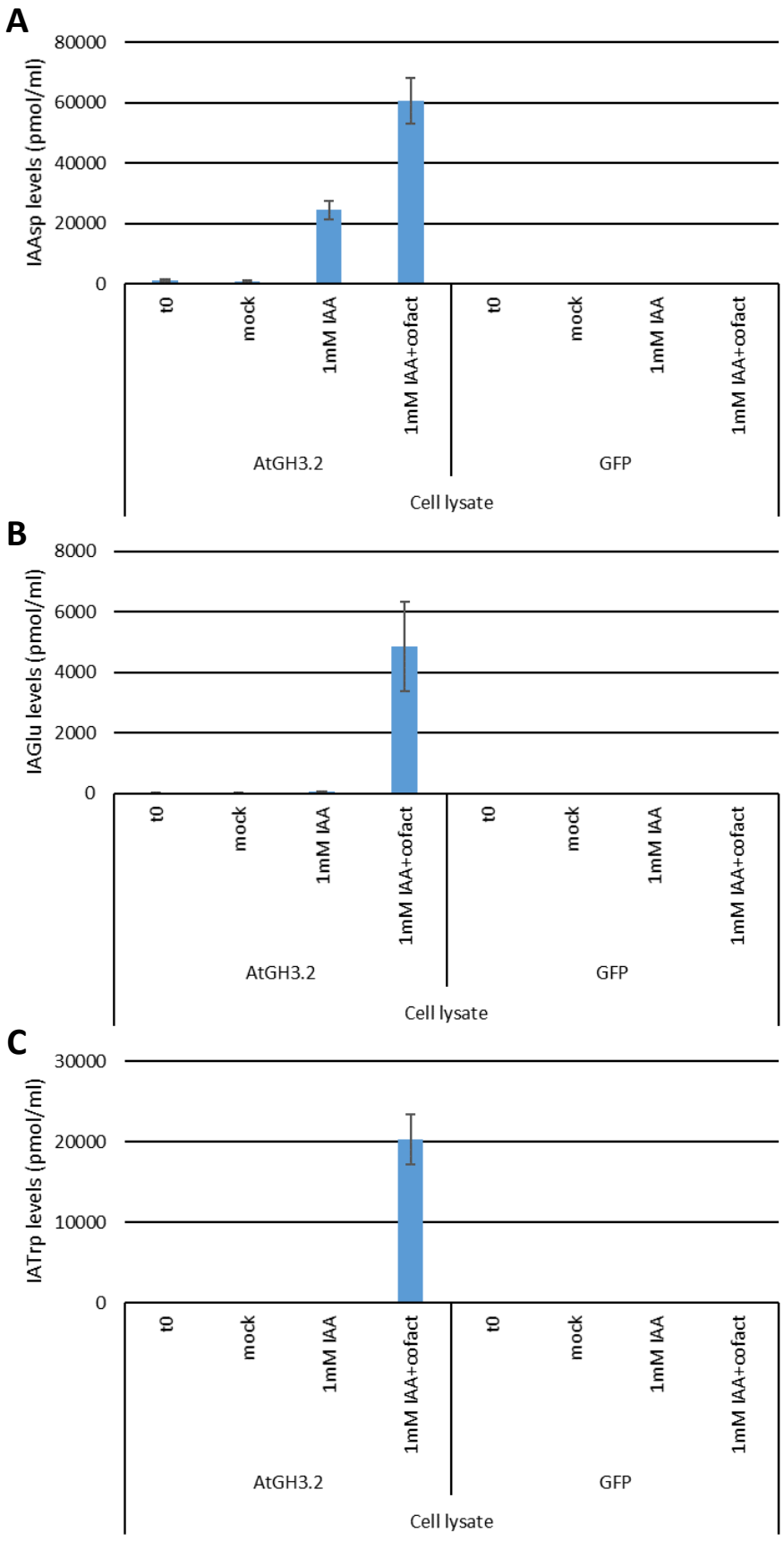

Fig. 80 Concentrations of IAAsp (A), IAGlu (B) and IATrp (C) in cell lysates. Bar graphs show mean values ± standard deviation (n=3).

Levels of the rest of conjugates in bacterial culture samples were decreasing in following order: IAPhe, IALeu, IAGly, IAVal and IAAla (Fig. 11A). Interestingly, the result of conjugation with Gly (Fig. 11B) was the only one, where nearly no activity of studied enzyme was observed in cell lysate even with addition of cofactors.

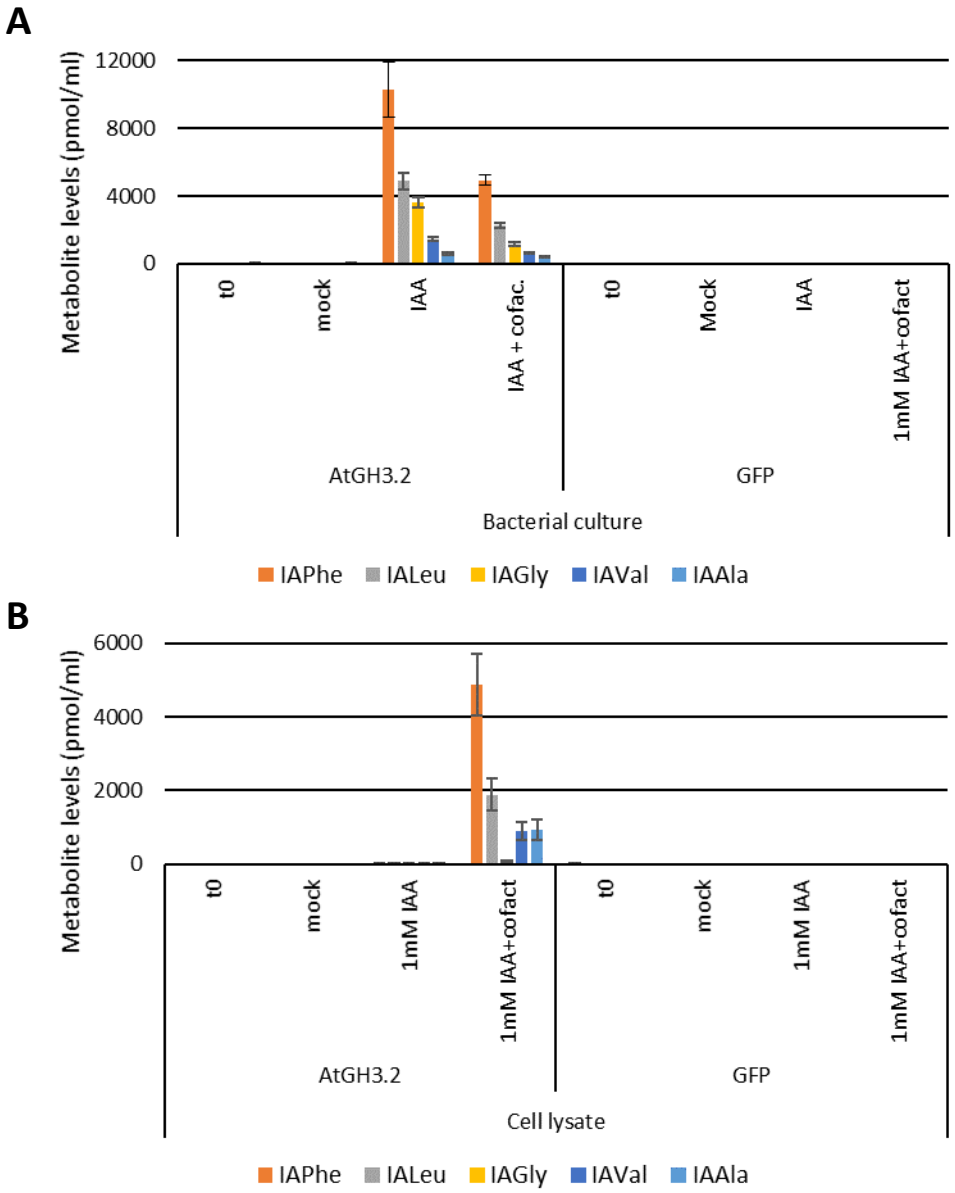

Fig. 11 Concentrations of IAGly, IAAla, IAVal, IALeu and IAPhe in bacterial cultures (A) and cell lysates (B). Bar graphs show mean values ± standard deviation (n=3).

5 DISCUSSION

The endogenous level of IAA in plants is very carefully regulated by a combination of its biosynthesis, conjugation and degradation. For further study of plant growth and development, it is important to characterize the enzymes involved in IAA metabolism. Studied enzyme AtGH3.2 is directly involved in conjugation of IAA to amino acids. This enzyme belonging to the family GH3 was characterized using recently published method using its heterologous expression in *E. coli* (Brunoni *et al.*, 2019). Compared to previously used *in vitro* methods, no protein purification was required because enzymatic reaction takes place directly in the bacterial culture providing rapid characterization of studied enzyme.

In the first part of presented work, experiments were performed to prepare recombinant bacterial cultures expressing the coding sequence of *GH3.2* from *Arabidopsis thaliana*. *E. coli* was chosen as the host organism, inter alia because it is incapable of IAA biosynthesis and does not have an endogenous response to IAA, making it an ideal host organism for the study of enzymes involved in IAA metabolism (Brunoni *et al.*, 2019).

First, RNA was extracted from plant material. Due to its low stability, its concentration, purity and integrity was checked (Tab. 5, Fig.2). It is necessary to obtain RNA in sufficient purity as well as not fragmented. In the presented experiment, an A_{260} / A_{280} average ratio 2.0 was achieved, which is generally considered to be pure RNA (Desjardins and Conklin, 2010).

Further, cDNA was synthesized and amplified using PCR. Its products were checked using agarose gel electrophoresis, which confirmed presence of DNA molecules with corresponding number of base pairs to the target gene (Fig. 4). Using overhanging primers, 6×His-tag and restriction site for *BamHI* were added to the 5' end and restriction site for *NotI* to the 3' end (Fig. 3). In order to remove small DNA fragments, another experiment focused on optimisation of purification of PCR products for downstream application by cleaning up, among the others, from primer dimers that would inevitably interfere with the cloning of the specific PCR product in the final expression vector. A decrease in unwanted fragments was achieved with minimal loss of target DNA by testing different dilution ratios of the buffer used for the PCR product clean-up (Fig. 4).

For expression in *E. coli*, vector pETM11 was chosen (Fig. 5). Its advantage is presence of bacteriophage T7 promoter placed right upstream of the target gene.

In addition, this system is under the control of IPTG-inducible *lac* operator. Generally, this system provides synthesis of large amounts of mRNA and recombinant protein is accumulated in high concentration (Baneyx, 1999). Previously, for example, the pET-32 vector, which has the same mechanism of recombinant protein expression as the vector used herein, was used to express the GH3.5 enzyme in *E. coli* (Zhang *et al.*, 2007).

Presence of the *AtGH3.2* gene in the plasmid of the host cells was verified by colony PCR and then plasmid was isolated from the positive colonies and subsequently sequenced. Obtained data show that the cloned sequence corresponds to the desired one (Fig. 6).

The correct expression of the recombinant protein in bacterial culture was confirmed by SDS-PAGE followed by Western blot with immunodetection using mouse anti His-Tag antibody including horseradish peroxidase. The experiment proved that after overnight induction at 20 °C with IPTG, the target gene was successfully expressed (Fig. 7). This corresponds to the theoretical principle of this expression vector. Expression systems based on this principle of gene expression regulation are commonly used to produce recombinant proteins in *E. coli* (Tabatabaee *et al.*, 2013; Margawati *et al.*, 2017). Obtained bands correspond to the assumptions, because *GH3* genes generally encode proteins of about 70 kDa (Böttcher *et al.*, 2010; Yu *et al.*, 2018).

Conversion of IAA to its conjugates with amino acids is catalysed by members of group II of GH3 enzyme family (Staswick *et al.*, 2005). Generally, different conjugates can be formed, but IAAsp and IAGlu are the most common IAA-amino acid conjugates in *Arabidopsis* (Novák *et al.*, 2012). In performed experiment, conjugation with 12 amino acids was tested including Ala, Asp, Glu, Gln, Gly, Ile, Leu, Met, Phe, Trp, Tyr and Val (Pěnčík *et al.*, 2009). The enzymatic products were analysed by LC–MS/MS using direct injection of a small supernatant fraction from the bacterial culture. Due to limited sensitivity of LC-MS/MS method used, accurate quantification was possible for eight of amino acid conjugates with IAA.

Presented results can be compared with *in vitro* activity assays of GH3.2 and other GH3 enzymes published by Staswick *et al.* (2005) using recombinant glutathione *S*-transferase:GH3 fusion proteins in *E. coli*. IAA conjugates formed by activity of GH3.2 were then analysed by thin-layer chromatography. Their results showed that GH3.2 may participate in formation of conjugates with Asp, Glu, Phe, Gly, Ile, Leu, Met, Pro, Tyr and Trp, which partly corresponds with results presented in this work. My results show

that Asp, Glu and Trp are three suitable substrates for IAA conjugation reaction (Fig. 9, Fig. 10). However, obtained data show that Asp is preferred over the other two substrates. Lower concentration was determined for IAA conjugate with Phe and compared to IAAsp, very low concentrations were determined for IAGly, IAAla, IAVal and IALeu (Fig. 11). It is important to mention that purification of the recombinant protein from bacterial cultures could be a further step to more accurately determine substrate specificity of AtGH3.2.

IAAsp and IAGlu are, according to published data so far, the best substrates for GH3.2, GH3.3, GH3.4, GH3.5, GH3.6 and GH3.17 enzymes, but preference of one of these varies among them (Staswick *et al.*, 2005). According to these results, there is also substantial amount of product gained from conjugation with Met and Trp. These findings were confirmed in my work for Trp, whereas IAMet was one of those conjugates that was not possible to quantify accurately. High levels were found for IAPhe and low concentrations for IAGly and IALeu, which also is in agreement with the results published by Staswick *et al.* (2005). On the contrary, compared to previously published results, a small amount of IAAla and IAVal can also be found in presented results.

6 CONCLUSION

In theoretical part of this work, there are chapters dealing with the basics of enzymology and introduction to the molecular cloning. Chapters about the role of GH3 enzymes in metabolism of phytohormones. At the end of the theoretical part, methods for targeted profiling of phytohormones were also described.

In experimental part, experiments focused on preparation of the bacterial culture expressing the recombinant enzyme, GH3.2 from *Arabidopsis thaliana*. Engineered bacterial cultures were obtained by adopting a methodology that was previously developed in our laboratories (Brunoni *et al.*, 2019). Extraction of RNA from *Arabidopsis* seedlings allowed the preparation of cDNA that was subsequently used as template for PCR amplification of the gene of interest using overhanging primers to insert restriction sites required for the cloning and a His-tag to facilitate further isolation and detection of the recombinant protein. As a cloning vector, pETM11 plasmid was used. As a host organism *Escherichia coli* was chosen and its competent cells were transformed using heat shock method. The presence of the target gene with no mismatches, insertions or deletions was verified by colony PCR followed by sequencing. All these steps resulted in successful preparation of bacterial cultures containing the desired construct. In the next step, production of recombinant protein was verified. This was done using overnight incubation at 20 °C with IPTG, because recombinant gene was under the control of T7 promoter linked to the *E. coli lac* operator. Obtained results confirmed that the desired recombinant enzyme was successfully produced.

All these findings were used in the final experiment to determine which amino acids can be conjugated with IAA by the enzyme AtGH3.2. Concentration of conjugates was determined by LC-MS/MS method after incubation of bacterial cultures with IAA. Interestingly, higher concentrations were also achieved when cofactors (ATP, $MgCl_2$ and mix of amino acids) were added. The highest concentration was determined for IAAsp, indicating that Asp is preferred over other amino acids. High concentrations were also obtained for IAGlu and IATrp, but their levels were lower compared to IAAsp. Possible activity was found also in the presence of Phe, Ala, Gly, Val and Leu. For comparison, these conjugation reactions were also investigated using cell lysate extract. These results show similar trends, but determined levels of conjugates in lysates cannot be directly compared to results from bacterial cultures.

For further characterization of the studied enzyme, purification of the recombinant protein from the bacterial cultures would be necessary. Subsequently, it would be possible to determine the kinetic parameters of the AtGH3.2 and determine more precisely the substrate specificity. This could be followed by crystallographic analysis in order to elucidate arrangement of catalytic residues in the active site and structure of enzyme. Furthermore, the mechanism of the catalytic reaction and mechanisms of regulation could also be studied.

7 LITERATURE

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8 LIST OF ABBREVIATIONS AND SYMBOLS USED

cDNA	complementary DNA
ELISA	enzyme linked immunosorbent assays
ESI	electrospray ionization
dNTP	deoxynucleotide triphosphates
GC	gas chromatography
GFP	green fluorescent protein
GH3	Gretchen Hagen 3 enzyme family
IAA	indole-3-acetic acid
IAAla	indole-3-acetyl-L-alanine
IAArg	indole-3-acetyl-L-arginine
IAAsn	indole-3-acetyl-L-asparagine
IAAsp	indole-3-acetyl-L-aspartic acid
IACys	indole-3-acetyl-L-cysteine
IAGln	indole-3-acetyl-L-glutamine
IAGlp	indole-3-acetyl-L-pyroglutamic acid
IAGlu	indole-3-acetyl-L-glutamic acid
IAGly	indole-3-acetyl-L-glycine
IAHis	indole-3-acetyl-L-histidine
IAHyp	indole-3-acetyl-L-hydroxyproline
IALeu	indole-3- acetyl-L-leucine
IAIle	indole-3-acetyl-L-isoleucine
IALys	indole-3-acetyl-L-lysine
IAMet	indole-3-acetyl-L-methionine
IAPhe	indole-3- acetyl-L-phenylalanine
IAPro	indole-3-acetyl-L-proline
IASer	indole-3-acetyl-L-serine
IAThr	indole-3-acetyl-L-threonine
IATrp	indole-3-acetyl-L-tryptophan
IATyr	indole-3-acetyl-L-tyrosine
IAVal	indole-3- acetyl-L-valine
IPTG	isopropyl-beta-D-thiogalactopyranoside
LB	lysogeny broth medium
LC	liquid chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MRM	multiple reaction monitoring
NTI	binding buffer used with the NucleoSpin™ Gel and PCR Clean-up Kit
OD_{600}	optical density measured at 600 nm
oxIAA	2-oxindole-3-acetic acid
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
TBST	mixture of tris-buffered saline and Tween 20