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Master thesis

# Overexpression of indole-3-acetic acid-amido synthetases GH3 in *Arabidopsis thaliana*

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## Acknowledgments

I would like to express my gratitude…

… to my family, for their patience and support.

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## Abbreviations

| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| 4-Cl-IAA | 4-chloroindole-3-acetic acid |
| ABCB | ATP-binding cassette |
| ABP-1 | auxin-binding protein 1 |
| ARE | auxin-responsive element |
| ARF | auxin response factor |
| Col-0 | Columbia ecotype |
| DBD | DNA-binding domain |
| ER | endoplasmic reticulum |
| gDNA | genomic DNA |
| GH3 | Gretchen Hagen 3 |
| HDGS | homology-dependent gene silencing |
| HRP | horseradish peroxidase |
| Hyg | hygromicin B |
| IAA | indole-3-acetic acid |
| IaaH | IAM hydrolase |
| IaaM | tryptophan monooxygenase |
| IBA | indole-3-butyric acid |
| IPA | indole-3-pyruvic acid |
| JA | jasmonic acid |
| MDR | multidrug resistance |
| MOP | modulator of PIN |
| NAA | 1-naphtalenacetic acid |
| NIT1 | nitrilase 1 |
| oxIAA | oxindole-3-acetic acid |
| PAA | phenylacetic acid |
| PAT | polar auxin transport |
| PIN | PIN-formed proteins |
| PTGS | posttranscriptional gene silencing |
| RT | room temperature |
| SA | salicylic acid |
| SAUR | small auxin up RNA |
| TAA | tryptophan aminotransferase of Arabidopsis |
| TGS | transcriptional gene silencing |
| TIR1 | transport inhibitor response 1 |
| Trp | tryptophan |
| WS | Wassilewskya ecotype |
| YUC | yucca |

# 1. INTRODUCTION

Phytohormones affect multiple developmental processes in plants and their activities are highly intertwined. One of the hormones identified as a key actor in plant growth and defense is auxin. Auxin regulates expression of numerous genes and its effects are concentration-dependent. Some of the auxin-responsive genes code for enzymes responsible for auxin conjugation. Formation of auxin conjugates enables fine regulation of auxin levels by converting active auxin into inactive conjugates. This is presumabely the main way of storing this hormone since most of auxin (>90%) is present in plants in this form. Unlike oxidative inactivation pathways, this process is reversible and auxin can be converted back to active state through acitivity of hydrolases. Conjugate synthetases link main active form of auxin in higher plants, indole-3-acetic acid (IAA), to sugars, amino acids or peptides. Superfamily of GRETHEN HAGEN 3 (GH3) genes was identified as a main family of auxin conjugate synthetases. These genes were most thoroughly studied in arabidopsis, but their homologs were also detected in other plants species and microorganisms. For example, in the moss *Physcomitrella patens*, another important model organism for studying plant differentiation and development, two members of GH3 protein family were identified (PpGH3.1 and PpGH3.2), while one homolog was identified in plant pathogen *Plasmodiophora brassicae* (PbGH3). These enzymes were also proven to conjugate auxin, only with different preferences for attached amino acids.

Aim of this study was to generate homozygous transgenic plants with homologous (AtGH3.3) and heterologous (PpGH3.2 and PbGH3) overexpression of indole-3-acetic acid-amido synthetases. This was achieved through transformation of wild type plants via floral dip method, and selection and cultivation of transformants. Level of overexpression was calculated from qPCR results. Homozygous lines, identified in T3 generation of transformants, will be further used for examining the role of indole-3-acetic acid-amido synthetases in auxin metabolism and during abiotic stress response.

## 2. BIBLIOGRAPHIC STUDY

The concept of phytohormones was firstly proposed by german botanist Julius von Sachs in 1887. Phytohormones are signalling substances that regulate coordinated plant growth and are active at low concentrations. Plant hormones include steroids, peptides and small molecules which constitute five classical classes of phytohormones: auxins, abscisic acid, cytokinins, ethylene and gibberellins (Teale *et al*., 2006). Two key groups of hormones important for almost all aspects of plant growth and development are auxins and cytokinins. These hormones are connected in a regulatory loop meaning they regulate each others synthesis and can act synergistically or antagonistically, depending on the context and their respective levels (Jones and Ljung, 2011). During the past century, plant physiology researches focused on plant hormones, identified auxins as critical hormones playing a central role in diverse developmental processes.

Auxins are a small class of molecules with a powerful ability to induce growth responses in plants. Charles Darwin was the first to document the effects of auxins when he published *The power of movement in plants* in 1880. Charles and Francis Darwin conducted the earliest experiments with auxin, observing the effects of a hypothetical substance modulating shoot elongation to allow tropic growth towards light. They noted that after the perception of light in one part of a grass coleoptile, an influence was transported, causing the bending towards the light in another part (Darwin, 1880). Initially, three types of auxins were identified in plants, one of which was also found in human urine. Today, auxin is a generic name representing a group of important molecules in plants, which are also found in humans, animals and microorganisms (Teale *et al*., 2006).

### 2.1. Auxin role and biosynthesis

Auxin drives plant growth and morphogenesis by controlling plant cell division, elongation and differentiation in a concentration-dependent manner. It modulates diverse processes such as tropic responses to light and gravity, general root and shoot architecture, organ patterning and vascular development (Davies, 2004). Elucidating auxins activity was greatly influenced by two theoretical concepts; those of hormones and morphogen. Auxin formally matches the classical definition of a hormone as extracellular signalling molecule which acts on target cells distant from the site of synthesis, if only non-polar transport is taken into consideration. However, the most investigated form of auxin movement, polar transport,

contrasts with the passive motion of mammalian hormones through blood. On the other hand, auxin also partially corresponds to the term morphogen as a substance that forms a concentration gradient and is involved in developmental patterning. This is due to its short-distance activity in regulating the pattern of cell division and differentiation (Friml, 2003).

To this day, multiple compounds which elicit an auxin response have been identified. These can be assorted into two remote groups, including naturally occuring active auxins such as indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-Cl-IAA) and phenylacetic acid (PAA), as well as synthetic compounds, 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthalenacetic acid (NAA), to name a few (Korasick *et al*., 2013).

Indole-3-acetic acid (IAA) is the main auxin in higher plants and the best-studied naturally occuring active auxin. Both plants and some plant pathogens produce IAA to modulate plant growth (Zhao, 2010). IAA biosynthesis in plants can occur through two major routes: tryptophan (Trp)-dependent and Trp-independent pathways. Biosynthesis of aromatic amino acids, including tryptophan, takes place in plastids, while most data suggest that Trp-dependent IAA biosynthesis is localized in cytoplasm. Multiple IAA biosynthetic pathways have high level of redundancy and may contribute to regulation of IAA production (Woodward and Bartel, 2005).

### *2.1.1. Trp-dependent IAA biosynthesis*

Several Trp-dependent pathways have been proposed, generally named after an intermediate. Potential IAA biosynthetic pathways are shown in Figure 1. The IPA pathway is the main contributor to free IAA and is the only pathway in which every step has been identified (Zhao, 2012). This two-step process is catalyzed by two enzyme families: TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family which convert Trp to INDOLE-3-PYRUVIC ACID (IPA), and YUCCA (YUC) family of flavin monooxygenases, which convert IPA to IAA using NADPH and oxygen (Dai *et al*., 2013). TAA1 is an aminotransferase with a wide distribution throughout the plant kingdom, suggesting that the IPA pathway is highly conserved (Zhao, 2010).

The IAM pathway is a second important pathway active in microorganisms, that may also contribute to IAA production in plants. In *Agrobacterium tumefaciens* and *Pseudomonas syringae* Trp monooxygenase (IaaM) converts Trp to IAM, and IAM hydrolase (IaaH) converts IAM to IAA (Patten and Glick, 1996). However, IAM hydrolase lacks auxin activity in

arabidopsis and IAA is probably produced through the activity of AMIDASE1 that converts IAM to IAA *in vitro* (Pollman *et al*., 2003).

The IAOx pathway involves CYP79B2 and CYP79B3, cytochrome P450 enzymes that convert Trp to IAOx (Zhao *et al*., 2002), which is further converted to IAN and to active IAA. Although all enzymatic steps between IAOx and IAN haven't yet been identified, IAN can be converted to active IAA through the activities of the NIT1 family of nitrolases (Normanly *et al*., 1997). IAOx pathway is largely used for production of defense compounds such as glucosinolates or camalexins, but is probably not the main IAA biosynthesis pathway in plants (Korasick *et al*., 2013).

### *2.1.2. Trp-independent IAA biosynthesis*

Not much is known about potential intermediates and none of the genes involved in the Trp-independent IAA biosynthetic pathway have been identified. However, it is postulated that this pathway branches from either indole or indole-3-glycerol phosphate (Ouyang *et al*., 2000).

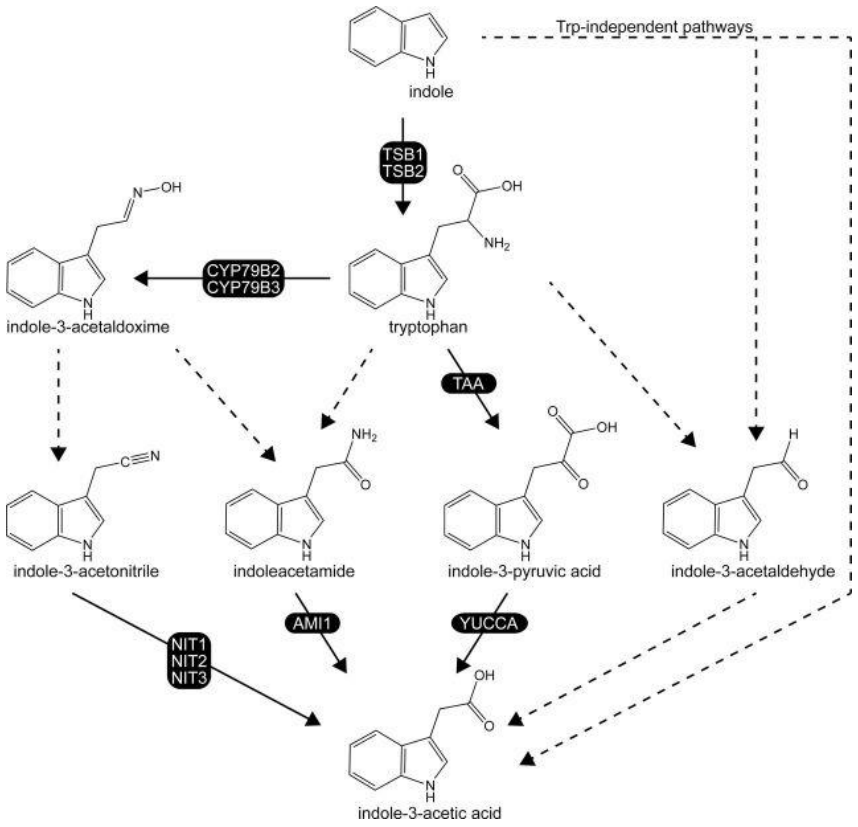



**Figure 1.** *Potential IAA biosynthetic pathways. (adapted from Korasick et al., 2013)*

## 2.2. Auxin transport pathways and regulation

Auxin is a highly mobile signalling molecule, unusual among other phytohormones since it is specifically and actively transported. Auxin is generally transported by two distinct pathways. Most IAA is passively transported away from the source tissues (young leaves and flowers) by an unregulated bulk flow in the mature phloem. Another way of auxin transport is a slower, regulated, carrier-mediated cell-to-cell directional transport which transfers auxin in the vascular cambium from the shoot towards the root apex (Goldsmith, 1977), and mediates short-range movement in different tissues. This type of regulated polar transport (PAT) hasn't been detected for any other signalling molecule in plants and therefore is considered to be a unique feature of auxin. These two pathways seem to be connected at the level of phloem in leaves and roots (Petrášek and Friml, 2009).

### *2.2.1. Polar auxin transport*

Polar auxin transport (PAT) proved to be essential for generation and maintenance of local auxin maxima and gradients. PAT is mediated by plasma membrane-based influx and efflux carriers with asymmetrical cellular localization which has functional significance. AUX1/LIKE AUX1 (AUX1/LAX) family of transmembrane proteins, similar to amino acid permeases, has been identified as auxin influx carriers. Four auxin influx carriers have been described in arabidopsis to date, as well as some homologs in other plants (Petrášek and Friml, 2009). The asymmetrical subcellular localization of AUX1 is dependent on AXR4, an endoplasmic reticulum protein which proved to be responsible for AUX1 distribution (Dharmasiri *et al*., 2006).

Some members of PIN-FORMED (PIN) protein family were identified as auxin efflux carriers. To date, eight members of the PIN family, PIN1 to PIN8, have been isolated from arabidopsis. These proteins have two transmembrane regions separated by a hydrophilic loop and are expressed at distinct times and locations (Gälweiler *et al*., 1998). First subgroup of PIN proteins involves PIN5, PIN6 and PIN8 proteins with a reduced middle hydrophilic loop, which regulate the auxin exchange between the endoplasmic reticulum and cytosol (Mravec *et al*., 2009). Proteins PIN1, PIN2, PIN3, PIN4 and PIN7 make the second subgroup of PIN proteins localized at the plasma membrane where they act as efflux carriers (Mravec *et al*., 2008). PIN homologs in other plants were also identified and functionally characterized (Zažímalová *et al*., 2007).

The localization of PIN proteins is very dynamic and changes rapidly during the important developmental events such as embryonic development and the response to gravity. This rapid relocalization is enabled by the recycling of PIN-containing endocytotic vesicles to and from plasma membrane, a process that is directly influenced by auxin (Teale *et al*., 2006). Other proteins that play a role of efflux carriers are certain MULTIDRUG RESISTANCE-like (MDR) proteins, plant orthologs of the mammalian ATP-binding cassette subfamily B (ABCB)-type transporters (Noh *et al*., 2001).

Canalization hypothesis postulates that auxin itself is one of the most important regulators of its own transport. According to this theory, auxin acts to polarize its own transport, meaning that the initial diffusion away from a source positively reinforces transport (Sachs, 1981). The carrier-mediated, polar auxin transport can be regulated at three levels. The first level is controlling the abundance of carriers by regulation of their transcription, translation and degradation. The abundance of some PIN proteins is controlled by degradation via the vacuolar targeting pathway requiring proteasome-mediated steps (Abas *et al*., 2006) and regulation of the MODULATOR OF PIN (MOP) proteins (Malenica *et al*., 2007). The second level of regulation is by controlling the subcellular trafficking and targeting of auxin carriers to a specific position on plasma membrane. Important factor here is also auxin, which inhibits PIN internalization by an unknown mechanism and thus increases the amount of PIN proteins at the cell surface (Paciorek *et al*., 2005). Third level of regulation is by altering transport activity through post-translational modification of carriers, activity of endogenous inhibitors, regulating the composition and pH gradient of plasma membrane, etc (Petrášek and Friml, 2009).

### 2.3. Auxin-mediated regulation of gene expression

Genes that act to regulate the auxin homeostasis are mostly hormone-responsive genes. Several classes of auxin-responsive genes have been identified to date. Most important gene groups regulated by endogenous auxin are AUXIN/INDOLE-3-ACETIC ACID INDUCED (Aux/IAA), SMALL AUXIN UP RNA (SAUR) and GRETCHEN HAGEN 3 (GH3) gene families. These genes are considered to be early or primary-response genes, considering their transcription is rapidly activated by auxin (Abel and Theologis, 1996).

The expression of PIN and AUX1 carrier proteins is also modulated by auxin, as well as some other plant hormones, such as ethylen and cytokinins. Furthermore, the transcription of all known carrier proteins is influenced by auxin triggering a signalling cascade that involves the

F-box protein TRANSPORT INHIBITOR RESPONSE 1 (TIR1) auxin receptor (Petrášek and Friml, 2009).

### *2.3.1. Aux/IAA genes regulation*

The first identified auxin-inducible genes were members of large Aux/IAA superfamily (Abel and Theologis, 1996). This early-response genes, together with AUXIN RESPONSE FACTOR (ARF) genes, mediate complex auxin responses. This family includes 29 genes in arabidopsis which encode transcriptional repressors of auxin response. Aux/IAA proteins are short-lived nuclear proteins with four conserved domains (I-IV), which form homo- and heterodimers not only with one another, but also with ARF factors (Teale *et al*., 2006).

### *2.3.2. ARF genes regulation*

ARF genes compose a large family in arabidopsis with 23 members (*AtARFs*). ARF proteins are transcription factors, typically composed of a DNA-binding domain (DBD) in the N-terminal region, and domains III and IV which make a protein-protein interaction domain, similar to those in the C-terminus of Aux/IAA proteins (Wang *et al*., 2007). The sequence of DNA-binding domain is highly conserved and ARFs probably have redundant and compensatory functions. The amino acid composition of the variable middle region between the DNA-binding domain and domains III/IV determines whether a protein will function as an activator or a repressor; glutamine-rich middle region activates transcription, while the proline-rich middle region is associated with repression of transcription (Tiwari *et al*., 2003).

### *2.3.3. Auxin-responsive elements*

Auxin-responsive elements (AuxREs; AREs) are *cis*-acting elements which regulate expression of auxin-responsive genes. They represent binding sites for *trans*-acting regulative factors, ARFs. AREs are located in promoters of auxin-responsive genes and contain a conserved TGTCTC motif (Chapman and Estelle, 2009).

### *2.3.4. Central model for auxin-mediated gene regulation*

The effects of auxin are dependent on its concentration. At basal auxin levels, early auxin-response proteins Aux/IAAs are relatively stable and form homo- and heterodimers with

ARFs, thus controlling the amount of free transcription factors and inhibiting ARE-mediated gene transcription. An increase in auxin levels causes the proteasome-mediated degradation of Aux/IAA, resulting in an increase in number of functional ARF proteins and transcriptional activation of auxin regulons (Teale *et al*., 2006). In order to be selected for proteasome-mediated degradation, Aux/IAA protein firstly needs to be recognized by an F-box protein, TIR1. TIR1 interacts directly with Aux/IAA proteins and this interaction forms a pocket in which auxin binds. Auxin stabilizes this interaction and is continuously required for its maintenance (Tan *et al*., 2007). TIR1 and other F-box proteins serve as the specificity determinants for the SCF class of E3 ubiquitin ligases, which target proteins for ubiquitylation and degradation by 26S proteasome (Chapman and Estelle, 2009).

#### *2.3.5. Alternative signalling pathways*

Although the described model for auxin regulation of gene expression places the $SCF^{TIR1}$-auxin-Aux/IAA complex in the center of action, this is probably not the only pathway through which auxin functions. Auxin controls numerous cellular processes, some of which occur too rapidly to be the subject of transcriptional control. Many of these mechanisms depend on proteins that have been proven to bind auxin directly, such as AUXIN-BINDING PROTEIN1 (ABP-1). ABP1 is a soluble, ER-located, dimeric glycoprotein involved in cell expansion, stomatal closure, plasma-membrane hyperpolarization and cell division (Teale *et al*., 2006) .

### 2.4. Auxin storage forms

Auxin storage forms exist to regulate auxin homeostasis: to influence its sensitivity, transport and compartmentalization. Higher plants can store auxin either in the form of IAA conjugates or as indole-3-butyric acid (IBA), a naturally occuring auxin precursor in variety of plant species. IBA-derived IAA, produced by β-oxidation in peroxisomes, proved to be important for seedling growth and development in arabidopsis. Similar to IAA, IBA can also form conjugates whose role hasn't yet been elucidated (Korasick *et al*., 2013).

### *2.4.1. Auxin conjugates*

Although distinct plant species exhibit diverse IAA conjugate profiles with various conjugate compositions, conjugate forms proved to be predominant over free, active forms of auxin in all examined plants. Three major groups of auxin conjugates are identified in plants: ester-linked sugar conjugates, amide-linked amino acid conjugates and amide-linked peptide and protein conjugates. Ester-linked IAA sugar conjugates are formed by the activity of UDP glucosyltransferases and have been identified in both monocots and dicots. However, most dicots including arabidopsis, primarily produce and store amide-linked amino acid conjugates (Korasick *et al*., 2013).

### *2.4.2. IAA-amino acid conjugates*

Amide-linked IAA conjugates constitute about 90% of the total auxin found in arabidopsis, approximately 10% are ester-linked conjugates, while only 1% of auxin is present in the free form. Although conjugates of many different amino acids have been identified in plants to date, the functions of those formed with Alanine, Leucine, Aspartate and Glutamate are best understood. Based on their activity and susceptibility to hydrolysis, these conjugates can be classified into two groups. IAA-Ala and IAA-Leu both inhibit root elongation and are readily hydrolysable in arabidopsis, which indicates they probably function to supply free IAA. In contrast, IAA-Asp and IAA-Glu conjugates are not substrates of amidohydrolases and therefore are more likely to act as intermediates in IAA catabolism (Woodward and Bartel, 2005). It was also demonstrated that IAA-Asp increases pathogen progression in plant by regulating the transcription of virulence genes, confirming the presumed role of auxin signalling in disease promotion (Gonzalez-Lamothe *et al*., 2012).

### *2.4.3. GRETCHEN HAGEN 3 gene family*

IAA-amino acid conjugation enzymes belong to the GH3 family of auxin-induced genes. The first GH3 gene described was isolated from soybean (*Glycine max*) as an early auxin-responsive gene (Hagen and Guilfoyle, 1985). Subsequently, these genes were identified in arabidopsis where they are present as a large multigene family of 19 members, of which at least seven were shown to catalyze the synthesis of IAA-amino acid conjugates (Staswick *et al*., 2005). The arabidopsis GH3 proteins are classified in three groups. Group I includes proteins AtGH3.11 which adenylates jasmonic acid, and AtGH3.10 protein with an unclear role

(Staswick and Tiryaki, 2004). Group II members act as indole-3-acetic acid-amido synthetases catalyzing the ATP-dependent conjugation of amino acids to IAA (Staswick *et al*., 2005). No adenylation activity was recorded with the members of group III (Terol *et al*., 2006).

Arabidopsis GH3 genes exhibit different responses to a variety of stimulus. Some GH3 genes are induced by exogenous auxin while others are regulated by light. GH3 proteins have a role in adventitious root initiation (Gutierrez *et al*., 2012) as well as stress responses. GH3.5 (WES1) protein is a unique member of group II in that it adenylates both IAA and salicylic acid (SA). GH3.5 plays a role in hypocotyl growth by mediating phytochrome B-perceived signals and probably has a role in environmental stress response (Park *et al*., 2007). In addition to model plant species, GH3 gene family was also identified in fruit plants, with 11 members discovered in citrus, 15 in apple and 9 in grapevine, as well as in bacteria. These GH3 genes presumably have roles in fruit setting, growth and ripening (Liu *et al*., 2016). In moss, *Physcomitrella patens*, auxin conjugates were also detected although their formation is slower than in arabidopsis, indicating they might play a different role in homeostasis. To date, only two GH3 homologs from moss were described, named PpGH3.1 and PpGH3.2. Both enzymes convert IAA, IBA and JA to amide conjugates, only with different preferences for amino acids (Ludwig-Müller *et al*., 2008).

### 2.5. Auxin inactivation pathways

Auxin conjugation is often a reversible process and many conjugates can be converted back to the active auxin by the activity of amidohydrolases. However, some storage forms are not susceptible to hydrolysis and are included in catabolic pathways responsible for protection against auxin toxicity in the presence of excess auxin. For example, IAA-Asp and IAA-Glu are not appreciably hydrolysed in arabidopsis and are probably included in auxin inactivation. This assumption is additionally confirmed by the fact that IAA-Asp can further be oxidized to oxIAA-Asp through oxindole-3-acetic acid (oxIaa) pathway responsible for permanent IAA inactivation (Östin *et al*., 1998).

## 3. RESULTS

### *3.1. Selection of primary transformants (T1)*

*Arabidopsis thaliana* plants, ecotypes Col-0 and WS, were previously transformed with pMDCAtGH3.3His, pMDCPbGH3His and pMDCPpGH3.2His vectors. T1 seeds were collected from transformed plants and seeded on 50 mg/l hygromicin B (Hyg) selective plates in 3 horizontal rows (Fig. 2). Following a 2 days stratification period and 8 days of vertical cultivation in long-day conditions, transformed seedlings were easily distinguished according to normal root and hypocotyl growth. Transformants were picked and planted to Steckmedium for further cultivation and T2 seed production.

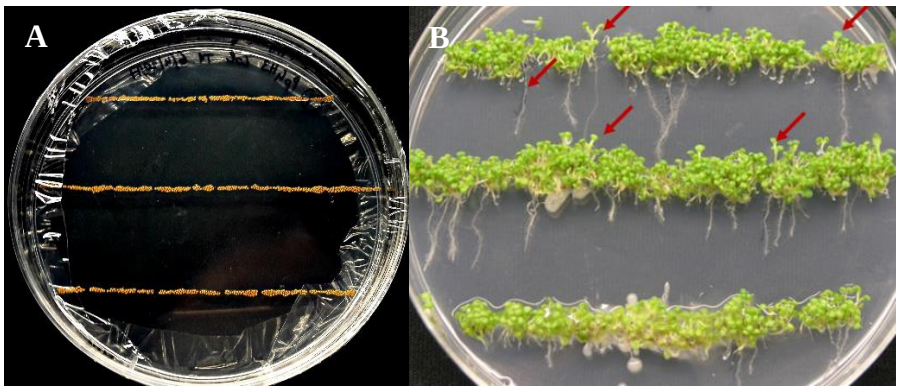



**Figure 2.** *Selection of transgenics. T1 seeds were seeded in uniformal horizontal rows (A) for easier detection of transformants. (B) Transformants (indicated with arrows) are easily distinguished from non-transformant seedlings by normal root development and hypocotyl growth.*

### *3.2. Detection of AtGH3.3, PbGH3 or PpGH3.2 transgenes in primary transformants*

Genomic DNA isolated from primary transformants (24 plants, 4 per line) was used as a template in PCR reaction. PCR reaction was conducted using RevHis6JLM reverse primer and either FwAtGH3.3, FwPpGH3.2 or FwPbGH3 forward primer for amplification of each transgene. Expected length of the amplified genes was 1803 bp for *AtGH3.3*, 1815 bp for *PpGH3.2* and 1713 bp for *PbGH3* transgene. Results demonstrate that AtGH3.3 and PbGH3 primary transformants indeed carry the desired transgene (Fig. 3). However, no bands were detected in any of the 8 lines tested for the presence of *PpGH3.2* transgene (Fig. 3), suggesting that either the transgene didn't successfully amplify with chosen primers or the selected plants aren't actually transgenic.

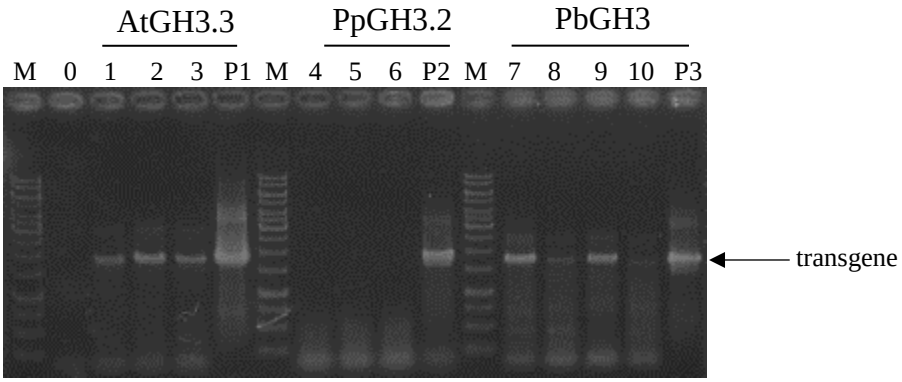



**Figure 3.** *PCR amplification of AtGH3.3, PpGH3.2 and PbGH3 transgenes. Gene specific forward and RevHis6JLM reverse primers were used. PCR fragments were analyzed in 1% agarose gel. Templates for 0-3 were gDNAs isolated from AtGH3.3, 4-6 are from PpGH3.2, and 7-10 are from PbGH3 primary transformants. M – marker (GeneRuler 1 kb DNA Ladder), 0 - negative control (water), P – positive controls (binary vectors used for plant transformation). 1,5,6,7,8 – WS; 2,3,4,9,10 – Col-0.*

Another control PCR reaction was carried out using 35S-3PRIM forward and RevHis6JLM reverse primer. *AtGH3.3* and *PbGH3* transgenes were detected but neither this time was the expected *PpGH3.2* transgene amplified (Fig. 4). Analyzed transformants with no *PpGH3.2* transgene were excluded from further analysis and in order to obtain PpGH3.2 transgenic plants, transformation procedure was conducted from the beginning.

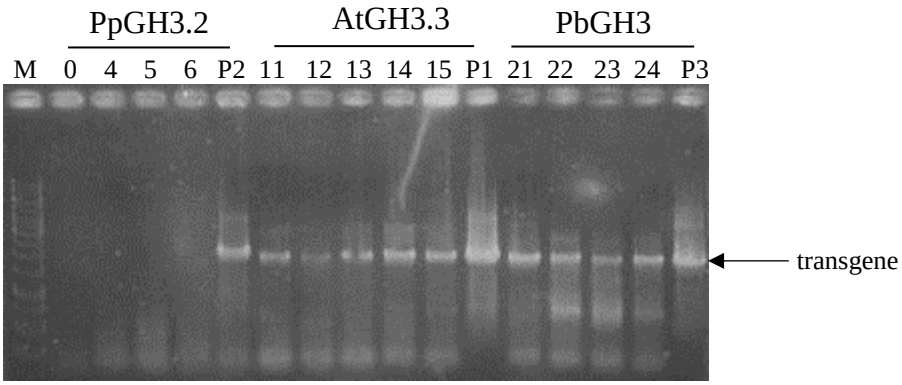



**Figure 4.** *PCR amplification of transgenes by 35S-3PRIM forward and RevHis6JLM reverse primers. Transgenes were analyzed in 1% agarose gel, following PCR amplification. Templates for 4-6 were gDNAs isolated from PpGH3.2, 11-15 are from AtGH3.3, and 21-24 are from PbGH3 primary transformants. M – marker, 0 – negative control, P – positive controls (binary vectors used for plant transformation). 4,14,15,23,24 – Col-0; 5,6,11,12,13,21,22 – WS.*

### *3.3. Transformation of Agrobacterium tumefaciens*

Bacteria *Agrobacterium tumefaciens*, strain GV3101 (pMP90), were transformed with vector pMDC32-PpGH3-2K6 by electroporation. From colonies of transformed agrobacteria, 3 ml overnight cultures were prepared. Samples of overnight cultures were collected, transgene amplified and PCR products were analyzed by electrophoresis on 1% agarose gel. Bands of approximately 2000 bp were detected in each of the analyzed samples, which coincides with the expected fragment size (1815 bp). This results suggest that all 3 samples anaylzed were suitable for further procedures. From selected overnight culture, 200 ml culture was prepared and used for floral dip transformation. Aliquots of large culture used for plant transformation were additionaly analyzed by PCR (Fig. 5). Amplified transgene detected on 1% gel confirmed its suitability for floral dip transformation of arabidopsis plants.

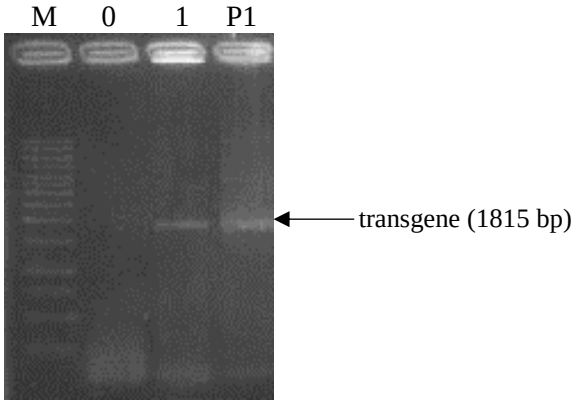



**Figure 5.** *Detection of PpGH3.2 transgene in samples from large agrobacterial culture, on 1% agarose gel following PCR amplification. Samples were amplified with FwPpGH3.2 and RevHis6JLM primers. M – marker, 0 – negative control, 1 – large culture sample, P – positive control (binary vector).*

### *3.4. Verification of successful floral dip transformation*

Proposed PpGH3.2 primary transformants were analyzed to verify the presence of transgene. Genomic DNA was isolated and amplified with PCR reaction using FwPpGH3.2 forward and RevHis6JLM reverse primers. Gel electrophoresis showed that only 3 out of 8 analyzed plants are actual transformants (Fig. 6).

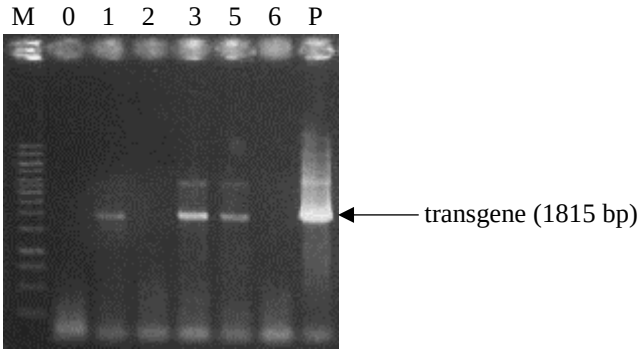



**Figure 6.** *Detection of PpGH3.2 transgene in primary transformants, on 1% agarose gel following PCR amplification. Samples were amplified with FwPpGH3.2 and RevHis6JLM primers. M – marker, 0 – negative control, 1-3 – PpGH3.2 Col-0, 5,6 – PpGH3.2 WS, P – positive control.*

### *3.5. Selection of transformants with one T-DNA insertion*

One-week-old T2 seedlings, seeded uniformly on Hyg selective plates, were counted and examined in order to distinguish lines bearing one T-DNA from those with multiple T-DNA insertions. For each separate plate, overall number of seedlings was counted and percentage of wild type plants was calculated. Plates with ~25% of wild type seedlings, recognized by abnormal root and hypocotyl growth (Fig. 7), were determined as lines bearing one T-DNA insertion. Several plates containing only wild type plants were also observed. Total number of analyzed lines was 36, of which 19 lines had one T-DNA insertion (Table 1) and were selected for further experiments. T2 seedlings (8 per line) with one T-DNA insertion were transferred to Steckmedium and cultivated in long-day conditions. After self polination, T3 seeds were collected for determination of homozigots and for further analysis.

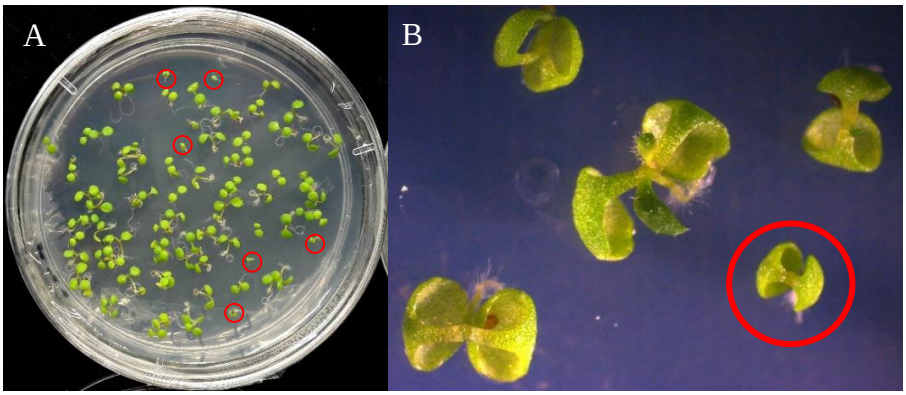



**Figure 7**. *Selection of T2 transformants. Wild type plants (indicated with red circles) are easily distinguished from transformants on Hyg selective plates (A). Wild type plants are significantly smaller than transformants and have shorter hypocotils and roots (B).*

**Table 1.** *Total number of analyzed transgenic T2 lines, lines with multiple T-DNA insertions and single copy T-DNA lines.*

| Line name | Number of lines analyzed | Lines with multiple T-DNAs | Lines with one T-DNA |
|---|---|---|---|
| AtGH3.3 WS | 11 | 4 | 7 |
| AtGH3.3 Col | 9 | 6 | 3 |
| PbGH3 WS | 11 | 4 | 7 |
| PbGH3 Col | 5 | 3 | 2 |
| **Total number** | 36 | 17 | **19** |

### *3.6. Selection of homozygous plants*

From each T2 plant with one T-DNA insertion, seeds were collected and after sterilization seeded on Hyg selective plate. Ten-days-old seedlings (T3) were screened. Lines without wild type seedlings were determined as homozygous (Fig. 8). All homozygous lines are listed in Table 2. Several plates without transformants were also detected in T3 generation.

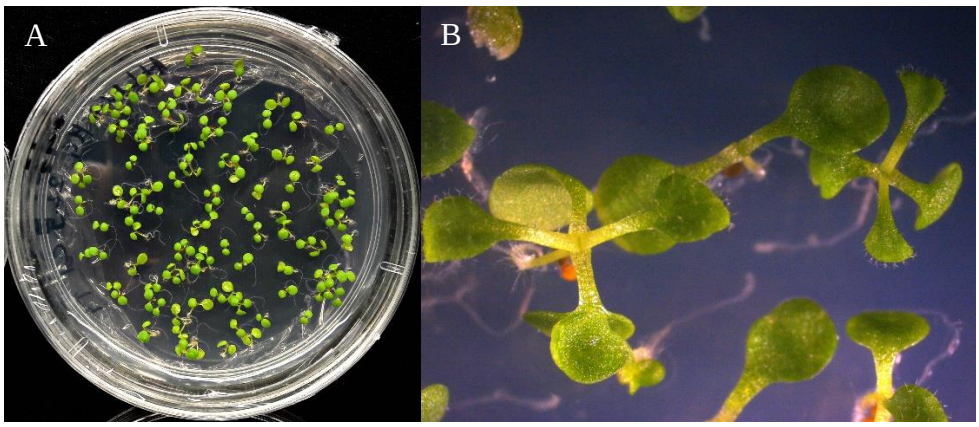



**Figure 8**. *Selection of homozygous plants. Selective plate with homozygous seedlings (A). All seedlings exhibit normal root and hypocotyl growth and are of equal size (B).*

***Table 2**. Homozygous transgenic lines with overexpression of AtGH3.3 and PbGH3 in Col-0 and WS ecotype of Arabidopsis thaliana.*

| Label | Line | Label | Line |
|---|---|---|---|
| 34D | PbGH3 WS | 52E | PbGH3 Col-0 |
| 34E | | 52B | |
| 38B | | 52G | |
| 47A | | 64E | |
| 47E | | 37E | AtGH3 WS |
| 47F | | 60B | AtGH3 Col-0 |
| 47H | | 60F | |

### *3.7. Measuring overexpression of transgene by qPCR*

To measure extent of transgene overexpression, primers for quantitative PCR were designed. Five PCR primer sets were designed: EXP primer set which amplifies reference gene, nAtGH3.3 set for amplifying native *AtGH3.3* gene, n+tAtGH3.3 for amplifying both native and transgene, tAtGH3.3 for amplification of *AtGH3.3* transgene and tPbGH3 set for *PbGH3* transgene. In order to amplify only transgenes, reverse primers in these sets were designed to bind to His-tags. Due to the low melting temperatures of designed primers, annealing temperature was lowered to 55 °C.

To test primer pairs, RNA was isolated from 2-weeks-old transgenic T2 seedlings, as well as wild type WS seedlings. Reverse transcription was conducted with 0.66 µg of RNA. To confirm the efficacy of reverse transcription, obtained cDNA samples were used for PCR amplification of *Actin3* gene. PCR reaction was conducted with 1 min elongation and 40 cycles. Ac3-fw and Ac3-rev primer set amplified 787 bp fragment from genomic DNA and 655 bp fragment from cDNA (Fig. 9) indicating that despite the treatment with DNase, there were still some traces of remaining genomic DNA in the samples which was co-isolated with RNA. Based on melting curves for each primer set (Fig. 10), I concluded that the designed primers were suitable for further analysis.

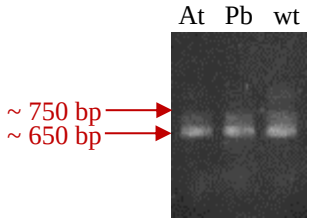



**Figure 9.** *Amplification of Actin3 gene by PCR following reverse transcription and detection on 1% agarose gel. Two bands are visible, sized 655 bp corresponding to cDNA and 787 bp corresponding to the remaining DNA. At – AtGH3.3, Pb – PbGH3, wt – wild type.*

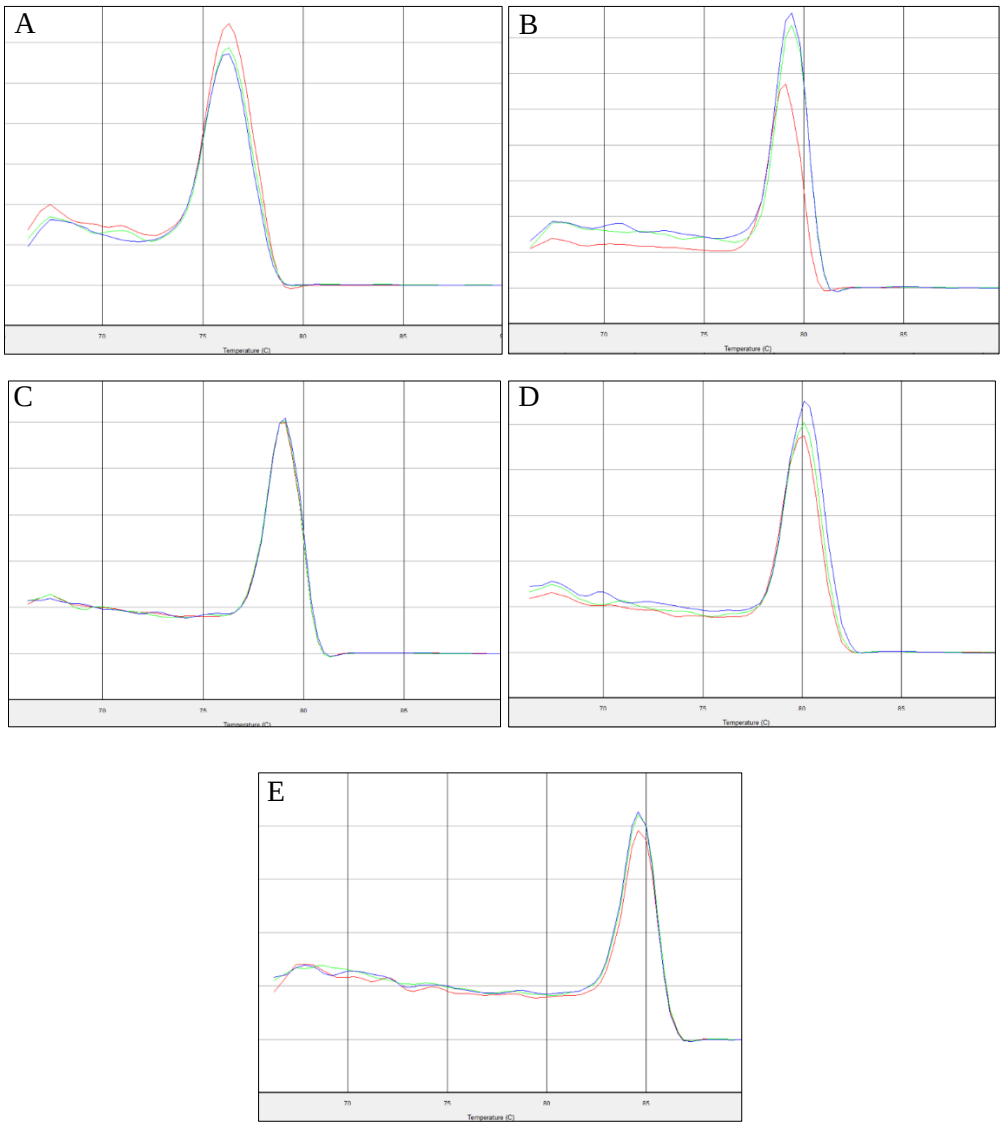



**Figure 10.** *Melting curves for each qPCR primer set. Single peak observed in each curve suggests that primers do not form primer-dimers and are suitable for qPCR reaction. (A) EXP, (B) nAtGH3.3, (C) n+tAtGH3.3, (D) tAtGH3.3, (E) tPbGH3.*

Six homozygous lines (60B, 37E, 52E, 64E, 34D, 38B) were chosen for quantification of transgene overexpression. For this purpose, sterilized seeds were plated on non-selective MS plates and cultivated in long-day conditions for 10 days. For each line, RNA isolated from 8 seedlings was converted to cDNA. To test RNA purity, PCR with primers for *Actine3* gene was conducted and analyzed with agarose gel electrophoresis (Fig. 11). Regardless of DNase treatment, results indicate the presence of genomic DNA in cDNA samples in majority of transgenic lines. According to these results, it was concluded that only 64E line sample was suitable for determination of overexpression, while for other lines procedure should be repeated.

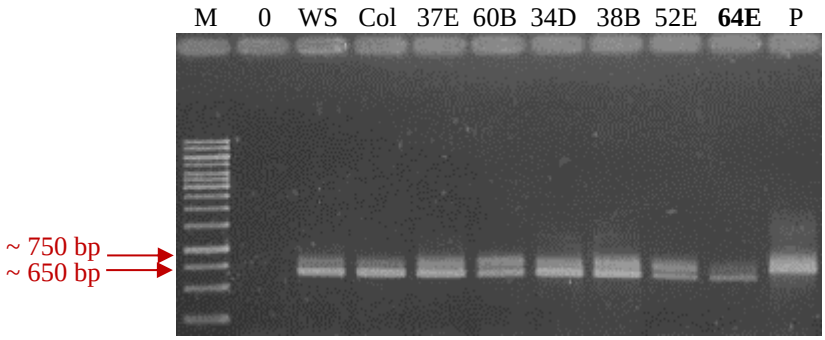



**Figure 11.** *Detection of Actine3 gene on 1% agarose gel following PCR amplification. Ac3-fw and Ac3-rev primer set was used for amplification. Two bands are visible, one corresponding to cDNA (655 bp) and one corresponding to the remaining genomic DNA (787 bp). M – marker, WS – WS wild type, Col – Col-0 wild type, 37E – AtGH3.3 WS, 60B – AtGH3.3 Col-0, 34D – PbGH3 WS, 38B – PbGH3 WS, 52E – PbGH3 Col-0, 64E – PbGH3 Col-0, P – positive control (gDNA sample).*

Mean ΔΔCt values calculated indicate that the highest overexpression level was present in 64E PbGH3 Col-0 sample (58 times higher than the native *AtGH3.3* gene). Although this is the single relevant result, overexpression for other samples was also calculated. AtGH3.3 samples exhibited 52 (60B) and 13 times (37E) higher overexpression, while PbGH3 WS samples 34D and 38B had 10 and 3 times higher levels. The other PbGH3 Col-0 (52E) sample showed no overexpression but instead had higher expression of the native *AtGH3.3* gene, probably due to the gDNA contamination. The rest of the samples showed lowered native gene expression.

### *3.8. Immunodetection of AtGH3.3-His$_6$ and PbGH3-His$_6$ transgenic proteins*

Soluble proteins were extracted from leaf tissue of 8-weeks-old primary transformant plants. Proteins with His-tag were purified on Ni-NTA agarose beads and denaturated. After seperating by SDS-PAGE, proteins were transferred to PVDF membrane and detected with anti-His antibodies.

After first extraction, presence of PbGH3-His$_6$ transgenic protein was detected in 3 out of 4 samples analyzed. Observed bands correspond to the expected size of the protein, which is around 62 kDa (Fig. 12). Since no AtGH3.3-His$_6$ transgenic protein was detected, extraction was repeated from more plant material, and His-tagged transgenic proteins were enriched on Ni-NTA agarose. After washing, two elution steps were performed and transgenic proteins were observed only in first eluats (Fig. 13). Presence of PbGH3-His$_6$ transgenic protein was proved for 4 additional transgenic lines, in both WS and Col ecotype (Fig. 13A). Proteins purified from AtGH3.3 transgenic lines, WS and Col-0 ecotype, seemed to be truncated forms of the expected AtGH3-His$_6$. Although signals were recorded in all tested samples (Fig. 13B), size of the visible bands did not match the expected AtGH3-His$_6$ protein size except for one sample (14) where faintly visible band of the expected size (68 kDa) was detected. The predominant protein appearing in all plants with AtGH3-His$_6$ overexpression had around 30-35 kDa. In addition, in samples 14 and 25 proteins of around 20, 30, 45 and 50 kDa were detected, suggesting that maybe AtGH3.3-His$_6$ protein was degraded during extraction and purification procedure.

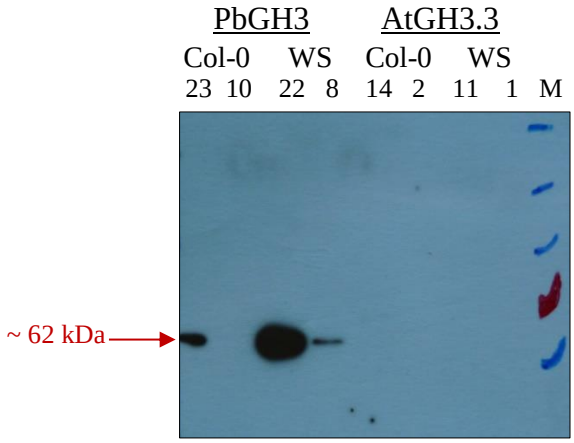



**Figure 12.** *Soluble proteins from A. thaliana plants transformed with AtGH3.3 and PbGH3 genes. Proteins were separated by SDS-PAGE and transferred to PVDF membrane. After blocking, the membrane was probed with anti-his for detection of transgenic proteins with His-tag. M – marker (PageRuler Plus Prestained Protein Ladder). 1, 11 – AtGH3.3 WS T1; 2, 14 – AtGH3.3 Col-0 T1; 8, 22 – PbGH3 WS T1; 10, 23 – PbGH3 Col-0 T1.*

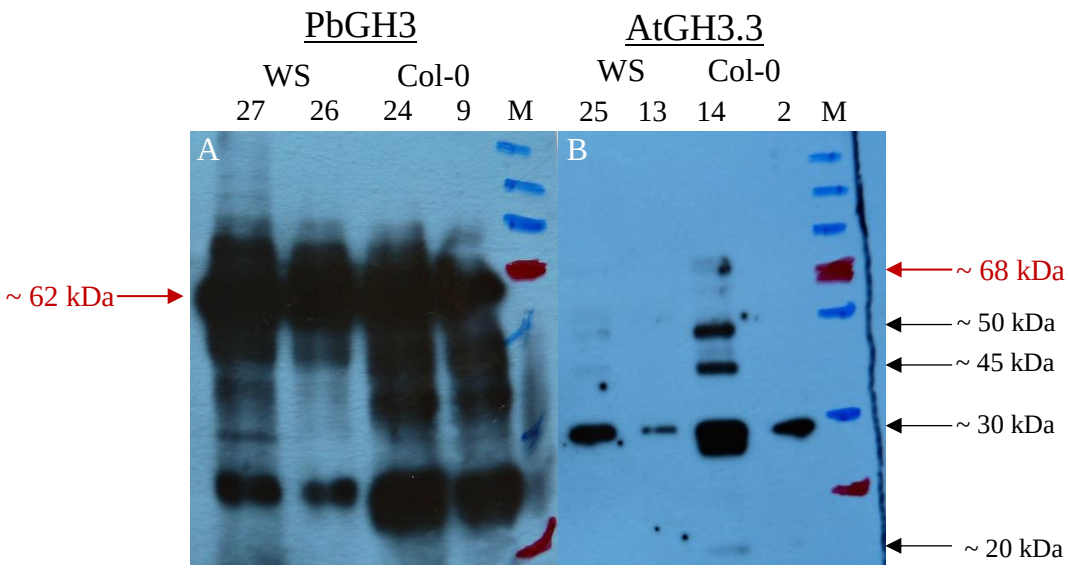



**Figure 13.** *Detection of PbGH3-His$_6$ (A) and AtGH3.3-His$_6$ (B) transgenic proteins after elution. After purification on Ni-NTA agarose and elution with 400 mM imidazole, samples were concentrated with Centrifugal filter units (Millipore). Approximate size of the detected proteins is indicated with arrows. M – marker; 25, 13 – AtGH3.3 WS; 2, 14 – AtGH3.3 Col-0; 27, 26 – PbGH3 WS; 24, 9 – PbGH3 Col-0.*

Some of the lines analyzed by qPCR were also detected by immunodetection. For example, line 64E with highest calculated overexpression level, was selected from line 23 of secondary transformants. HRP signal of this sample is clearly visible in Figure 12, confirming the overexpression detected by qPCR. The same is observed for samples 34D (sample 22) (Fig. 12) and 37E (sample 25) (Fig. 13B).

# 4. DISCUSSION

## *4.1. Floral dip transformation efficacy and transgene silencing*

Until 1990, arabidopsis was mainly transformed using *in vitro* methods, root or leaf explant transformation. Since these tissue-culture dependent methods were not efficient for transformation of all arabidopsis ecotypes, they were gradually exchanged for more suitable *in planta* methods, vacuum infiltration and floral dip. Labor-intensive vacuum infiltration process was soon replaced with floral dip as a main transformation method for arabidopsis plants. Floral dip is a simple, quick and cheap transformation method whose protocole constitutes of dipping of developing floral tissues into agrobacterial solution containing 5% succrose and 0.03% Silwet L-77 to reduce surface tension. Plants are subsequently wrapped with plastic cover to maintain high humidity which increases transformation efficacy (Clough and Bent, 1998). Although efficacy of arabidopsis floral dip transformation is relatively high, in order to generate desired transgenic plants numerous factors have to be taken into account. First of all, floral dip efficiency depends on physiology and the developmental stage of the plant, meaning that the number of developing flowers, floral level and morphology highly influence the outcome (Ghedira *et al*., 2013). Ecotypes WS and Col-0, transformed in this research showed obvious phenotypical differences, with Col-0 plants exhibiting better leaf rosette development and slower flower and seed setting compared to the WS ecotype. This differences apparently didn't affect the process since both AtGH3.3 and PbGH3 primary transformants were successfully obtained. However, several plates containing only wild type plants were detected when examining transformant seedlings, regardless of the ecotype. This can be explained by clarifying one of the main known disadvantages of floral dip method. Namely, despite its simplicity, floral dip has low frequency of single-copy transformants, with an average of 4-6 T-DNA copies per transformant (De Buck, 2009). Multiple transgene integration often results in low and unstable transgene expression, and can even lead to transgene silencing, as observed in the conducted research.

Introduction of transgene into plants has been recorded to cause multiple unwanted modifications, one of the best investigated being transgene silencing. There are various explanations to why this phenomenon occurs, but the most widely accepted theory is homology-dependent gene silencing (HDGS). HDGS phenomenon suggests that homology, between transgene and native gene or transgene and transgene, serves as a signal that triggers gene silencing. This explains why multiple T-DNA copies enhance transgene silencing frequency

(Meyer and Saedler, 1996). Homology-mediated silencing is thought to act through two molecular mechanisms: transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS). Although biological function of homology-scanning systems is still not fully elucidated, multiple roles have been proposed, one of the main being plant host defense (Matzke *et al*., 2002). Silencing mechanisms are various and single-copy transgenes can also undergo silencing when highly expressed. However, their expression is mostly stable and this is why single-copy transformants are prefered for agricultural and commercial purposes (De Paepe *et al*., 2009).

Therefore, considering the previous discussion, floral dip transformation of plants with vectors carrying homologs of the arabidopsis native *GH3* gene is expected to yield several lines with silenced transgenes. Transgene silencing mostly occurs after several generations. Therefore, it can be concluded that while T3 lines exhibiting wild type phenotype possibly result from transgene silencing, this is probably not the cause for hygromicin non –resistant lines observed in T2 generation. These lines can rather be explained by a simple mistake of selecting wild type plant instead of transformant. Since not all primary transformants were tested for the presence of transgene, these plants were further cultivated and handled as transformants. Furthermore, *PbGH3* is a gene originating from *Plasmodiophora brassicae*, an obligate biotrophic protist causing clubroot disease in arabidopsis and other brassicas. PbGH3 protein conjugates auxin to amino acids and has a role in altering the host hormone metabolism during infection (Jahn *et al*., 2013). Silencing of this transgene can also serve as a verification of the role of homology-mediated gene silencing in plant defense responses.

However, all of the previous statements do not explain the low number of transgenics with *PpGH3.2* transgene. One possible explanation to why this problems might occur is the T-DNA transfer disorder. Transfer can be altered because some parts of the *PpGH3.2* gene sequence are homologous to the T-DNA right border repetition. This causes VirD proteins, responsible for transfer and integration of T-DNA, to recognize homologous sequence in *PpGH3.2* gene instead of right border sequence and transfer only part of the transgene. This explanations is further supported by the fact that all transformants were hygromicin resistant despite the absence of transgene.

### *4.2. Overexpression levels for heterologous and homologous expression*

Overexpression was calculated using data obtained from qPCR analysis of 6 homozygous lines. However, the given results have to be taken with reserve since analyzed samples exhibited high gDNA contamination.

Both transgenic lines with *AtGH3.3* transgene showed higher expression of transgene compared to the native *AtGH3.3*, as expected. However, measured expression levels between these two lines significantly differ, with Col-0 line showing 52 times higher overexpression compared to the native gene, while in WS ecotype overexpression is 13 times higher.

From 4 analyzed PbGH3 homozygous lines, 3 showed overexpression of transgene. Highest overexpression was calculated for one Col-0 line (64E), which had 58 times higher overexpression, while WS lines showed 3 and 10 times higher expression. One Col-0 line (52E) showed no overexpression but instead exhibited higher expression of the native gene. This is probably due to the gDNA contamination of analyzed samples and in order to obtain more reliable results, experiment should be repeated. In all other lines, expression of native gene was lowered compared to the wild type samples. This could be clarified by previously explained homology-dependent gene silencing phenomenon, since introduction of transgene can not only cause transgene silencing, but can also alter the regulation of expression and transcription of native genes, an effect known as cosupression (Furner *et al*., 1998).

Although it would be expected that expression of heterologous genes is higher than homologous, calculated levels do not completely support that hypothesis. While highest overexpression level was calulated for one PbGH3 homozygous line (64E), other PbGH3 lines showed no or low levels of overexpression (3 and 10 times higher), which is lower than those calculated for AtGH3.3 lines (52 and 13 times higher). Several factors could have led to this outcome. First of all, in pMDC32 vector both transgene and hygromicin resistance gene are under constitutive CaMV 35S promoters. These promoters have the same orientation, which might cause transcription deregulation and lead to inaccurate results. This could be avoided by using another vector with hygromicine gene under different promoter, for example nopaline synthase promoter. Furthermore, all of the analyzed samples, except for 64E, contained gDNA contamination. This could have affected transgene expression. Therefore, in order to calculate the overexpression levels more accurately, whole procedure should be repeated, preferably using some other RNA isolation method and DNase in solution digestion to eliminate the gDNA contamination more efficiently.

### *4.3. Immunodetection and proteolytic degradation of AtGH3.3-His$_6$ and PbGH3-His$_6$ transgenic proteins*

Immunodetection of transgenic proteins indicated that heterologous overexpression was higher than homologous, as expected. However, it was concluded that both PbGH3-His$_6$ and AtGH3.3-His$_6$ proteins were degraded during extraction and purification since the strongest visible bands appeared to belong to truncated protein forms. This is especially obvious in AtGH3.3 samples in which complete protein form was barely detected. This is probably due to the proteolytic degradation of proteins rather than premature termination of translation since transgenic proteins have the detected His-tag on C-terminus. Stronger degradation of AtGH3.3-His$_6$ protein compared to the PbGH3-His$_6$ can be explained by the fact that proteases better recognize and control expression of homologous proteins. Degradation occurred despite the use of PMSF protease inhibitor, confirming its already proven instability and pH and temperature change-dependent inactivation (James, 1978). In order to obtain higher recombinant protein yield during purification, additional protease inhibitors with more specific activity should be used in the future.

## 5. CONCLUSION

This study provides data on homologous and heterologous overexpression of indole-3-acetic acid-amido synthetases in *Arabidopsis thaliana*. Floral dip method, used in research, proved to be suitable for transformation of both, WS and Col-0 ecotype. Although all ecotypes were equally efficiently transformed, generation of *PpGH3.2* transgenic plants proved to be challenging. However, issues observed while transforming plants with *PpGH3.2* transgene are probably not caused by inefficiency of floral dip method. It is more likely that these difficulties appeared due to the homology of *PpGH3.2* transgene and T-DNA border sequence, or because of the use of pMDC32 vector with transgene and selection gene under same constitutive promoters. In order to obtain higher number of *PpGH3.2* transformants, transformation should be repeated with transgene cloned in a different vector. Furthermore, despite the satisfying number of obtained primary transformants, floral dip transformation also yielded a significant number of lines with silenced transgene, probably due to the multiple T-DNA insertions. This proves previously recorded phenomenon of homology-dependent gene silencing.

Overexpression levels suggest strong effect of ecotype on both heterologous and homologous expression, since calculated levels were significantly higher in Col-0 plants. On the other hand, obtained results did not confirm the hypothesis which postulates that heterologous expression levels are higher than homologous. Furthermore, one PbGH3 line showed no detectable transgene overexpression while expression of native *AtGH3.3* gene was elevated. The remaining samples showed lowered native gene expression, confirming the effect of cosupression. However, these conclusions have to be taken with caution considering all samples analyzed contained notable gDNA contamination.

Immunodetection of AtGH3.3-$His_6$ and PbGH3-$His_6$ proteins confirmed that complete recombinant proteins are successfully synthesized in transgenics. However, since detected proteins didn't match the expected size, it was concluded that transgenic proteins are degraded by action of proteases. In order to obtain complete transgenic proteins, additional protease inhibitor treatment should be included in future extraction procedures.

# 6. ANNEXE

## 6.1. Materials

### *6.1.1. Plant materials*

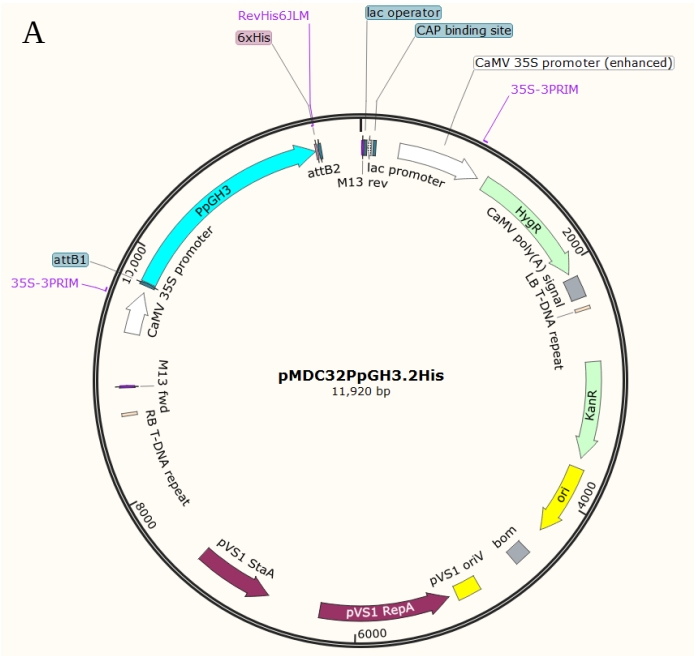
For the purpose of generating transgenic plants overexpressing homologous (*AtGH3.3*) or heterologous (*PpGH3.2 and PbGH3*) genes, wild type *Arabidopsis thaliana*, ecotypes Columbia (Col-0) and Wassilewskya (WS) were used. For all analyses, transgenic and wild type arabidopsis seedlings and plants were used.

### *6.1.2. Agrobacterial strain*

*Agrobacterium tumefaciens* strain GV3101 (pMP90) was used for plant transformation. This rifampicin resistant strain carries a pMP90 helper plasmid (with *vir* region) bearing a gene for gentamicin resistance.

### *6.1.3. Plasmid maps*

Genes *AtGH3.3* (At2g23170), *PbGH3* and *PpGH3.2* were cloned in pMDC32 vector (Fig. 14) and used for transformation of *Agrobacteria*. Transgenes have a C-terminal 6xHis-tag.



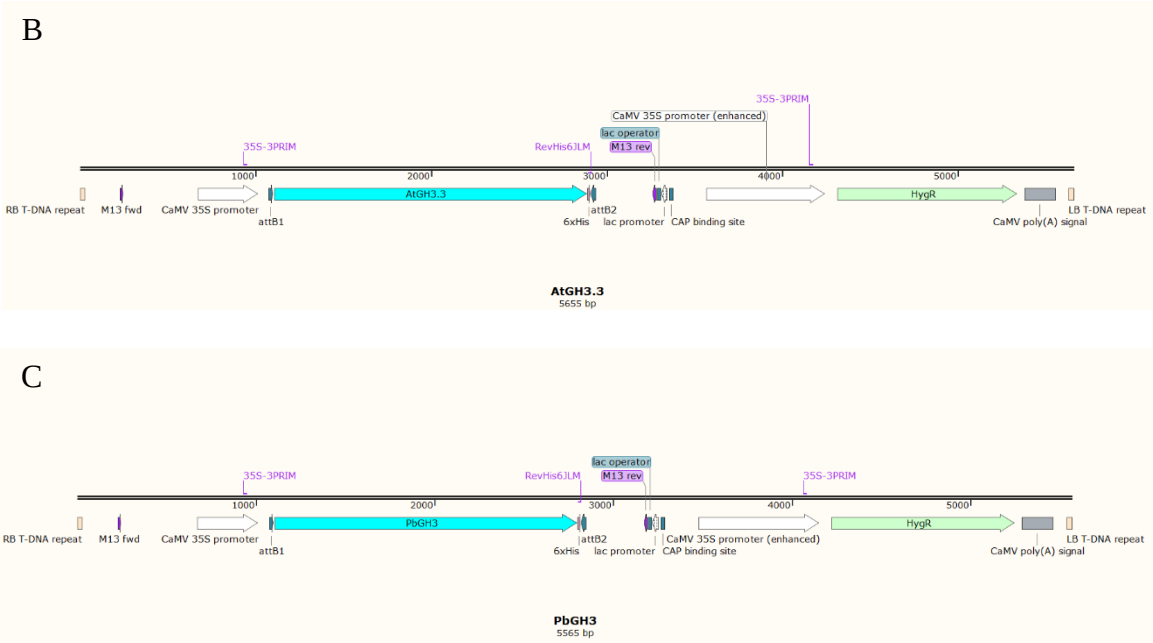



**Figure 14.** *Plasmid and T-DNA maps for overexpression of transgenes designed in SnapGene Viewer. Genes were cloned into pMDC32 plasmid bearing kanamycin resistance gene. (A) Plasmid map of pMDC32PpGH3.2His vector. (B) AtGH3.3His T-DNA map. (C) PbGH3His T-DNA map. RB – T-DNA right border repeat, CaMV 35S promoter – constitutive promoter from cauliflower mosaic virus, attB1, attB2 – recombination sites, PpGH3.2, AtGH3.3, PbGH3 – transgenes, 6xHis – His-tag, HygR – gene for hygromicin resistance, KanR – gene for kanamycin resistance, ori – origin of replication, 35S-3PRIM – forward primer, RevHis6JLM – reverse primer, LB – left border repeat.*

### *6.1.4. Growth media*

**LB medium** (pH 7):

tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l (agar 15 g/l for solid medium)

**Selective LB medium**

For selection of transformed *Agrobacteria*, LB medium with addition of antibiotics was used: 50 mg/ml gentamicin, 50 mg/ml kanamycin, 50 mg/ml rifampicin.

**MS medium** (pH 5.7 – 5.8)

macronutrients: $KNO_3$ (1900 mg/l), $NH_4NO_3$ (1650 mg/l), $CaCl_2$ x $H_2O$ (755 mg/l), $KH_2PO_4$ (170 mg/l), $MgSO_4$ x $7H_2O$ (370 mg/l);

micronutrients: $H_3BO_3$ (6.2 mg/l), $CoCl_2$ x $6H_2O$ (0.025 mg/l), KI (0.83 mg/l), $Na_2MoO_4$ x $2H_2O$ (0.25 mg/l), $CuSO_4$ x 5 $H_2O$ (0.025

mg/l), $MnSO_4$ x $4H_2O$ (16.9 mg/l), $ZnSO_4$ x $7H_2O$ (8.6 mg/l), $FeSO_4$ x $7H_2O$ (27.8 mg/l), $Na_2EDTA$ (37.3 mg/l);

vitamins and organics: succrose (20000 mg/l), myo-inositol (100 mg/l), nicotinic acid (0.5 mg/l), pyridoxine x HCl (0.5 mg/l), thiamine x HCl (0.1 mg/l), glycine (2 mg/l), agar (8000 mg/l).

**Selective MS medium**

For selection of transgenic plants, selective MS medium with addition of hygromicin B (50 mg/l) was used.

**SOC medium:**

tryptone 20 g/l, yeast extract 5g/l, NaCl 0.5 g/l, $MgCl_2$ 0.95 g/l, KCl 0.186 g/l, glucose 2 g/l

### *6.1.5. Gel electrophoresis marker*

GeneRuler 1 kb DNA ladder (Thermo Scientific) (https://www.thermofisher.com/order/catalog/product/SM0312) was used as a reference for analysing DNA fragments in agarose gel electrophoresis.

### *6.1.6. Protein electrophoresis marker*

PageRuler Plus Prestained Protein Ladder (Thermo Scientific) (https://www.thermofisher.com/order/catalog/product/26619 ) was used as a standard for protein electrophoresis.

### *6.1.7. PCR and qPCR primers*

Primers used for PCR reactions are listed in the Table 3. Primer stocks (100 μM) were diluted 10 x. Final concentration of primer solutions used in PCR reaction was 10 μM.

**Table 3.** *Sequences and melting temperatures for primers used in PCR reactions*.

| **Primer name** | **Sequence (5'→3')** | **Tm** [°C] |
|---|---|---|
| FwAtGH3.3 | ATGACCGTTGATTCAGCTCTGC | 62.1 |
| FwPpGH3.2 | ATGGCGCTTAGAATAGAGAACCCA | 63.5 |
| FwPbGH3 | ATGGCGACGGGGGAGG | 58.4 |
| 35S-3PRIM | CACTGACGTAAGGGATGACGCAC | 58.8 |
| Ac3-fw | GATTTGGCATCACACTTTCTACAATG | 54.8 |
| RevHis6JLM | GTGGTGGTGGTGGTGGTG | 60.7 |
| Ac3-rev | GTTCCACCACTGAGCACAATG | 54.4 |

Sequences of primers designed for qPCR reaction are listed in Table 4. AtGH3.3 set of primers will amplify native and transgenic *AtGH3.3* gene, nAtGH3.3 primer set amplifies only endogenous *AtGH3.3* gene, and tAtGH3.3 set only transgene. PbGH3 set was designed for amplification of *PbGH3* transgene and EXP amplifies reference gene (At4g26410).

**Table 4.** *Sequences, melting temperatures(Tm), and expected product sizes for qPCR primers.*

| **Primer name** | **Sequence [5'→3']** | **Tm** [°C] | **Product size** [bp] | **Predicted product Tm** [°C] |
|---|---|---|---|---|
| AtGH3.3qfw | CAATGGTGACCGGTCAATG | 57.3 | 79 | 79.1 |
| AtGH3.3qrev | CCATGTCTTCATCAATGGTT | 54.3 | | |
| nAtGH3.3qfw | TACCATTTTAATCATGACCG | 52.3 | 113 | 79.4 |
| nAtGH3.3qrev | CGACGTTACGTGTCATCTC | 57.3 | | |
| tAtGH3.3qfw | CACGCCAATAATGGAGCTTC | 58.4 | 96 | 80.1 |
| tAtGH3.3qrev | TGGTGGTGACGACGACGTT | 59.5 | | |
| PbGH3qfw | AGTTCTTCCAGGACGCGCT | 59.5 | 112 | 84.6 |
| PbGH3qrev | TCGCCATAGCTGCTGAAAAT | 56.4 | | |
| EXPqfw | GAGCTGAAGTGGCTTCCATGAC | 62.1 | 81 | 76.3 |
| EXPqrev | GGTCCGACATACCCATGATCC | 63.3 | | |

## 6.2. Methods

### *6.2.1. Agrobacterium transformation*

*Agrobacterium* cells were transformed with electroporation. To 50 µl of electrocompetent agrobacteria, 50 ng of plasmid, pMDC32-PpGH3-2K6 (Fig. 14A), was added and incubated on ice for 15 min. The mixture was then transferred into a cuvette that has been kept on ice. Cuvette was placed into electroporator and electroporation was conducted at 2200 V and 3.7 ms pulse. Mixture was transferred back into 1.5 ml tube, 1 ml of SOC medium was added and mixed by pipetting. Cells were incubated on shaker for 2 h at 28 °C before plating. After incubation, 50 µl of cells was plated on ∅90 mm selective LB medium plate. After 3 days of incubation at 28 °C, individual colonies were picked from plate and transferred into liquid LB selective medium. Three tubes containing 3 ml of liquid culture were prepared and incubated on shaker for 24 h at 28 °C.

Large liquid culture for floral dip transformation was prepared by adding 2 ml of the overnight culture to 200 ml of liquid LB medium without antibiotics. The mixture was placed into 1 l flask, sealed with aluminium foil and incubated on shaker overnight, at 28 °C and 160 rpm.

### *6.2.2. Floral dip transformation*

After an overnight incubation, 200 ml agrobacterial suspension was centrifuged for 15 min at 5000 rpm. Supernatant (medium) was discarded and bacterial precipitate was mixed with 200 ml of freshly prepared transformation solution (5% sucrose and 0.03% Silwet L-77 in distilled water). For transformation, floral buds and flowers of *A. thaliana* Col-0 and WS ecotype (6 plants of each) were dipped in the agrobacterial suspension for 2 min, wrapped individually in plastic bags to increase humidity and laid down on a tray. Plants were kept in this position in the dark for 24 h, after which the plastic bags were removed, plants straightened and further incubated in long-day conditions (16 h day, 6000-8000 lux/8 h night). The procedure was repeated after 7 days, so each plant was transformed two times. After last transformation plants were watered for 3 weeks and left to dry until the seeds were ready for harvesting.

### *6.2.3. Collection and sterilization of seeds*

Seeds from *A. thaliana* plants transformed in this work, as well as plants transformed previously (35S:AtGH3.3-His; 35S:PbGH3-His and 35S:PpGH3.2-His, genetic maps in *Materials*) were used for selection of transgenics. Seeds from plants transformed with floral dip were collected and sterilized with 1 min incubation in 1 ml of 70% ethanol, followed by 10 min incubation in 1 ml of sterilization solution (1% Izosan G and 0.1% mucasol in distilled water) on shaker and 5 times washing with sterile water in laminar flow cabinet. After the final wash, seeds were sown on selective MS plates supplemented with hygromicin B.

### *6.2.4. Selection of primary transformants (T1)*

Sterile seeds were resuspended by pipetting and seeded on medium for germination containing hygromicin B at a concentration of 50 mg/l. Seeds were uniformly arranged on a plate in 3 horizontal rows and incubated for 48 h on +4 °C. Following a stratification period, plates were transferred to a climate chamber and incubated in vertical position for 8 days in long-day conditions. Selection of hygromicin-resistant transformants from non-transformants was made based on their elongated hypocotyls and primary root growth. Transgenic seedlings were transferred into Steckmedium substrate, abundantly watered with distilled water and grown in short-day conditions (10 h day/14 h night) for a week, after which they were transferred to long-day conditions. Plants were watered until the seeds were ready for harvesting (approximately 6 weeks).

T2 seeds were used for selection of plants with one T-DNA insertion and T3 seeds for selection of homozygous plants. Selections were conducted in a similar way. After sterilization T2 or T3 seeds were uniformly distributed on selective MS plates for easier counting. Lines with one T-DNA insertion were supposed to give 25% of wild type progeny, and homozygotes are supposed to give 0% wild types.

### *6.2.5. Genomic DNA (gDNA) isolation*

Genomic DNA was isolated according to the following protocole. Small part (less than 1 $cm^2$) of a leaf tissue was collected in 1.5 ml Eppendorf tubes containing glass beads. After freezing in liquid nitrogen, samples were grinded 2 x for 8 sec using Silver Mix. Four hundred µl of extraction buffer (200 mM TRIS pH 8, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was

added and samples were incubated for 10 min at 95 °C. After 5 min centrifugation at 13,200 rpm and room temperature (RT), approximately 300 µl of supernatant was collected and transferred to new Eppendorf tubes. For gDNA precipitation 2.5 volumes (750 µl) of 96% ethanol and 0.1 volume (30 µl) of sodium acetate (3 M, pH 5.2) was added, samples were mixed by inversion and incubated for 1 h at -20 °C. After incubation, samples were centrifuged (5 min, 13,200 rpm, RT). Supernatant was discarded and 1 ml of 70% ethanol was added to wash gDNA. After centrifugation (5 min, 13,200 rpm, RT), ethanol was removed and samples were dried at 37 °C. DNA precipitate was dissolved in 100 µl of distilled water and stored at +4 °C.

### *6.2.6. PCR amplification*

From overnight agrobacterial cultures, template for PCR reaction was prepared to examine their suitability for further procedures. Five hundred microliters of overnight culture was centrifuged for 5 min on maximum speed (13,200 rpm) at room temperature. Supernatant was discarded and bacteria were resuspended with 200 µl of distilled water. Samples were then denaturated for 5 min at 95 °C and aliquot used for PCR amplification. Large agrobacterial culture prepared from a single overnight culture and used for plant transformation was analyzed by PCR in a same way.

In order to confirm transgene incorporation, gDNA isolated from selected seedlings was used as a template for amplification of transgene with a PCR reaction.

cDNA obtained after reverse transcription was tested by PCR amplification of *Actin 3* gene.

For each PCR reaction, Master Mix was prepared by mixing 12.5 µl EmeraldAmp Max PCR Master Mix (2x Premix) (TaKaRa), 9.5 µl water, 0.5 µl forward (10 µM) and 0.5 µl reverse (10 µM) primer per sample. In each PCR reaction, 23 µl of Master Mix and 2 µl of template (water, gDNA, plasmid or denaturated agrobacterial suspension) was added. Reactions were conducted in conditions listed in Table 5. PCR samples were stored at -20 °C.

**Table 5.** *Temperature, duration and number of repetitions for each step of PCR reaction.*

| **Stage** | **Repetition** | **Temperature** [°C] | **Duration** |
|---|---|---|---|
| Initial denaturation | 1 | 98 | 3 min |
| Denaturation | 40 | 98 | 10 sec |
| Annealing | | 60 | 30 sec |
| Extension | | 72 | 1-2 min |
| Final extension | 1 | 72 | 7 min |

### *6.2.7. Gel electrophoresis assay*

PCR samples were analysed using agarose gel electrophoresis. One percent (1%) agarose gel was prepared in 1x TAE buffer (40 mM Tris pH 7.6, 20 mM acetic acid, 1 mM EDTA) and 3 μl of marker *GeneRuler 1kb DNA Ladder* was added to the first well. In each of the remaining wells, 8 μl of each PCR sample was added. Electrophoresis was conducted at the following conditions: 5 min 25 V, 30 min 100V. After electrophoresis, gel was stained in ethidium bromide solution (10 mg/l) and bands were visualized under UV light.

### *6.2.8. RNA isolation and reverse transcription*

For qPCR primer test, RNA was first isolated from seedlings using *RNeasy plant mini kit* (Qiagen). Five seedlings were dried on a paper towel before putting into 1.5 ml tubes with glass beads. Samples were freezed in liquid nitrogen and grinded 2 x for 8 sec with Silver Mix. The isolation procedure was conducted according to the protocol and with chemicals provided in the kit, including the optional on-column DNase digestion for efficient elimination of genomic DNA. Concentration of isolated RNA was measured on nanodrop, and appropriate volume was used for reverse transcription. Reverse transcription was conducted with *RevertAid First Strand cDNA Syntheis Kit* (Thermo Scientific) following the enclosed protocole.

RNA for quantification of overexpression was isolated from 10-days-old homozygous seedlings (8 per line) following the same protocole.

### *6.2.9. Quantitative PCR (qPCR)*

Used primers are listed in *Materials*. Primer master mix with 250 µM forward and 250 µM reverse primer was prepared for each primer set. cDNA samples were dilluted 5 x with water. Each cDNA Master Mix was prepared by mixing 7.5 µL SYBR Green (Power SYBR Green PCR Master Mix), 3.5 µL miliQ water and 2 µl of dilluted cDNA. Reaction was conducted in triplicates, with each well containing 13 µL of cDNA Master Mix and 2 µL of suitable primer mix. Reaction volume was 15 µL. Reaction was conducted with Applied Biosystems 7300 Real-Time PCR system in conditions listed in Table 6.

Mean ΔΔCt values were calculated. Values were normalized according to reference gene (EXP) expression and overexpression level was expressed in regards to native *AtGH3.3* gene expression.

**Table 6.** *Temperature, duration and number of repetitions for each stage of qPCR reaction.*

| **Stage** | **Repetition** | **Temperature** [°C] | **Duration** |
|---|---|---|---|
| 1 | 1 | 50 | 2 min |
| 2 | 1 | 95 | 10 min |
| 3 | 40 | 95 | 15 sec |
| | | 55 | 1 min |
| 4 (Dissociation) | 1 | 95 | 15 sec |
| | | 60 | 1 min |
| | | 95 | 15 sec |
| | | 60 | 15 sec |

### *6.2.10. Protein extraction and Ni-NTA purification*

Fresh leaf tissue was collected in 1.5 ml Eppendorf tubes, freezed in liquid nitrogen and grinded 2 x for 8 sec using Silver Mix. Extraction buffer (20 mM $NaH_2PO_4$ pH 8, 500 mM NaCl, 10 mM β-mercaptoethanol, 1% Triton X-100, 10 mM imidazole, 1 mM PMSF) was prepared and kept on ice. One mililiter of extraction buffer was added to each tube, samples were mixed by inversion and incubated on ice for 30 min. After centrifugation (15 min, 13,200 rpm, 4 °C), supernatant was transferred to new 2 ml tube and centrifuged again (5 min, 13,200 rpm, 4 °C). Supernatant was transferred to ~300 µl of Ni-NTA agarose beads (Qiagen) (1:1

distilled water: beads, washed 2 x with distilled water and 1 x with extraction buffer) and incubated on a rotor at +4 °C overnight.

After incubation, samples were centrifuged (1 min, 2,000 rpm, 4° C), supernatant was carefully pipetted to a new tube and beads were washed 3 x with washing buffer (20 mM $NaH_2PO_4$ pH 8, 500 mM NaCl, 10 mM β-mercaptoethanol, 1% Triton X-100, 20 mM imidazole, 1 mM PMSF). The method was conducted multiple times, firstly by denaturing proteins directly on the Ni-NTA beads with sample buffer (125mM Tris pH 6.8, 4% SDS, 10% β-mercaptoethanol, 32% glycerol, 0.5% bromphenol blue) (5 min on 80 °C) or samples were eluted from columns before denaturation. The elution was performed by adding 800 µl of elution buffer (20 mM TRIS pH 7.5, 500 mM NaCl, 5% glycerol, 1 mM DTT, 400 mM imidazole) to Ni-NTA beads and incubating for 10 min on a rotor at +4 °C. Samples were concentrated with *Centrifugal filter units* (Millipore). Eluted proteins were stored at +4 °C.

### *6.2.11. SDS polyacrylamide gel electrophoresis*

Polyacrylamide gels for stacking (4%, pH 6.8) and resolving (12%, pH 8.8) were prepared (according to Balen *et al*., 2011). *Page Ruler Plus Prestained Protein marker* (3 µl) was added to the first well. Protein eluates (20 µl) were loaded on gel and electrophoresis was started. Running conditions were 100 V for 35 min and 200 V for 70 min.

### *6.2.12. Western-blotting and immunodetection of transgenic proteins*

Proteins from polyacrylamide gel were transferred onto PVDF membrane (Immobilon). Transfer was conducted in cooled transfer buffer (28 mM Tris, 192 mM glycine, 10% methanol; pH 8.3) at constant current of 200 mA for 2 h. Afterwards, membrane was washed with 1x TBS buffer (20 mM Tris, 73 mM NaCl; pH 7.6) and blocked in 2% milk (prepared in 1x TBS pH 7.6 buffer) overnight at +4 °C. After blocking, membrane was washed with 1x TBS buffer and incubated on a shaker for 1.5 h in 10 ml of primary antibody solution (AntiHis, Proteintech in 2% milk, 1:1000). Membrane was washed 3x for 10 min with 30 ml of 1x TBS buffer before incubation in secondary antibody solution (Antimouse horseradish peroxidase (HRP), Sigma in 2% milk, 1:5000). Washing with 1x TBS was repeated and 500 µl of *Luminata Forte Western HRP substrate* (Millipore) was added for 2 min. The membrane was exposed to film for 10 min for the detection of HRP activity.

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## Abstract

The GH3 family of acyl-acid-amidosynthetases catalyze the formation of amino acid conjugates to modulate levels of active auxins in plants. The aim of this study was to regenerate plants with overexpression of different *GH3* genes. Wild type *Arabidopsis thaliana* plants, ecotypes WS and Col-0, were previously transformed via floral dip with *Agrobacterium tumefaciens* (strain GV3101, pMP90) to obtain *AtGH3.3*, *PbGH3* and *PpGH3.2* overexpressing plants. Primary transformants (T1) were analyzed for the presence of transgenes by DNA isolation, PCR amplification and gel electrophoresis. Because PpGH3.2 transgenic plants could not be obtained, floral dip transformation was conducted with new plant material. Secondary transformants (T2) were grown on hygromicin selective plates and lines with one T-DNA insertion were selected. AtGH3.3-$His_6$ and PbGH3-$His_6$ transgenic proteins were extracted from T2 plants and purified on Ni-NTA agarose. Western-blotting and immunodetection showed successful purification of transgenic PbGH3-$His_6$, while AtGH3.3-$His_6$ was degraded. T3 seedlings were grown on selective plates and homozygous lines were determined. These lines were used for RNA isolation and qPCR for measuring transgene overexpression. Seeds with overexpression of *GH3* genes, regenerated in this research, will be further used to study the auxin metabolism in plants.

**Keywords: auxin, IAA, GH3, floral dip, transformation, overexpression**

## Resumé

La famille GH3 d'acyl-acid-amido synthétases catalyse la formation de conjugués des acides aminés pour moduler les niveaux des auxines actives dans les plantes. L'objectif de cette thèse était de régénérer des lignées surexpresseurs des différents gènes *GH3*. Les plantes de type sauvage d'*Arabidopsis thaliana* (écotypes WS et Col-0) ont été transformés au préalable avec les souches d'*Agrobacterium tumefaciens* GV3101, pMP90 pour obtenir les lignées de surexpression de *AtGH3.3*, *PbGH3* et *PpGH3.2*. Les transformants primaires (T1) ont été analysés pour détecter la présence de transgènes par l'isolation de l'AND, l'amplification par PCR et l'électrophorèse sur gel. Comme les lignées transgéniques PpGH3.2 ne pouvaient pas être obtenues, des nouvelles plantes ont été transformées avec *A. tumefaciens*. Les transformants secondaires (T2) ont été cultivés sur l'hygromycine et les lignées avec juste une insertion d'ADN-T ont été sélectionnées. Les protéines transgéniques AtGH3.3-$His_6$ et PbGH3-$His_6$ ont été extraites des plantes (T2) et elles ont été purifiées par affinité sur l'agarose Ni-NTA.

Le Western Blot et l'immunodétection ont montré une purification réussie de la protéine PbGH3-$His_6$, mais la protéine AtGH3.3-$His_6$ a été dégradée. La descendance de transformants secondaires (T3) a été cultivé sur l'hygromycine et les lignées homozygotes pour chaque transgène ont été sélectionnées. Ces lignées ont été utilisées pour l'isolation de l'ARN et la surexpression de transgènes a été mesuré par qPCR. Les surexpresseurs des gènes *GH3* générées dans cette recherche seront utilisés pour étudier le métabolisme des auxines dans les plantes.