



**PALACKÝ UNIVERSITY OLOMOUČ**

**Faculty of Science**

**Laboratory of growth regulators**

**Study of subcellular localization of auxin-inactivating  
enzymes in *Arabidopsis thaliana*: from cloning to fusion  
protein expression in plants**

**MASTER THESIS**

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Abstrakt	<p>Auxiny jsou rostlinné hormony, které hrají klíčovou roli v růstu a vývoji rostlin. Některé aspekty metabolismu auxinů však stále vyžadují další zkoumání. Jedním z takových aspektů je inaktivace pomocí konjugace s aminokyselinami prostřednictvím enzymů patřících do rodiny GRETCHEN HAGEN 3 (GH3). Cílem práce je určit prostorovou lokalizaci enzymů GH3s řízených jejich nativním promotorem v rostlinách <i>Arabidopsis thaliana</i>. Za tímto účelem byly enzymy GH3s fúzovány se zeleným fluorescenčním proteinem (GFP) a vytvořeny expresní konstrukty. Pomocí konfokální laserové skenovací mikroskopie byla analyzována subcelulární lokalizace konstruktů AtGH3s-GFP. Připravené vektory pro transformaci rostlin a výsledné transgenní linie by mohly přispět k dalšímu výzkumu zaměřenému na pochopení metabolismu auxinu v rostlinách.</p>
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Abstract	<p>Auxin is a plant hormone that plays a key role in plant growth and development. Several mechanisms, such as auxin biosynthesis, transport, and inactivation regulate cellular auxin homeostasis in plants. Conjugation of auxin with amino acids by amido synthetases belonging to the GRETCHEN HAGEN 3 (GH3) family is one of the main inactivation pathways. This thesis aims to uncover the spatial localization of GH3 enzymes in <i>Arabidopsis thaliana</i> plants. This was achieved by expressing GH3 enzymes as fusion proteins with green fluorescent protein (GFP) to create expression constructs. The subcellular localization of the GH3-GFP fusion protein was analyzed using confocal laser microscopy. Prepared plant transformation vectors and resulting transgenic lines could contribute to setting up a baseline for further research to understand how GH3 enzymes contribute to auxin metabolism in plants.</p>
Keywords	<i>Arabidopsis thaliana</i> ; auxin; GH3s; cloning; protein localization; fluorescent tags; confocal laser microscopy
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„Prohlašuji, že jsem předloženou diplomovou práci vypracovala samostatně za použití citované literatury.“

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## List of abbreviations

4-Cl-IAA - 4-chloroindole-3-acetic acid	JA - Jasmonate
AMI - Amidase	JA-Ile - Jasmonoyl-isoleucine
AMP - Adenosine Monophosphate	KO - Knockout
ARF - Auxin Response Factor	LB - Luria-Bertani
ATP - Adenosine Triphosphate	MES - 2-(N-morpholine)ethane sulfonic acid
AtNIT - <i>Arabidopsis thaliana</i> nitrilase	MCS - Multiple Cloning Site
<i>A.thaliana</i> - <i>Arabidopsis thaliana</i>	mQH2O - Milli-Q water
BSA - Bovine serum albumin	MPK - Mitogen-Activated Protein Kinase
bp - base pairs	MS - Murashige and Skoog medium
CYP - Cytochrome	MW - Molecular weight
dNTPs - Deoxynucleotide triphosphates	NAP - Nuclear Auxin Pathways
<i>E. coli</i> - <i>Escherichia coli</i>	ORI - Origin of Replication
EDTA - Ethylenediaminetetraacetic acid	oxIAA - 2-oxindole-3-acetic acid
ELFO - Electrophoresis	PAT - Polar auxin transport
ER - Endoplasmic reticulum	PBC - Pelleted bacterial culture
ERF13 - Ethylene Responsive Factor 13	PCR - Polymerase Chain Reaction
Fw - Forward	PAA - Phenylacetic acid
<i>g</i> - gravitational force	RT - Room temperature
gDNA – genomic DNA	rpm - Revolutions per minute
GFP - Green fluorescent protein	TAA - Tryptophan aminotransferase
GH3s - GRETCHEN HAGEN 3 enzymes	TARs - tryptophan aminotransferase-related proteins
GMOs - Genetically Modified Organisms	T-DNA - Transferred DNA
HDGS - homology-dependent gene silencing	TAE - Tris-acetate-EDTA
IAA - Indole-3-acetic acid	Ti plasmid - Tumor-inducing plasmid
IAA-aa - IAA-amino acid conjugates	Tris - 2-amino-2-hydroxymethylpropane-1,3-diol
IAOx - Indole-3-acetaldoxime	Trp - Tryptophan
IAM - Indole-3-acetamide	TMK - Transmembrane Kinases
IAMT1 - IAA Carboxyl Methyltransferase	UMAMiT – Multiple Acids Move In And Out proteins
IAN - Indole-3-acetonitrile	WAT - Walls Are Thin group of proteins
IBA - Indole-3-butyric acid	X-gal - 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
ILR/ILL - IAA-Leu-Resistant1/ILR1-like	
IpyA - Indole-3-pyruvic acid	
INS - Indole Synthase	



## 1. Introduction

Phytohormones are small molecular weight signaling molecules that are produced within the plant. In extremely low concentrations, they can regulate all aspects of plant growth and development. One of the most important plant hormones is the auxin indole-3-acetic acid (IAA) that is essential for optimal plant growth, development and it responds to environmental stimuli such as light and gravity. Auxin exert its function through concentration gradients within organs, tissues, and cells. These gradients result from coordinated local biosynthesis, metabolism, and transport.

Subgroup II members of the GRETCHEN HAGEN 3 (GH3) protein family typically conjugate amino acids to IAA. This reaction is the plant's primary inactivation response to excess auxin cellular concentrations and takes place in the cytoplasm. As a small molecule, IAA could potentially enter the nucleus via diffusion through the nuclear pores without restriction to activate the auxin-signaling cascade. However, auxin fluxes are instead mediated by specific efflux and influx carriers that are localized at the endoplasmic reticulum (ER) membrane, where they are predicted to regulate cellular IAA homeostasis *via* their transportation in and out of the ER compartment. While this is an emerging model for regulating auxin movement to the nucleus, it still needs to be fully understood how inactivation pathways contribute to maintaining auxin balance within the cell.

Determining the GH3 spatial localization could help advance our understanding of GH3-mediated mechanisms. To date, synthesis of IAA-amino acid conjugates is believed to occur in the cytosol, as cytosolic localization was demonstrated for GH3.17 by isopycnic glucose gradient. While this method allows inspecting the proteins' presence in a specific cellular compartment, getting comprehensive information about the protein's subcellular localization may be challenging as each subcellular compartment needs to be individually verified. If not, it may result in incomplete, misleading information. Therefore, in this work we have utilized commonly used GFP tagging of the protein approach. The main advantage of this method is the *in vivo* localization of the protein of interest. The main goal of this work is to determine the subcellular localization of GH3 proteins by generating *Arabidopsis thaliana* stable transgenic lines expressing GFP-fusion proteins under their native genomic locus.

## 2. Goals of the thesis

The goals of the thesis:

- Literature review that is focused on molecular cloning techniques, as well as auxin synthesis, metabolism, and signaling;
- Design and preparation of plant transformation vectors for the expression of *Arabidopsis* GH3-GFP fusion proteins for subcellular localization studies in *planta*;
- Generation of *Arabidopsis* transgenic lines by *Agrobacterium*-mediated transformation with transgenic line selection;
- Subcellular localization study of GFP-tagged proteins by confocal laser scanning microscopy.

### 3. Theoretical part

#### 3.1 Auxin role and biosynthesis

The existence of auxin was famously inferred by Darwin's work on coleoptile phototropism. He showed that the seedling's response to unidirectional light producing a growth stimulus originated at the coleoptile tip. When the stimulus was traveling downward, it caused lower cells on the shaded side to grow faster compared to those on the illuminated side. The mobile growth regulator hypothesized by Darwin with these experiments was later identified by Went in 1928 as indole-3-acetic acid (IAA), the most abundant active auxin form in plants<sup>1</sup>. Besides IAA, several other naturally occurring molecules exhibit auxin-like activity: indole-3-butyric acid (IBA), 4-chloroindole-3-acetic acid (4-Cl-IAA), and phenylacetic acid (PAA) (Figure 1). Auxin and its derivatives act like morphogens; their gradient determines developmental patterns. At the same time, auxin is transported from cell to cell, similar to mammalian hormones, and triggers plants' response to environmental stimuli<sup>2</sup>. Auxins regulate various physiological processes during plant development, including the formation of bilateral symmetry in the embryo, root emergence, apical dominance, as well as environmental responses, such as gravitropism and phototropism<sup>2,3</sup>.

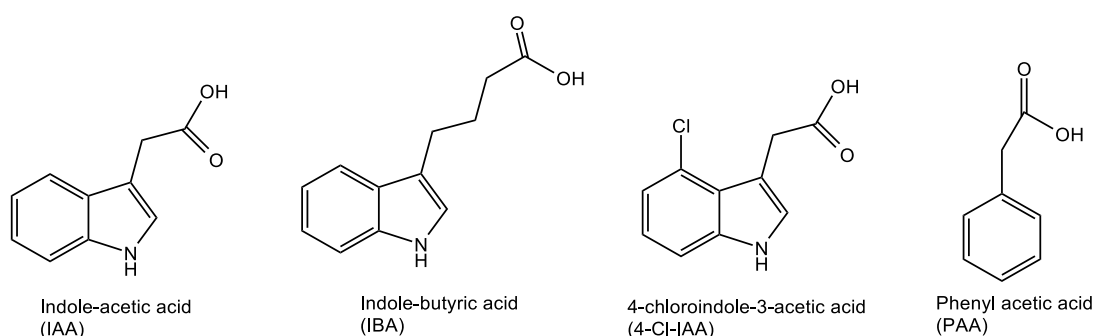


Figure 1. Chemical structures of most common natural auxins in plants.

The phenylacetic acid (PAA) (Figure 1) is a less studied type of auxins. The PAA is synthesized from amino acid phenylalanine *via* the phenylpyruvate pathway<sup>4</sup>. Some data show its involvement in the formation of root primordial cells in some terrestrial plants<sup>5</sup>. Next, 4-chloroindole-3-acetic acid (4-Cl-IAA) was exclusively found in ovaries without growing embryos. These findings indicate that 4-Cl-IAA is a key signaling molecule in floret senescence<sup>6</sup>. The particular mechanisms of action of these hormones are unknown. While 4-Cl-IAA is involved in fruit and seed formation, IAA and indole-butyric acid (IBA) affect mainly root development<sup>7</sup>. IBA is a precursor molecule that serves as a storage form of IAA<sup>8</sup>. Furthermore, IBA produces plant responses independent of IAA or it is converted to IAA, where it contributes to IAA function in plant development, stress responses, or both<sup>7</sup>.

As auxin plays a pivotal role in almost all aspects of plant growth and development, regulating optimal levels of active auxin within cells is necessary. The levels of free IAA can be controlled by transport, interconversion of modified auxin forms, and biosynthesis<sup>7</sup>. The aromatic amino acid L-tryptophan (Trp) has been solidly established as a key precursor of auxin biosynthesis. However, two main IAA production routes exist: Trp-dependent and Trp-independent<sup>9</sup>. The existence of multiple IAA biosynthetic pathways ensures redundancy and robustness in auxin production. If one pathway is disturbed or inhibited, plants could still produce auxin and maintain essential growth and developmental processes in non-optimal conditions.

### **3.1.1 Tryptophan-dependent pathway**

The tryptophan-dependent pathway occurs in two distinct compartments of the cell. First, aromatic amino acids, including tryptophan, are synthesized in plastids. Then tryptophan is translocated to the cytoplasm where downstream reactions can occur<sup>9</sup>. Trp-dependent auxin biosynthesis includes several parallel pathways (Figure 2) named after intermediate molecules: IAOx (indole-3-acetaldoxime), IAM (indole-3-acetamide), and IpyA (indole-3-pyruvic acid)<sup>4</sup>.

Tryptophan deamination results in the synthesis of IpyA by tryptophan aminotransferase (TAA1) and TAA1-related proteins (TARs). Afterward, the YUCCA (YUC) family of flavin monooxygenases converts IpyA to IAA<sup>4</sup>.

The conversion from Trp to IAOx is mediated by two cytochrome isozymes of the Monooxygenase P450 (CYP) family, CYP79B2 and CYP79B3<sup>9</sup>. IAOx is converted to IAN (indole-3-acetonitrile) by another enzyme of the P450 family – CYP71A13. AtNIT1, AtNIT2, and AtNIT3 are gene products with nitrilase activity. They take part in the conversion of IAN to IAA<sup>10</sup>. Specifically, they convert nitriles to carboxylic acids.

Although whether the conversion of Trp to IAM occurs is still unknown, IAM can be converted to active IAA through the action of AMIDASE1 (AMI1)<sup>7,11</sup>.

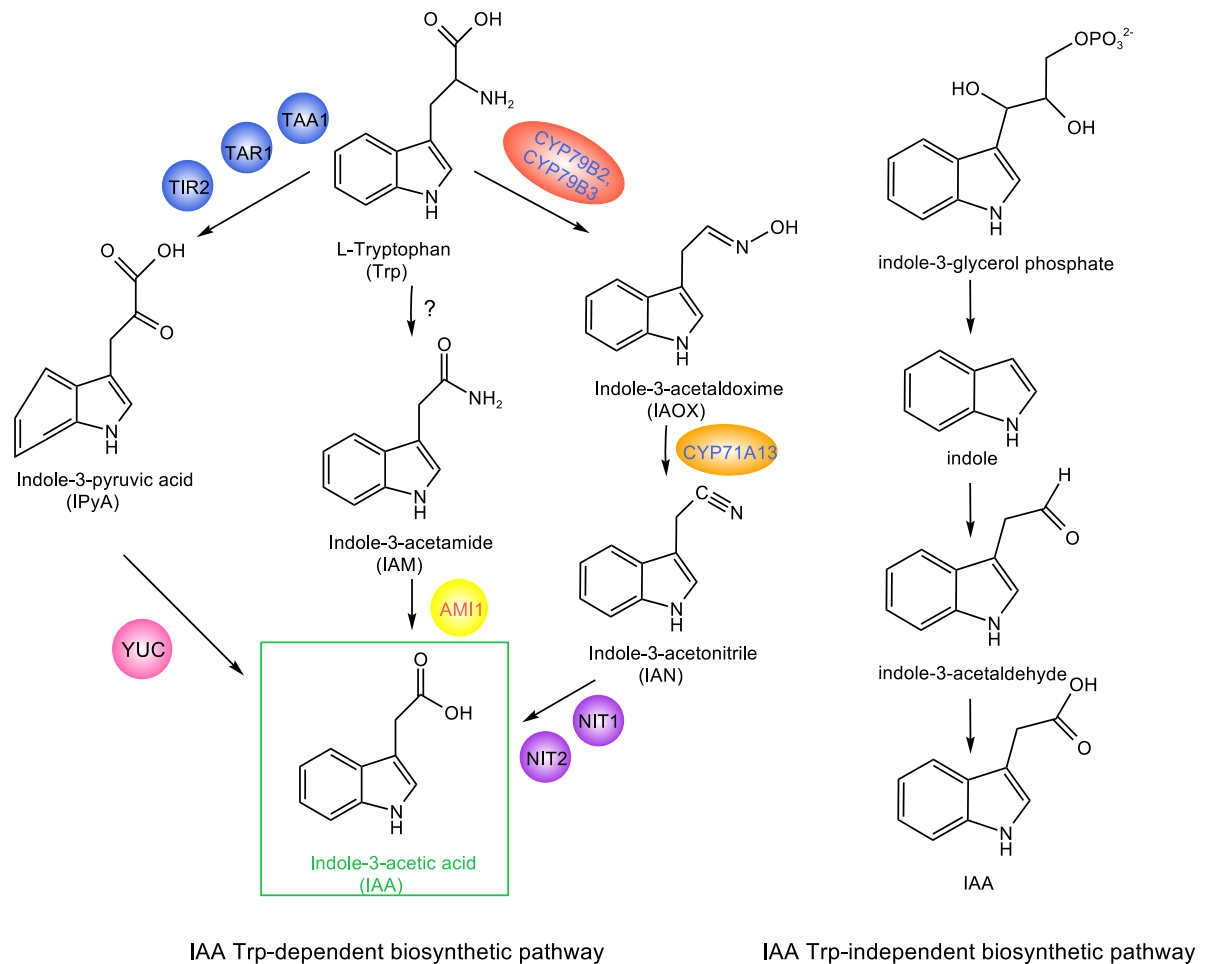


Figure 2. Potential IAA Trp-dependent and Trp-independent biosynthetic pathways<sup>7,9,12</sup>.

So far, IpyA pathway is the only complete Trp-dependent auxin biosynthesis pathway, and it is considered the main pathway for IAA synthesis in plants. The role of IAOx and IAM pathways still has to be further elucidated<sup>9</sup>.

### 3.1.2. Tryptophan-independent pathway

After discovering that maize and *Arabidopsis* mutants lacking in Trp biosynthesis were still producing IAA, a Trp-independent mechanism for auxin production was proposed<sup>13</sup>. Later research<sup>14</sup> suggested that cytosolic indole synthase (INS) mediates Trp-independent IAA production by converting indole-3-glycerolphosphate to indole (Figure 2). However, the molecular mechanism behind the indole to IAA conversion is unknown<sup>12</sup>.

### 3.2. Auxin transport in plants

Typically, auxin is synthesized in flowers and leaves and then is transported by phloem to target tissues. One type of transport unique to auxin is polar auxin transport (PAT)<sup>15</sup>. This transport includes both active and passive ways of auxin movement and is mediated through influx and efflux carriers<sup>16</sup>. According to the chemiosmotic hypothesis, all auxins are weak acids in either proton-dissociated or nondissociated state<sup>17</sup>. Therefore, their movement

between cells depends on pH, and the transport of cellular IAA relies on the collective action of auxin transporters. In the apoplast's slightly acidic conditions (around pH 5.5), a small portion of IAA is in the nondissociated form (IAAH) and can enter cells by passive lipophilic diffusion. Most IAA exists in a proton-dissociated form (IAA<sup>-</sup>), requiring transporters for active uptake into cells. Since the cytoplasm has a higher pH than the apoplast (around pH 7.0), the equilibrium shifts towards the proton-dissociated form, which cannot passively diffuse across the plasma membrane. To exit the cell, IAA relies on efflux transporters. Since then, candidates for auxin carrier proteins have been identified<sup>15,18</sup>.

The PIN-formed (PIN) family of proteins is a significant class of efflux transporters. They are frequently distributed polarly within cells, resulting in directed auxin transport only *via* membranes with PINs. The PIN family consists of eight members divided into two subclasses based on the lengths of the hydrophilic loop<sup>19</sup>. Canonical or "long" PINs (PIN1, PIN2, PIN3, PIN4 and PIN7 proteins) have subcellular polar localization finely corresponding to the directionality of auxin movement and explaining auxin asymmetrical distribution at intercellular level<sup>20-23</sup>. It seems that auxin alone is one of the most important regulators of its transport, but regulation of carrier-mediated transport can take effect on a few different levels<sup>17,24</sup>. The role of PINs in various developmental processes, such as vascular differentiation, apical dominance, patterning, organ polarity, embryogenesis, organogenesis, phyllotaxis, and tropisms, have been reported and widely discussed<sup>25,26</sup>.

Another group of proteins involved in auxin transport is the AUX1/LIKE AUX1 family of permeases. In *Arabidopsis*, this small gene family consists of four members: *AUX1*, *LAX1*, *LAX2* and *LAX3*. Unlike PINs, AUX proteins facilitate the influx of auxin into cells. Similarly to PINs, AUX protein activity and localization are tightly regulated to ensure proper auxin transport and response. These proteins typically contain several transmembrane domains that ensure selective transport of auxin molecules from the extracellular space into the cytoplasm of cells. The expression and activity of AUX proteins are influenced by various factors such as hormones, environmental cues, and developmental signals. One of the critical regulators of AUX protein activity is auxin itself. Mutants that lack functional AUX proteins often exhibit defects in various auxin-dependent processes, further highlighting the importance of these proteins in auxin transport and plant development<sup>27</sup>.

### **3.2.1. Auxin transport on subcellular level**

Contrary to cell-to-cell auxin transport driven by plasma membrane PINs, the ER-localized PIN5 and PIN8 („short“, noncanonical PINs) ensure the compartmentalization of intracellular auxin pools<sup>28,29</sup>. Studies have shown that PIN5 and PIN8 act antagonistically.

Opposite values in analogous experiments indicate that PIN5 may convey auxin transport from the cytoplasm to the ER, whereas PIN8 conveys transport in the opposite direction<sup>28,29</sup>.

PIN-like transporters (PILS) are yet another ER-localized family of transporters contributing to subcellular auxin distribution<sup>30-32</sup>. It is believed that auxin transport in the ER would sequester molecules in the cell compartment, restricting the abundance of signaling molecules in the nucleus<sup>31</sup>.

WALLS ARE THIN1 (WAT1/UmamiT5) of the UmamiT (USUALLY MULTIPLE ACIDS MOVE IN AND OUT) family of proteins is considered to harbor auxin transport, which was demonstrated in *Arabidopsis* vacuoles<sup>33</sup>.

### **3.3 Auxin signaling**

The understanding of auxin signaling has significantly improved in the past decades. Auxin effects through the nuclear auxin pathways (NAP) consist of protein-protein interactions. There are three groups of them: TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) family of coreceptors, AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) family of transcriptional repressors, and AUXIN RESPONSE FACTORS (ARF) transcription factors family<sup>34</sup>.

One of the most critical pathways in auxin metabolism is the inhibition pathway, which prevents overstimulation by auxin in plants. The TIR1/AFB/IAA pairs act as coreceptors for auxin. The binding of the TIR1/AFBs pair to auxin triggers ubiquitination and further degradation of Aux/IAA transcriptional repressors. Without Aux/IAAs, ARFs (ARF10, ARF16) are no longer inhibited and start the transcription of auxin target genes<sup>35</sup>.

Auxin has alternative signaling pathways, controlled by TRANSMEMBRANE KINASEs (TMKs) and MITOGEN-ACTIVATED PROTEIN KINASE (MPKs). TMK1 protein plays a role in extracellular auxin signaling by massive protein phosphorylation<sup>34</sup>. However, the precise roles of TMK2 and TMK3 in auxin signaling remain unclear. Another key protein for plant development and later root growth is Ethylene Responsive Factor 13 (ERF13). It is proved<sup>36</sup> that its degradation, carried by MPK14-mediated phosphorylation, is essential to regulating the auxin signaling cascade.

TMKs and TIR1/AFBs participate in general auxin signaling and are also crucial in keeping the balance between apoplastic acidification and alkalization<sup>34</sup>. The right balance is the main condition for effective auxin-mediated cell proliferation and plant growth.

### 3.4 Auxin metabolism

The main IAA inactivation mechanisms include oxidation, glycosylation, amino acid conjugation, and methylation. The last three processes are reversible. Therefore, they allow adjustable auxin release without the necessity for *de novo* auxin production<sup>37</sup>. However, some auxin forms, such as 2-oxindole-3-acetic acid (oxIAA), are irreversibly changed and safeguard against auxin toxicity in the presence of auxin overflow<sup>38</sup>.

The key catabolic mechanism for inactivating auxin is the oxidation of IAA to oxIAA, which is mediated by DIOXIGENASE FOR AUXIN OXIDATION (DAO) enzyme, with further glycosylation to oxIAA-glc<sup>22</sup>. This pathway is considered a major route for auxin inactivation, as oxIAA-glc is the most prevalent IAA metabolite in *A.thaliana*<sup>39</sup>. A recent study showed<sup>39</sup> that IAA inactivation is coordinately regulated by a GH3-ILR1-DAO framework. According to this model, GH3 proteins first conjugate IAA. This reaction is reversible as ILR/ILL (IAA-Leu-Resistant/ILR1-like) amidohydrolases can hydrolyze conjugates back to the active form. Alternatively, IAA amino acid conjugates can be irreversibly oxidized by DAO and oxIAA-aa can be further hydrolyzed to form oxIAA<sup>39</sup>.

In the past, it was thought that linking IAA with either aspartate (Asp) or glutamate (Glu) would mark these molecules for degradation. In contrast, conjugation with other amino acids would create storage forms of the hormone<sup>40</sup>. However, it has been recently shown that the production of IAA-Asp and IAA-Glu does not lead to degradation; instead, these conjugates also serve as storage forms of auxin<sup>39</sup>.

Furthermore, the differences in expression levels between *DAO* and *GH3* genes suggest that DAO has slower enzyme kinetics compared to GH3 proteins<sup>42</sup>, which helps to keep basal auxin concentrations in standard growth conditions. In contrast, GH3 proteins rapidly increase cellular IAA concentrations in response to environmental factors<sup>43</sup>.

The last proposed type of auxin inactivation is methylation *via* IAA CARBOXYL METHYLTRANSFERASE 1 (IAMT1), which converts IAA to IAA methyl ester (MeIAA). However, the exact metabolism and function of MeIAA remain unknown<sup>44</sup>.

#### 3.4.1. Gretchen Hagen 3 (GH3) protein family

*Gretchen Hagen 3 (GH3)* genes encode acyl acid amido synthetases that catalyze ATP-dependent conjugation of phytohormones. Their mechanism consists of two steps. Firstly, acidic phytohormone is adenylated, forming an intermediate molecule with AMP. Then, amino acid nucleophilically attacks AMP, creating conjugated amino acid product (Figure 3)<sup>45,46</sup>. This mechanism contributes to maintaining active phytohormone levels by balancing



the synthesis of the hormone and formation of amide-linked conjugates. The genome of *Arabidopsis* consists of 19 *GH3* genes, the exact role of which is difficult to determine due to their genetic redundancy<sup>45</sup>. However, these 19 members of *GH3* family can be classified into three subgroups based on their sequence similarity and substrate preference. Subgroup I consists of two members, GH3.10 and GH3.11, which catalyze the conjugation of jasmonate (JA) with isoleucine, resulting in the formation of jasmonoyl-isoleucine (JA-Ile)<sup>47</sup>. Next, subgroup II consists of eight members: *GH3.1*, *GH3.2*, *GH3.3*, *GH3.4*, *GH3.5*, *GH3.6*, *GH3.9* and *GH3.17*. All subgroup II proteins effectively conjugate auxin with amino acids, resulting in temporary auxin inactivation<sup>48,49</sup>. Lastly, subgroup III comprises nine members: *GH3.7*, *GH3.8*, *GH3.12*, *GH3.13*, *GH3.14*, *GH3.15*, *GH3.16*, *GH3.18*, *GH3.19*<sup>50</sup>. Some members, such as GH3.12, have been demonstrated to conjugate isochlorismate with glutamate, that can be converted to salicylic acid<sup>49</sup>.

Three members belonging to Subgroup II of the GH3 protein family, such as GH3.3, GH3.4 and GH3.17, will be investigated in this thesis. Recent studies<sup>45</sup> showed that GH3.17 may play prominent role in root elongation, while GH3.3 and GH3.4 may contribute to lateral root formation and affect the primary root growth<sup>45</sup>.

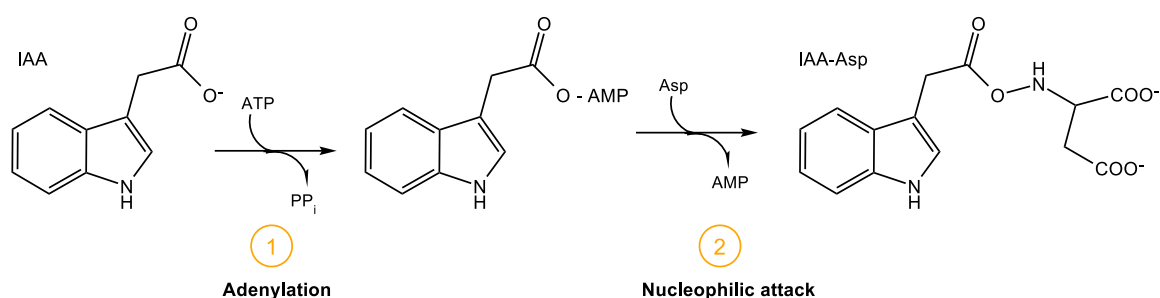


Figure 3. General reaction catalyzed by IAA-amido synthetases belonging to the GH3 family<sup>47</sup>.

### 3.5. Molecular cloning

Gene cloning comprises the *in vitro* creation of new DNA molecules containing unique combinations of genes or oligonucleotides and the propagation of recombinant DNA molecules *in vivo* via the replication mechanisms of bacteria and other microorganisms. The term "clone" comes from the Greek word for "bud" or "twig"<sup>51</sup>. It was initially used in biomedical sciences as a term for a set of genetically identical organisms. As they derive from a single organism, clones are supposed to be identical. The ability to make numerous identical clones of a DNA molecule ("molecular cloning", achievable both *in vivo* or *in vitro*) or a cell is referred to as cloning<sup>52</sup>.

Molecular cloning includes specific, unique tools and terminology. A DNA construct is created by a segment of DNA carried on a vector that can be used to insert genetic material into a target tissue or cell. A vector is any particle (such as plasmids, cosmids, Lambda phages) used to transport a foreign nucleic sequence artificially - usually DNA - into another cell, where it can be duplicated and/or expressed<sup>53</sup>. Recombinant DNA is a vector that contains foreign DNA. Plasmids are the most widely used vectors. Typically, the vectors used in DNA constructs contain several essential features: an origin of replication (ORI), a multiple cloning site (MCS), and a selectable marker<sup>53</sup>.

In general, there are five main steps of molecular cloning: preparation of the insert and vector, ligation, transformation, screening of the clones, and selection of the transformants<sup>51</sup>.

### **3.5.1. Cloning vectors**

The most well-known double-stranded vectors are plasmids. They can replicate independently of the host chromosome. Naturally, plasmids contain genes that benefit the survival of the organism<sup>54</sup>. A plasmid cloning vector is commonly used to clone DNA fragments of up to 15 kbp. The pBR322 plasmid was one of the first commonly used as a cloning vector<sup>55</sup>. There are five main categories of plasmids by original function. However, one plasmid can belong to more than one group<sup>56</sup>: fertility F-plasmids, resistance plasmids (R), Col plasmids, degradative plasmids, and virulence plasmids.

One of the main parts of vector is the ORI, which is responsible for the self-replication and sharing plasmid copies to the daughter cells. MCS is a specific DNA sequence for restriction enzymes used to insert the target DNA into the plasmid. MCS plays a significant role in the cloning methods. The promoter region is a sequence located upstream of inserted gene and directs to start of transcription. Promoters come in various types: native, constitutive, inducible and repressible<sup>54</sup>. Native promoters initially consist of a single fragment from the 5' region of a given gene. An inducible promoter controls the expression of the target gene in specific circumstances. They activate gene transcription when exposed to a certain small chemical, such as lactose and galactose. Opposite to inducible promoters, repressible promoters are inhibiting gene expression in the presence of specific small chemicals, such as tryptophan or ethanol. The next part of the vector is the terminator. It defines the end of transcription. Therefore, only the gene of interest will be transcribed. Another type of the vector sequence is the primer binding site, which is complementary to the sequence of a PCR (polymerase chain reaction) primer used for amplification of the region of interest<sup>54</sup>. Last but not least, is a selectable marker. It is a gene that brings

identifiable characteristic to a vector. The most used ones are antibiotic resistance genes and genes encoding fluorescent proteins.

### 3.5.2. GreenGate cloning

GreenGate is a cloning technique for the fast building of plant transformation constructs. It relies on the Golden Gate technique. Ready-to-use plant transformation vectors are constructed from six pre-cloned entry modules and a destination vector in a single-tube reaction. The method includes release of DNA fragments from entry modules using the type IIS restriction endonuclease *BsaI*<sup>57</sup>. Each DNA fragment is flanked at its 5'-end by the same overhang as the 3'-end of its previous neighbor. All overhangs vary from each other by at least two of the four nucleotides. Once DNA fragments are released, they can be ligated in specific order between the destination vector's left and right border sequences by *T4* DNA ligase (Figure 4). These six pUSC19-based modules include plant promoter, N-terminal tag, coding sequence (*i.e.* the gene of interest), C-terminal tag, plant terminator and plant resistance cassette<sup>57</sup>. All modules can be modified, and the gene's coding region should be inserted in the module designed for coding sequence. In addition, the pGreen-IIS-based destination vector encodes a different antibiotic resistance than the entry vectors. It carries recognition sites in an orientation that removes them from the backbone after type IIS digestion, exposing overhangs compatible with those of the outer insert modules<sup>57</sup>.

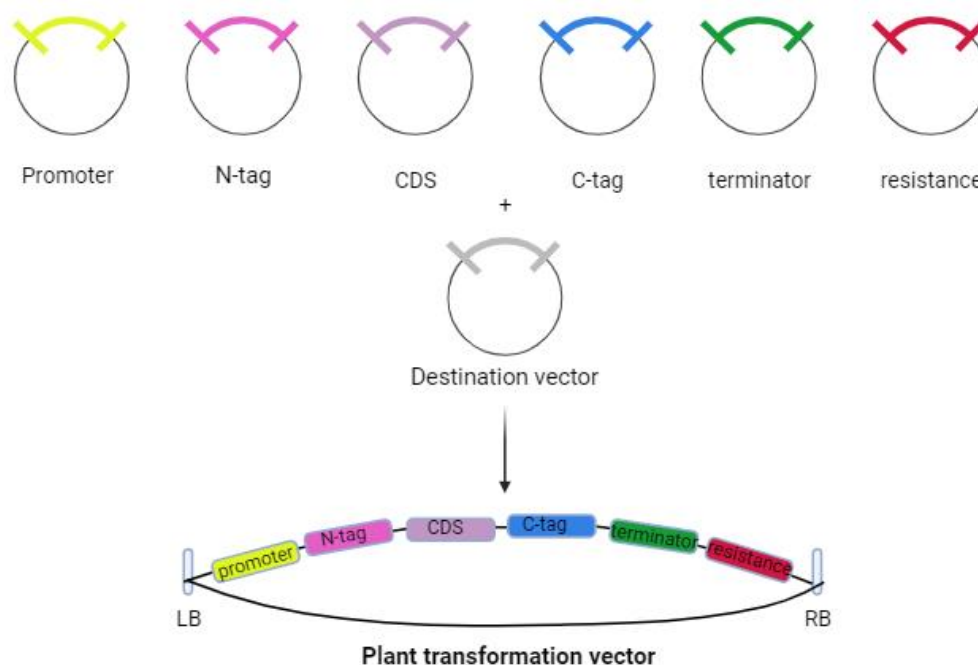


Figure 4. The process of generating a plant transformation vector by GreenGate cloning strategy, created in Biorender.

### 3.5.3. TA cloning

TA cloning is a technique that creates complementary single-stranded overhangs between the insert and vector using Taq polymerase's terminal transferase activity. Taq polymerase adds a single deoxyadenosine (dA) to the 3'-ends of double-stranded DNA, a unique feature not found in all thermostable DNA polymerases<sup>58</sup>. Vectors with T overhangs (overhangs with the deoxythymidine on 3'-end) can be bought or prepared individually by using a blunt-end restriction enzyme, mixing with terminal transferase enzyme and dideoxythymidine triphosphate (ddTTP), which lacks the 3' hydroxyl group. The lack of the hydroxyl group guarantees that only one T is attached to the vector ends. Alternatively, the vector can be cleaved using restriction enzymes that produce T overhang straightly<sup>58</sup>.

The TA cloning process starts with generating the insert in a PCR reaction using Taq polymerase, which attaches a single A to the 3'-ends of the PCR product. Subsequently, the PCR products are fused with a vector containing complementary 3' deoxythymidine (T) overhangs. DNA ligase is then utilized to join the vector and insert together. The disadvantage of this method is that proofreading DNA polymerases cannot be used, as they are incapable of producing A-overhangs<sup>59</sup>. However, this limitation could be solved by incubating the PCR reaction with Taq polymerase after performing the PCR reaction with the DNA-proofreading polymerase.

### 3.5.4. PCR

A straightforward enzymatic procedure called PCR enables the *in vitro* amplification of a particular DNA region from a complex DNA pool. PCR can produce enough copies of DNA from minimal levels. Therefore, DNA can be examined with standard laboratory techniques. As a result, PCR is considered a sensitive assay<sup>60</sup>. The critical component of PCR is the Taq polymerase, a thermostable DNA polymerase, which utilizes DNA primers made especially for the target DNA region to be amplified. After first step, denaturation, which breaks double-stranded DNA into two separated strands, follows primers annealing. Thermostable Taq polymerase recognizes primers and production of DNA amplicons begins in 5' to 3' orientation<sup>61</sup>. The final phase is called elongation and occurs after the last cycle. Its purpose is to guarantee that every amplicon will have their second strand produced<sup>62</sup>. The target region can be created in significant quantities by the PCR process, as with every cycle, the number of DNA molecules is doubled<sup>63</sup>.

### 3.5.5. Screening Strategies

Selection and screening strategies are used to validate if transformation of foreign DNA into host cells was effective. There are two main types of selection: positive and negative.

The most often used negative selection method is blue-white selection.  $\beta$ -galactosidase is a naturally occurring protein in *E.coli* encoded by the *lacZ* gene of the *lac* operon. In its native form, it forms a homotetramer, which cuts lactose into glucose and galactose<sup>64</sup>. N-terminal deletion makes a mutant  $\beta$ -galactosidase from the M15 strain of *E.coli* inactive, as the  $\omega$ -peptide cannot form a tetramer. However, in the presence of the protein's N-terminal part, the  $\alpha$ -peptide, in this mutant form of the protein, may fully rebound to its active tetrameric structure. The  $\omega$ -peptide-containing *lacZ* deletion (*lacZ $\Delta$ M15*) is carried by the host *E.coli* strain in this screening approach, whereas the plasmids utilized carry the *lacZ $\alpha$*  sequence, which encodes the  $\alpha$ -peptide<sup>65</sup>. Both are ineffective, but when both peptides are expressed simultaneously, they combine to generate a functioning  $\beta$ -galactosidase enzyme. X-gal is a colorless lactose analog that may be cut down by  $\beta$ -galactosidase to produce an insoluble pigment that is vivid blue. Growing bacteria on X-Gal may produce blue and white colonies. Blue colonies suggest that the vector may have an unbroken *lacZ $\alpha$*  (no DNA insert present), but white colonies, where X-gal is not hydrolyzed, indicate the presence of an insert in *lacZ $\alpha$*  that prevents the  $\beta$ -galactosidase from being active<sup>66</sup>.

The other well-studied method is antibiotic selection. When the inserted plasmid has a marker for antibiotic resistance, selection is straightforward. Transformed cells are placed on a medium containing the appropriate antibiotic. The cells that proliferate should be those harboring plasmids containing selected antibiotic resistance<sup>67</sup>.

The most precise ways of selection are PCR methods, such as Colony PCR and Sanger sequencing. These techniques use specific primers to identify the presence of the inserted gene. Colony PCR is a technique for detecting an inserted construct or plasmid into bacteria or yeast. In colony PCR, primers are designed to produce a specific, known-size product only in the presence of the wanted construct. Ideally, the product will have a different size if the desired DNA insertion did not occur<sup>68</sup>. By using a very crude cell preparation, the tiny quantity of template DNA produces an easily visible band on an agarose gel after PCR amplification. Consequently, colony PCR is an effective method for quick and straightforward sorting through a potentially huge number of positive colonies from false ones<sup>69</sup>.

### **3.5.6. Competent cells transformation**

The majority of techniques for transforming bacteria are based on the findings of Mandel and Higa<sup>70</sup> and Cohen *et al*<sup>71</sup>, who demonstrated the possibility of transfecting bacteria with bacteriophage and DNA plasmids by incubating them with an ice-cold CaCl<sub>2</sub> solution and briefly heating them after that. Through this process, bacterial cells are brought into a brief stage of "competence" that allows them to absorb DNA. After transformation, cells are incubated into a recovery medium to refurbish the cell membrane and the cell wall<sup>72</sup>. Other methods of transformation are electroporation and heat shock.

The electroporation method uses an electric pulse, which produces an irregular electric field and creates pores in the plasma membrane. That helps the foreign DNA to move into a host cell. After that, the cell membrane locks itself again and traps the DNA inside. A comparison between electroporation and the heat shock method demonstrates that electroporation is more efficient, in terms of higher colony numbers and faster procedure<sup>73</sup>. One of the method's limitations is that it requires electroporation cuvettes and an electroporator, a specialized piece of instrumentation, while the heat shock method can be performed using standard Eppendorf tubes and a conventional thermoblock. Furthermore, a common issue with electroporation is the existence of air bubbles or salts lasted because of wrong competent cell preparation, which might lead to an arcing in the cuvette and in that case losing the sample<sup>73</sup>.

In the heat shock transformation, the membrane potential of competent cells is reduced by the temperature pulse, which reduces the potential barrier for negatively charged DNA to enter the cytoplasm<sup>74</sup>. Chemically competent cells are frequently formed in pellets by adding salt, such as CaCl<sub>2</sub>. The negative charge on the phospholipid and DNA is eliminated by applying salt, which helps the DNA to move toward the cell<sup>75</sup>.

### **3.5.7. Molecular cloning impact**

The advancement of genetic engineering techniques has enabled the manipulation of microbe genomes to produce substances with little intrinsic value but significant medical or commercial benefit to mankind. Small portions of DNA could be replicated with great precision using molecular cloning. The high number of copies makes it easier to analyze the DNA sequence, content, and genes within specific DNA fragments. Molecular cloning has excellent benefits in areas such as gene function analysis, recombinant protein production and generation of genetically modified organisms (GMOs).

To investigate the function of a gene, either gain-of-function or interference studies using microRNA can be performed by cloning the cDNA into an expression vector to induce overexpression in the target organism. Besides RNA interference, there are other ways to inhibit gene function, such as the CRISPR/Cas9 editing method, Zinc-Finger Nucleases (ZFNs), and transcription activator-like (TAL) effector nucleases (TALENs)<sup>76</sup>. Furthermore, adding particular mutations can determine genes' effectiveness in gene function, whether through site-directed mutations or the creation of protein mutants<sup>53</sup>.

Molecular biology and the production of relevant proteins have been revolutionized by cloning. By cloning the gene that encodes a specific protein into an appropriate expression system, scientists can generate substantial quantities of this protein for research, therapeutic or industrial purposes. For example, extraneous genes have been inserted into the DNA of *E.coli* to enable the creation of beneficial proteins such as interferons, human hormones insulin, somatostatin, and somatotropin<sup>52</sup>.

Molecular cloning techniques have enabled scientists to insert certain genes into organisms, creating GMOs. This has resulted in agricultural breakthroughs such as the producing crops with better features such as insect resistance, disease resistance, and nutritional value<sup>77</sup>.

### **3.6. *Agrobacterium*-mediated plant transformation**

Among the various techniques used to introduce foreign genetic material into plants, *Agrobacterium*-mediated plant transformation, known as the floral dip method, is commonly used. With this method, a naturally occurring bacteria in soil, *Agrobacterium tumefaciens*, is used as vector to deliver foreign DNA into the plant cells leading to heritable changes in the plant genome. The process includes growing *Arabidopsis* to the flowering stage, dipping plant floral buds in a *Agrobacterium* growth culture medium containing sucrose or hormones, gathering seeds several weeks later, and identifying transformed progeny by selection on media containing antibiotic or herbicide<sup>78</sup>. The optimal period for conducting the floral dip is when plants have a substantial number of unopened flower buds. The fact that transformed progeny are usually hemizygous for the transgene at a particular locus indicates that transformation takes place subsequent to the divergence of the ovarian and anther cell types<sup>79</sup>. *Agrobacterium*-mediated transformation is suitable not only for introducing certain gene constructs into plants, but also for a random mutagenesis technique for gene-tagging as transformed plants can be obtained at high numbers<sup>80</sup>. Beside sucrose media there is a second crucial component - surfactant Silwet - which allows chemicals and *Agrobacterium* to enter a plant tissue, particularly female gametes<sup>81</sup>.

In detail, the plant is infected by *Agrobacterium* via its *Ti* plasmid. The *Ti* plasmid incorporates T-DNA, a portion of its DNA, into the chromosomal DNA of its host plant cells. The periplasmic space contains a *chvE* protein, which is encoded by a chromosomal gene and serves for recognizing sugars<sup>82</sup>. Attachment occurs in two stages. This mechanism is mediated by four major genes: *chvA*, *chvB*, *pscA*, and *att*. The first three genes' proteins appear to be responsible for the creation of cellulose fibrils. These fibrils also serve as anchors for the bacteria, assisting in forming a microcolony. *VirC2* ensures proper processing and is also essential for single-copy system integration<sup>83</sup>.

### **3.7. Confocal microscopy**

Confocal laser scanning microscopy is an optical imaging technique that uses a spatial pinhole to exclude out-of-focus light during image generation to increase the optical resolution and contrast of a micrograph<sup>84</sup>. The optical sectioning method, which allows for reconstructing three-dimensional structures within an object, is made possible by taking many two-dimensional photographs of the object (z-stack) at different depths in the sample. A traditional microscope allows light to penetrate as far into the material as possible. In contrast, a confocal microscope only concentrates a smaller beam of light at one particular depth level. A confocal microscope focuses both illumination and detection lenses on a single, diffraction-limited point in the sample. This particular spot is the only spot imaged by the detector throughout a scan<sup>84</sup>. While the original design's pinholes, objective lenses, and low-noise detectors remain the fundamental elements of a conventional confocal microscope, fast scanning mirrors, wavelength selection filters, and laser illumination are also commonly included. Usually, the laser is focused on two scanning mirrors that move the beam in x and y directions over a single field of view<sup>85</sup>, creating an image.

In this diploma thesis, confocal microscopy was used to observe the subcellular localization of proteins fused to a green fluorescent protein (GFP). GFP is a fluorophore, originally isolated from the jellyfish *Aequorea victoria*<sup>86</sup>. The cell generates the fluorophore spontaneously by cyclizing the tripeptide Ser65-Tyr66-Gly67, forming a heterocyclic imidazolinone ring and oxidizing Tyr66 with molecular oxygen<sup>86</sup>.



## **4. Experimental part**

### **4.1 Material and equipment**

#### **4.1.1 Chemicals**

2-(N-morpholine)ethane sulfonic acid (MES) - Duchefa Biochemie (Netherlands)

2-amino-2-hydroxymethylpropane-1,3-diol (Tris) - Duchefa Biochemie (Netherlands)

5x Green GoTaq Flexi Reaction Buffer – Promega (Czech Republic)

Acetic acid – Penta (Czech Republic)

Agarose - Merck Life Science (Germany)

Alfa Aesar Gellan Gum - Thermo Fisher Scientific (USA)

10 mM ATP – Thermo Fisher Scientific (USA)

Bacteriological agar - Sigma-Aldrich (USA)

dNTPs - Thermo Fisher Scientific (USA)

DreamTaq Green PCR Master Mix (2x) - Thermo Fisher Scientific (USA)

E.Z.N.A. ® Plant DNA Kit – Omega Bio-tek (USA)

Ethanol - Lachner (Czech Republic)

Ethylenediaminetetraacetic acid (EDTA) - Penta (Czech Republic)

10x FastDigest Green buffer - Thermo Fisher Scientific (USA)

GelRed - Sigma-Aldrich (USA)

Glycerol - Sigma-Aldrich (USA)

Hydrochloric acid 37% (HCl) – Penta (Czech Republic)

Isopropanol - Lachner (Czech Republic)

LB Broth - Sigma-Aldrich (USA)

Magnesium Chloride - Penta (Czech Republic)

Milli-Q water (from Milli-Q Reference Water Purification System) - Merck Life Science (Germany)

Murashige & Skoog including vitamins - Duchefa Biochemie (Netherlands)

NucleoSpin® Gel and PCR Clean-up – Takara Bio (USA)

GeneRuler 1 kB Plus DNA Ladder - Thermo Fisher Scientific (USA)

Polyoxyethylene sorbitan monolaurate 20 (Tween 20) - Sigma-Aldrich (USA)

Phusion HF Buffer Pack - Thermo Fisher Scientific (USA)

Silwet L-77 - Sigma-Aldrich (USA)

Sodium hypochloride – Carl Roth (Germany)

Sucrose - Lachner (Czech Republic)

TriTrack DNA Loading Dye (6x) - Thermo Fisher Scientific (USA)

QIAprep Spin Miniprep Kit – Qiagen (USA)

#### **4.1.2 Enzymes**

DreamTaq DNA Polymerase - Thermo Fisher Scientific (USA)

Eco31I (*Bsa*I) 10 U- Thermo Fisher Scientific (USA)

GoTaq G2 Flexi DNA polymerase - Promega (Czech Republic)

Phusion High-Fidelity DNA Polymerase - Thermo Fisher Scientific (USA)

T4 DNA Ligase 30 U - Thermo Fisher Scientific (USA)

#### **4.1.3 Instruments**

Airstream Gen 3 Horizontal Laminar Flow Cabinet - Esco Micro Pte. Ltd. (Singapore)

Confocal microscope LSM 900 - Zeiss (Germany)

Electroporator ECM 399 - BTX (USA)

Fume cupboard - MERCI (Czech Republic)

ICT incubator - P-Lab (Czech Republic)

INCU-Line incubator - VWR International, Avantor (USA)

IKA IKAMAG Magnetic Mixer - IKA (Germany)

Mega Star 600R refrigerated centrifuge - VWR International, Avantor (USA)

Memmert incubator - VWR International, Avantor (USA)

Microcentrifuge/vortex Combi-spin PCV-2400 - Keison products (UK)

Microwave oven - Sencor (Japan)

Milli-Q Reference Water Purification System - Merck Life Science (Germany)

NanoDrop OneC - Thermo Fisher Scientific (USA)

pH Meter Ultra-Basic Bench-top Meter - Biotech (France)

POS-300 Orbital Shaking Platform - Keison products (UK)

Precision Balances - Mettler Toledo (USA)

Plant Growth Chamber Sanyo MLR-351H - SANYO Electric Co., Ltd. (Japan)

SANOclav Laboratory autoclave La-MCS - SANOclav (Germany)

Thermocycler Doppio - VWR International, Avantor (USA)

ThermoMixer Temperature Control Device - Eppendorf AG (Hamburg, Germany)

Transilluminator Gel Doc EZ System - Bio-Rad Laboratories (USA)

UV-1600PC Spectrophotometer - VWR International, Avantor (USA)

XSR analytical balances - Mettler Toledo (USA)

#### **4.1.4 Software**

Geneious Prime – Dotmatics (New Zealand)

ImageLab – Bio-Rad (USA)

ZEN blue/black – Zeiss (Germany)

#### **4.1.5 Biological material**

*Arabidopsis thaliana*, ecotype Columbia (Col-0)

*Escherichia coli* NEB5 $\alpha$

*Escherichia coli* TOP 10

*Agrobacterium tumefaciens* GV3101 (pSoup)

#### **4.1.6 Antibiotics**

Kanamycin - Duchefa Biochemie (Netherlands)

Carbenicillin - Duchefa Biochemie (Netherlands)

Rifampicin - Duchefa Biochemie (Netherlands)

Spectinomycin - Duchefa Biochemie (Netherlands)

Tetracycline - Duchefa Biochemie (Netherlands)

### **4.2 Methods**

#### **4.2.1 Genomic DNA extraction**

Genomic DNA (gDNA) was extracted by E.Z.N.A. ® Plant DNA Kit or DNA extraction buffer.

*Arabidopsis* leaves were ground in liquid nitrogen, and 100 mg of the sample was transferred to a nuclease-free 1,5-ml microcentrifuge tube. Six hundred  $\mu$ l of the P1 Buffer was added to the sample following vortexing until all clumps disappeared. Samples were incubated at 65 °C for 10 minutes, and mixed twice during the incubation by inverting the tube. Afterward, 140  $\mu$ l of the P2 Buffer was added, followed by thorough vortexing. Samples were centrifugated at 10,000 *g* for 10 minutes. The cleared lysate was transferred to a new 1,5-ml microcentrifuge tube, 0,7 volumes of 100 % isopropanol were added, and

samples were thoroughly mixed. Centrifugation at 14,000 g for 2 minutes was performed to pellet DNA. The supernatant was discarded. The tube containing DNA was inverted on a paper towel for 1 minute to allow any residual liquid to drain. Three hundred  $\mu\text{l}$  of sterile deionized water heated to 65 °C was added and pellet was resuspended by vortexing. Next, 4  $\mu\text{l}$  of RNase A was added, and samples were vortexed briefly. One hundred and fifty  $\mu\text{l}$  of P3 Buffer and 300  $\mu\text{l}$  of absolute ethanol were added, and samples were vortexed immediately to obtain a homogeneous mixture. A HiBind® DNA mini-column was inserted into a collection tube. The entire sample was transferred to the column and was centrifuged at 10,000 g for 1 minute. The flow-through was discarded, and 650  $\mu\text{l}$  DNA washing buffer was added. Centrifugation at 10,000 g for one minute was carried out, and the washing step was repeated. The column was centrifuged at 10,000 g for two minutes to dry. The HiBind® DNA mini-column was transferred into a new 1,5-ml microcentrifuge tube with the addition of 50  $\mu\text{l}$  mQH<sub>2</sub>O heated to 65 °C. Samples were incubated at room temperature for five minutes. The DNA was eluted by centrifugation at 10000 g for one minute.

Alternatively, DNA was extracted using a genomic DNA extraction buffer containing 50 mM of Tris-HCl (pH 7,2), 300 mM NaCl, and 10 % sucrose. The solution was topped up to ten ml with mQH<sub>2</sub>O and autoclaved on a liquid cycle (121 °C for 15-20 minutes). Approximately 100 mg of fresh leaves with 500  $\mu\text{l}$  of genomic DNA extraction buffer were homogenized with beadmill (3 beads, 27 Hz, 3 minutes) and incubated at 95 °C for one minute. Samples was centrifuged at 3000 g, at 22 °C for two minutes. One  $\mu\text{l}$  of supernatant was used for the PCR reaction.

#### **4.2.2 Plasmid isolation**

Isolation of plasmids was performed using a QIAprep® Miniprep kit. Selected colonies were inoculated in 6 ml of LB medium supplemented with the corresponding antibiotic, and grown at 37 °C overnight. The day after, the bacterial culture was pelleted at 6800 g for three minutes, and the pellet was resuspended in 250  $\mu\text{l}$  of buffer P1. Then, 250  $\mu\text{l}$  buffer P2 were added, and the mixture was mixed by inverting the tube 4–6 times. Next, 350  $\mu\text{l}$  buffer N3 was added, and the mixture was gently remixed. Samples were centrifuged for ten minutes at 17,900 g, forming a compact white pellet. The supernatant was transferred to the QIAprep spin column. Centrifugation at 17,900 g for one minute was carried out, and the flow-through was discarded. The column was washed by adding 700  $\mu\text{l}$  PE Buffer, followed by one minute centrifugation at 17,900 g. The flow-through was discarded, and centrifugation was performed for two minutes to remove residual washing buffer. The column was placed in a clean 1,5-ml microcentrifuge tube, and samples were incubated in a preheated thermoblock

at 70 °C for 5 minutes. Fifty µl of mQH<sub>2</sub>O water was added to the column, incubated at RT (room temperature) for five minutes, and then centrifuged for two minutes to elute.

### 4.2.3 Preparation of Luria-Broth (LB) medium

LB medium was prepared by dissolving 25 g of LB Broth in 900 ml of mQH<sub>2</sub>O. pH was adjusted to 7-7,2, and the solution was topped up to 1000 ml with mQH<sub>2</sub>O. To prepare LB agar plates, 1,5% bactoagar was added into the LB medium. The medium was autoclaved on a liquid cycle (121 °C for 15 minutes). LB medium containing bactoagar was supplemented with either 60 µg/ml spectinomycin, 100 µg/ml carbenicillin, or 50 µg/ml kanamycin. For *Agrobacterium* selection, LB agar plates were supplemented with 60 µg/ml spectinomycin, 50 µg/ml rifampicin, and 5 µg/ml tetracycline and poured into Ø 90mm round Petri dishes.

### 4.2.4 Preparation of MS agar plates

Half-strength MS medium was prepared by dissolving 4,4 g of MS salts + vitamins, 0,5 g of MES, and 10 g of saccharose in 900 ml of mQH<sub>2</sub>O. Reagents were stirred until completely dissolved. pH was adjusted on 5,7, and the solution was topped up to 1000 ml with mQH<sub>2</sub>O. After pH adjustment, 0,57 % of Gellan Gum was added, and the medium was autoclaved on a liquid cycle (121 °C for 15 minutes). The medium was supplemented with 30 µg/ml of Hygromycin B and poured in Ø145 mm round Petri dishes.

### 4.2.5 PCR amplification and PCR clean-up

PCR was used to amplify coding regions of *AtGH3.3*, *AtGH3.4* and *AtGH3.17* genes. Ten µl of gDNA were mixed with 0,5 µl of the Phusion DNA polymerase, 10 µl of 5xPhusion HF buffer, 1 µl of 10 µM dNTPs, 2,5 µl of 10 µM forward (Fw) and reverse (Rv) primer pairs (Table 2) and 21,5 µl of mQH<sub>2</sub>O. After mixing, samples were spun down and transferred to a thermocycler to initiate PCR amplification. The PCR amplification parameters are described in Table 1. If amplicons were used for TA cloning strategy, 0,7 units of GoTaq Polymerase were added to the mixture and additional ten-minute incubation at 72 °C was performed.

Table 1. Thermocycler parameters for PCR amplification

Temperature (°C)	Time (minutes)	Cycles (number)
98	0:30	1
98	0:30	35
55	0:30	35
72	4	35

72	10	1
8	60	

Table 2. Primers used for amplification of the coding region of GH3s from genomic DNA

Primer name	Primer sequence
AtGH3.3_GG_Fw	AACAGGTCTCAGGCTCAACAATGACCGTTGATTCAGCTCTGCG
AtGH3.3_GG_Rv	AACAGGTCTCTCTGAACGACGACGTTCTGGTGACCA
AtGH3.4_GG_Fw	AACAGGTCTCAGGCTCAACAATGGCTGTTGATTCGCTTCTTCA
AtGH3.4_GG_Rv	AACAGGTCTCTCTGAATGACGTCGTTCTGGCGACCA
AtGH3.17_GG_Fw	AACAGGTCTCAGGCTCAACAATGATACCAAGTTACGAC
AtGH3.17_GG_Rv	AACAGGTCTCTCTGAAGAATCTAAACCAAGTGG

The size of PCR amplicons was verified using agarose electrophoresis, and PCR products were processed using NucleoSpin® Gel and PCR Clean-up Kit.

The 40 µl of the PCR reaction were mixed with 80 µl of NTI buffer, loaded in the NucleoSpin® Column, and centrifuged for 30 seconds at 11,000 g. The flow-through was discarded, and 650 µl of NT3 buffer were added. The sample was centrifuged under the same conditions as in the previous step. To dry silica membrane, centrifugation was performed at 11,000 g for one minute to remove the remaining Buffer NT3. All columns were additionally incubated for five minutes at 70 °C. The NucleoSpin column was placed into a new tube and 35 µl of mQH<sub>2</sub>O were added to the center of the column, and further incubated for five-minute at RT. The samples were eluted by centrifugation at 11,000 g for two minutes.

#### 4.2.6 Electrophoresis

In 80 ml of the 1 % agarose in TAE buffer, 6 µl of GelRed was added. The agarose-GelRed mixture was poured into an electrophoresis gel caster, and wells for sample loading were prepared using a comb. The gel solidifies in approximately 10-15 minutes. The gel was placed into the electrophoresis chamber, and the chamber was filled with 1x TAE buffer. GeneRuler 1 kB Plus DNA Ladder was used as a marker. If a PCR reaction buffer did not contain any loading dye, 1,2 µl of TriTrack dye was added to 10 µl of each sample. The electrophoresis run was performed for 30 minutes at 100V. After 30 minutes, the gel was placed on a UV transilluminator tray, and the GelDoc EZ Gel Documentation system visualized the bands.

The 50x TAE contained 50 mM EDTA dipotassium salt, 2 M Tris-base, and 1 M glacial acetic acid. The pH was adjusted to 8,5 by KOH . TAE working buffer (1x) was prepared by mixing 20 ml of 50x TAE buffer with 980 ml of mQH<sub>2</sub>O.

#### 4.2.7 *Bsa*I/T4 ligation

The coding region of *AtGH3.3*, *AtGH3.4* and *AtGH3.17* was inserted into the *pGGC000* GreenGate entry vector by mixing 9  $\mu$ l of PCR product with 1,5  $\mu$ l of *pGGC000* plasmid, 1,5  $\mu$ l of 10x FD Buffer, 1  $\mu$ l of 10 mM ATP, 1  $\mu$ l of *Bsa*I and 1  $\mu$ l T4 DNA ligase. Samples were spun down and incubated in a thermocycler, with cycling parameters described in Table 3.

Table 3. Thermocycler parameters for *Bsa*I/T4 ligation

Temperature ( $^{\circ}$ C)	Time (minutes)	Cycles (number)
37	5	50
16	5	50
50	5	1
80	5	1
10	1 hour	

TOP 10 competent cells were transformed with the generated constructs, transformed bacterial cells were plated on LB media supplemented with carbenicillin and plates were incubated at 37  $^{\circ}$ C. Positive bacterial colonies were selected by colony PCR method (Table 4). Primers used for colony PCR were designed with the forward primer specific for each insert and the T7 reverse primer specific for the vector.

Table 4. The list of primers used for the colony PCR and Sanger sequencing of *pDrive* and *pGGC000* vectors harboring *AtGH3.3*, *AtGH3.4*, and *AtGH3.17* coding regions.

Primer name	Primer sequence
T7_prom_Rv	AATACGACTCACTATAGG
AtGH3.3_Fw	GACGTCCTAACGATCCGTACA
AtGH3.3_Rv	ATAACGGTTAAGCCCAGCATA
AtGH3.4_Fw	CTTCAGGACGTCGGATTCAGA
AtGH3.4_Rv	ACGGTAGAGCCCCGCGTAGGT
AtGH3.17_Fw	TTTCAGAAACAGACCATTCAA
AtGH3.17_Rv	ACAGAGTAGGCGATATTCTAA
SP6_prom_Fw	ATTTAGGTGACACTATAGA

#### 4.2.8 Colony PCR

For colony PCR, eight colonies were picked from selective plates to verify the presence of DNA insert in the plasmid harboring the resistance to the antibiotic. A small portion of the

colony surface was rubbed with the pipette tip. Then, the tip was incubated in the PCR tube containing the PCR reaction mixture: 4  $\mu\text{l}$  of 5x GoTaq Flexi Buffer, 3  $\mu\text{l}$  of  $\text{MgCl}_2$  (25  $\text{mmol}\cdot\text{l}^{-1}$ ), 0,4  $\mu\text{l}$  of dNTPs (10  $\text{mmol}\cdot\text{l}^{-1}$ ), 0,1  $\mu\text{l}$  of GoTaq G2 Flexi DNA polymerase, 10,5  $\mu\text{l}$  of  $\text{mQH}_2\text{O}$  and 1  $\mu\text{l}$  of each forward and reverse primers (Table 4). Pipette tips were removed and samples were incubated in a thermocycler following the cycling parameters described in Table 5.

Table 5. Thermocycler parameters for colony PCR program

Temperature ( $^{\circ}\text{C}$ )	Time (min)	Cycles (nr)
95	5	1
95	0:30	30
55	0:30	30
72	4	30
72	5	1
8	15	1

### 3.2.9 TA cloning

The amplified coding regions of *AtGH3.3*, *AtGH3.4* and *AtGH3.17* genes were cloned into a *pDrive* vector by TA cloning. Approximately 150 ng of amplified DNA (Table 8) was mixed with 1  $\mu\text{l}$  of *pDrive* vector (50 ng/ $\mu\text{l}$ ) and 5  $\mu\text{l}$  of 2x Ligation Master Mix. The mixture was spun down and incubated at 4  $^{\circ}\text{C}$  for 2 hours. After two hours, TOP10 competent cells were transformed with *pDrive* vectors containing coding regions of *AtGH3.3*, *AtGH3.4* or *AtGH3.17* genes. Transformed competent cells were plated on LB agar plates containing kanamycin, 1 mM IPTG and 20  $\mu\text{g}/\text{ml}$  X-Gal.

A colony PCR was carried out to verify the presence of *pDrive* plasmid carrying the desired insert in positive bacterial colonies. Primers used for colony PCR were designed with the forward primer specific for each insert and the T7 reverse primer specific for the vector (Table 4). For positive colonies, liquid cultures were prepared and corresponding plasmids were isolated. The plasmids, along with the primers listed in Table 4, were sent for Sanger sequencing at SEQme in Dobriř, Czech Republic. The sequencing reaction consisted of 5  $\mu\text{l}$  of purified plasmid, 2,5  $\mu\text{l}$  of  $\text{mQH}_2\text{O}$ , and 2,5  $\mu\text{l}$  of the specific primer.

### 3.2.10 Green Gate reaction

*pDrive-AtGH3.3*, *pDrive-AtGH3.4*, and *pDrive-AtGH3.17* plasmids were used as entry modules to assembly the final plant transformation vectors. The coding region of every gene was cloned under the control of the corresponding native promoter (pN, previously cloned),



followed by C-terminal GFP tag, plant terminator and plant resistance cassette (Table 6). For the assembly, modules and reagents were mixed in corresponding volumes: 1,5 µl of 10x FD Buffer, 1,5 µl of 10 mM ATP, 1,5 µl of *pGGA-pNAtGH3s* (100 ng/µl), 1,5 µl of *pGGB003* (100 ng/µl), 1,5 µl of *pDrive-AtGH3s* (concentrations are in Table 9), 1,5 µl of *pGGD001* (100 ng/µl), 1,5 µl of *pGGE001* (100 ng/µl), 1,5 µl of *pGGF005* (100 ng/µl), 1 µl of *pGGZ003* (100 ng/µl), 1 µl of *Bsal* (10 U/µl), 1 µl of T4 DNA ligase (30 U/µl). Afterward, samples were incubated in a thermocycler following the cycling parameters described in Table 3.

Table 6. Description of GreenGate modules

<b>GreenGate modules</b>	<b>Type</b>
pGGA-pNAtGH3s	Native promoter sequence of the specific gene
pGGB003	B-dummy (empty vector if no N-tag is needed)
pDrive-AtGH3s	coding region of the specific gene
pGGD001	GFP-linker
pGGE001	Terminator sequence
pGGF005	Plant resistance cassette
pGGZ003	Destination vector

Ligation reactions were used to transform competent cells, and transformed cells were plated on selective LB agar plates. To verify the correctness of the final assembly, colony PCR was performed on positive bacterial colonies using gene-specific Fw primer and eGFP Rv primer (Table 7). Confirmed positive colonies were grown in liquid LB medium, and corresponding plasmids were isolated. These plasmids, along with primers specified in Table 7, were subsequently sent for Sanger sequencing. The sequencing reaction consisted of 5 µl of purified plasmid, mixed with 2,5 µl of mQH2O and 2,5 µl of the primer.

Table 7. The list of primers used for colony PCR and Sanger sequencing of final transformation vectors

<b>Primer name</b>	<b>Primer sequence</b>
AtGH3.3_Fw	GACGTCCTAACGATCCGTACA
AtGH3.3_Rv	ATAACGGTTAAGCCCAGCATA
AtGH3.4_Fw	CTTCAGGACGTCGGATTCAGA
AtGH3.4_Rv	ACGGTAGAGCCCCGCGTAGGT
AtGH3.17_Fw	TTTCAGAAACAGACCATTCAA
AtGH3.17_Rv	ACAGAGTAGGCGATATTCTAA

Hygr_Fw	CTTGACCAACTCTATCAGAGC
Hygr_Rv	ATCGACCCTGCGCCCAAGCTG
E9_Fw	CGCTATCGAACTGTGAAATGG
E9_Rv	GCCTAGTGAATAAGCATAATGG
LB_T-DNA_Rv	TGGCAGGATATATTGTGGTGTA
RB_T-DNA_Fw	TTTACCCGCCAATATATCCTGTCA
eGFP_Fw	GCAGCGTGCAGCTCGCCGACC
eGFP_Rv	TTGTACAGCTCGTCCATGCCG
pUBQ10_Fw	AACAATACCCAAAGAGCTC
pUBQ10_Rv	ATACGAAATTGGGATCGAACG

#### 4.2.11 Competent cells transformation

TOP 10 competent cells were thawed on ice and gently mixed with 10 µl of the ligation reaction. After mixing, the tubes were kept in ice for 30 minutes. Afterward, the tube containing the mixture was heated at 42 °C for 45 seconds, placed on ice for two minutes, and resuspended with 800 µl of liquid LB medium. Bacteria were grown at 37 °C in a shaking incubator for 60 minutes. The competent cells were pelleted at 2000 g for 5 minutes. About 700 µl of supernatant was discarded, the remaining 100 µl was used to resuspend pellet and plated to LB agar plates supplemented with antibiotics. The plates were incubated at 37 °C overnight to allow bacterial growth. Single bacterial colonies were used for colony PCR and plasmid isolation.

*Agrobacterium*-competent cells were transformed by electroporation. Electroporation consisted of combining 50 µl of electrocompetent *agrobacteria* with 1 µl of either *pNAtGH3.3:GH3.3cdr:GFP:HygR*, *pNAtGH3.4:GH3.4cdr:GFP:HygR* or *pNAtGH3.17:GH3.17cdr:GFP:HygR* vectors. The mixture was transferred to an ice-cooled cuvette and subjected to electroporation at 1800 V. The cells were transferred to 1,5 ml tube with 1 ml of LB medium and incubated for 3 hours on a shaker at 28 °C. Subsequently, bacterial cells were plated on LB agar plates containing antibiotics (*i.e.*, spectinomycin, rifampicin, gentamicin, and tetracycline). After a 48-hour incubation at 28 °C, individual colonies were grown in liquid LB selective medium containing identical antibiotic mixture as mentioned above. A 10-ml liquid culture was prepared and incubated on a shaker for 24 hours at 28 °C.

#### 4.2.12 Floral dip method

Four-six weeks old *Arabidopsis* plants, ecotype Columbia (Col-0), still carrying unopened floral buds, were used for floral dip transformation. *Agrobacterium* cells harboring *pNAtGH3.3:GH3.3cdr:GFP:HygR*, *pNAtGH3.4:GH3.4cdr:GFP:HygR* or *pNAtGH3.17:GH3.17cdr:GFP:HygR* were grown overnight at 28 °C, 130 rpm in 200 ml of LB medium supplemented with spectinomycin, rifampicin, gentamycin, and tetracycline. The next day, the bacterial cultures were pelleted at 3000 g for 15 minutes, and pellet was resuspended in a 5% sucrose solution containing 0,03% Silwet L-77. Afterward, floral buds were dipped in the mixture for two minutes. Transformed plants were kept in the dark overnight. The next day plants were transferred to a growth chamber and grown under long-day conditions (16 h photoperiod; 22 ± 1 °C), and the floral dip was repeated after five days. Once siliques were dry, seeds were harvested.

#### 4.2.13 Selection of primary transformants (T<sub>1</sub>)

Seeds were sterilized by chlorine gas method. Tubes containing approximately 200 mg of seeds were placed with an open lid in a desiccator, and exposed to a mixture of 15 ml of mQH<sub>2</sub>O, 25 ml of bleach (NaClO) and 10 ml of 37% HCl for 2 hours. Sterilized seeds were transferred to the flowbox, and 1 ml of sterile mQH<sub>2</sub>O was added to each tube. Next, water was removed, 0,1 % agarose solution was added, and seeds were sowed on hygromycin B-containing half-strength MS plates. Plates were sealed and kept in the dark at 4 °C for 48 hours for stratification. Seeds were germinated under long-day conditions (16 h photoperiod; 22 ± 1 °C) in a growth cabinet. After ten days, the presence of hygromycin-resistant transformants was inspected. Hygromycin-resistant seedlings were larger than non-resistant seedlings, as hygromycin causes growth retardation of sensitive seedlings. Selected resistant seedlings were transferred to the soil, and grown under long-day conditions. Collected seeds represented the first generation of transformants (T<sub>1</sub>).

#### 4.2.14 Confocal microscopy

GFP signal from selected T<sub>1</sub> hygromycin-resistant seedlings harboring *pNAtGH3.4:GH3.4cdr:GFP:HygR* or *UBQ10:AtGH3.3-GFP:HygR* (positive control), and *A. thaliana* (Col-0) (negative control) was inspected by confocal microscopy. Ten-day-old seedlings grown under long-day conditions (16 h photoperiod, 22 ± 1 °C) were mounted on a glass slide with approximately 150 µl of mQH<sub>2</sub>O and with a coverslip. Visualization was conducted using a Zeiss confocal microscope with a 20x objective. The fluorescent signal was visualized as eGFP (excitation wavelength 488 nm, emission wavelength 509 nm).

## 5. Results

### 5.1 Genomic DNA extraction and PCR amplification

Genomic DNA extraction is a method aimed at isolating DNA from other cellular components. The isolated gDNA was quantified using a micro-volume Nanodrop spectrophotometer (Table 8). “gDNA\_1” and “gDNA\_2” samples were extracted by E.Z.N.A. ® Plant DNA kit, while “gDNA\_3” and “gDNA\_4” samples were extracted by genomic DNA extraction buffer.

The  $A_{260/280}$  and  $A_{260/230}$  values are critical to determine the DNA quality, as they refer to DNA purity and contaminants presence. The  $A_{260/280}$  ratio is used to evaluate residual protein contamination from the nucleic acid isolation process, as nucleic acids absorb at 260 nm and proteins absorb at 280 nm. Absorption at 230 nm can indicate contamination by organic compounds such as phenolate ions or thiocyanates. An  $A_{260/280}$  ratio of approximately 1,8 is considered indicative of "pure" DNA. Expected 260/230 absorbance values would typically range from 2,0 to 2,2.

The highest DNA concentrations were obtained for DNA extracted using genomic DNA extraction buffer (gDNA\_3 = 231,6 ng/µl, gDNA\_4 = 114,4 ng/µl), while “gDNA\_1” and “gDNA\_2”, isolated with E.Z.N.A. ® Plant DNA kit, exhibited approximately three times lower concentrations. The “gDNA\_2” sample displayed the best  $A_{260/280}$  ratio with a value of 1,74, whereas the lowest ratio was observed for “gDNA\_4” with 1,51. The  $A_{260/230}$  ratio values were relatively low for all samples, ranging from 0,22 for “gDNA\_4” to 1,3 for “gDNA\_2”.

Table 8. gDNA concentrations and purity, isolated from *A.thaliana Col-0* plants.

Name	Concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
gDNA_1	50,2	1,87	0,59
gDNA_2	78,7	1,74	1,30
gDNA_3	231,6	1,65	0,26
gDNA_4	114,4	1,51	0,22

PCR amplification was carried out to amplify the coding regions of *GH3.3*, *GH3.4*, *GH3.17* genes. Primers contained *BsaI* restriction sites, and the stop codon was removed for the reverse primers (Figure 5). To check the integrity of the isolated DNA, electrophoresis

on agarose gel was performed (Figure 6). The expected sizes of the *GH3.3*, *GH3.4*, and *GH3.17* amplicons were 2410 bp, 2154 bp, and 2446 bp, respectively. Figure 6 displays bands corresponding to the estimated sizes of the DNA amplicons. However, there are also additional bands, which could correspond to non-specific amplification, DNA contamination, or other experimental artifacts.

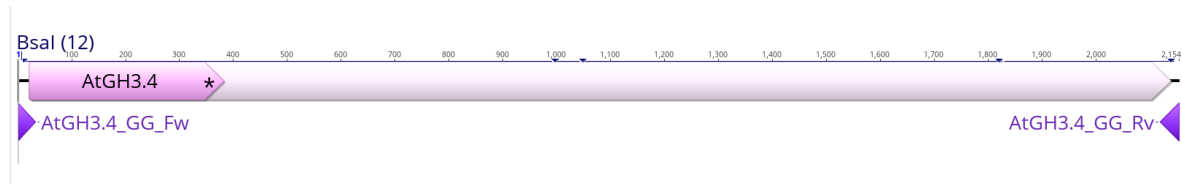


Figure 5. Representation of the *GH3.4* PCR product, created in Geneious Prime. Overhanging primers were used for the addition of the *Bsal* restriction site to both ends of the gene.

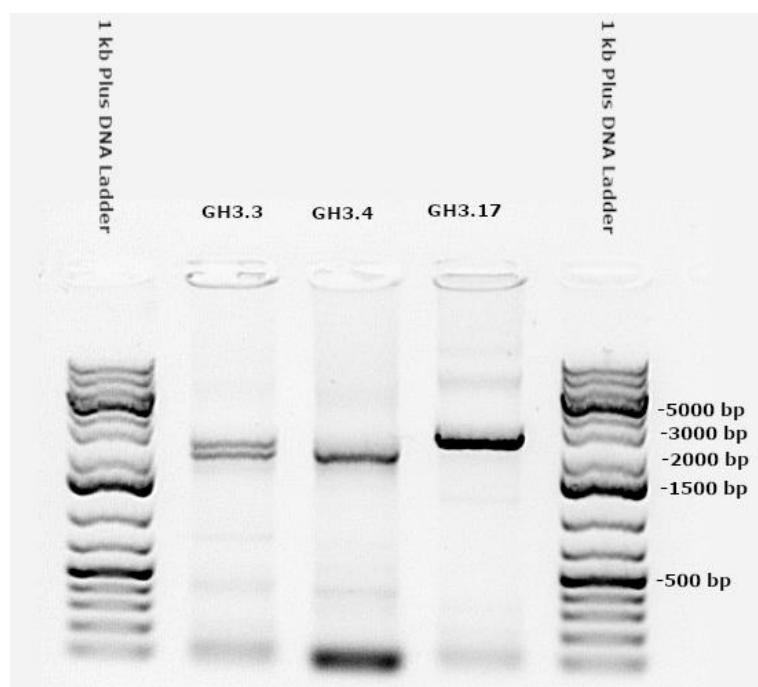


Figure 6. Agarose gel electropherogram of amplified coding regions. Lane 2, *AtGH3.3*; Lane 3, *AtGH3.4*; Lane 4, *AtGH3.17*. Lane 1 and 5, 1 kb Plus DNA Ladder (marker).

## 5.2 *Bsa*I/T4 ligation in *pGGC000* Green Gate module

Plasmid *pGGC000* carries resistance to ampicillin and was used as the GreenGate module to carry inserts. Insertion of the three genes *GH3.3*, *GH3.4*, *GH3.17* in respective vectors was performed by *Bsa*I/T4 ligation (Attachments 1-2, Figure 7).

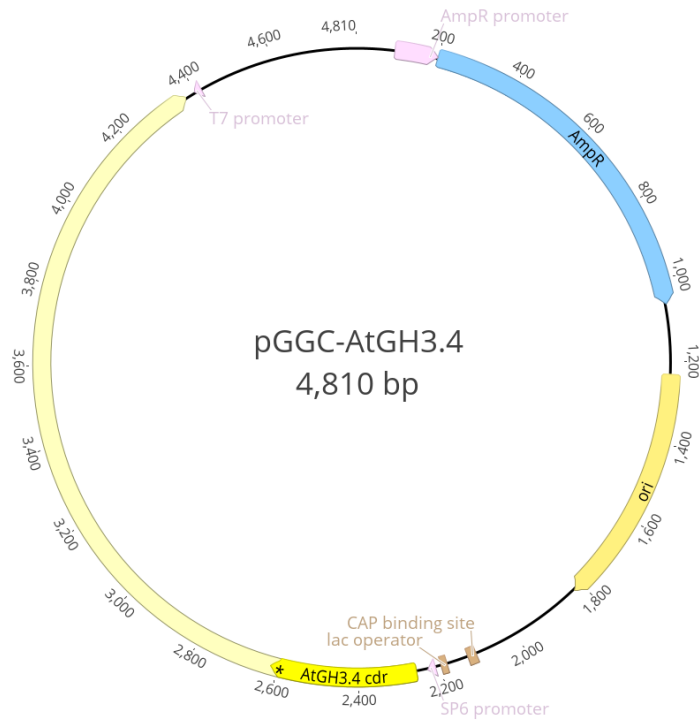


Figure 7. Representation of pGGC000 plasmid with inserted AtGH3.4 gene, created in Geneious Prime. The plasmid is 4,810 bp and carries the origin of replication ORI, lac operator (required for lactose metabolism), CAP binding site (positive regulator of lac operon), SP6 promoter, AtGH3.4 inserted gene sequence, T7 promoter, and ampicillin resistance cassette.

Colony PCR was conducted on transformed bacteria to confirm the presence of the target gene in the recombinant plasmid. Results of colony PCR were visualized by electrophoresis (Figure 8). The expected size of the amplicons was around 2000 bp. Multiple bands corresponding to the expected size were detected for all three constructs on the agarose gel. Besides them, there were additional bands around 5000 bp and 500 bp. However, Sanger sequencing confirmed unsuccessful transformation.

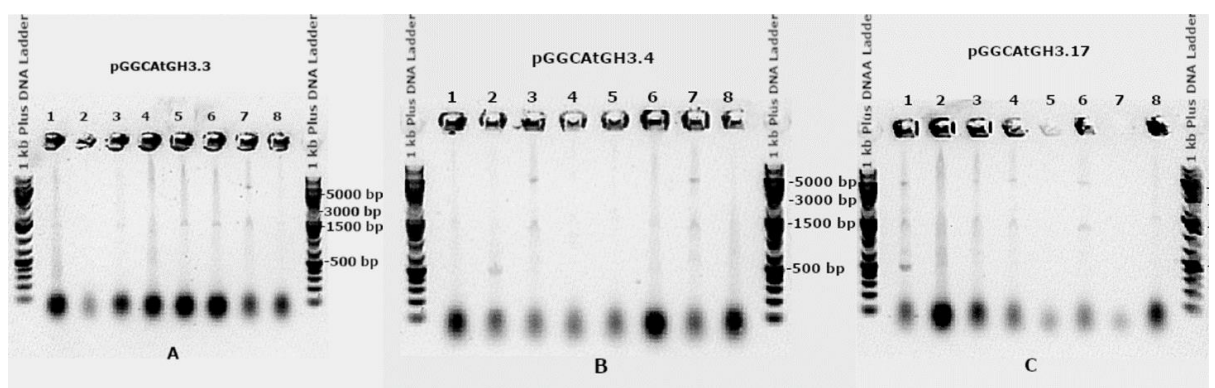


Figure 8. Agarose gel electropherograms of pGGCAtGH3.3 (A), pGGCAtGH3.4 (B), and pGGCAtGH3.17 (C) PCR amplicons. In each electropherogram, the 1 kb Plus DNA Ladder (marker) was loaded in the first and last lanes. Lanes 2-9 exhibit colony-PCR products obtained from transformed TOP 10 E.coli colonies grown on LB medium.

### 5.3 TA cloning with *pDrive*

TA cloning method is used to insert amplified DNA fragments harboring A-overhangs directly into a linearized vector. Coding regions of three distinct genes *AtGH3.3*, *AtGH3.4*, and *AtGH3.17* were inserted into *pDrive* vectors (Attachments 3-4, Figure 9).

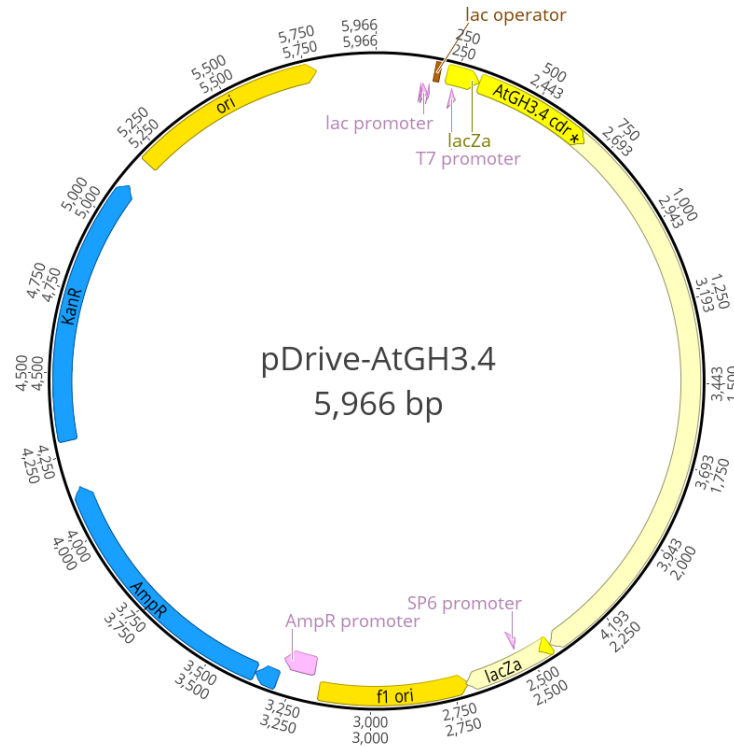


Figure 9. Representation of *pDrive* vector with inserted *AtGH3.4* gene, created in *Geneious Prime*. The plasmid is 5,966 bp and carries the origin of replication *ORI*, *f1 ORI*, *lac operator* with *LacZa* (required for lactose metabolism), *CAP binding site* (positive regulator of *lac operon*), *SP6 promoter*, *AtGH3.4* inserted gene sequence, *T7 promoter*, *ampicillin*, and *kanamycin resistance*

To verify the presence of target genes in a plasmid, transformed bacteria were subjected to colony PCR. Eight white colonies from every construct (*pDrive-AtGH3.3*, *pDrive-AtGH3.4*, *pDrive-AtGH3.17*) were inspected (Figure 10). Results of colony PCR were visualized by electrophoresis (Figure 11). The transformation was successful, as amplicons of the expected size (around 2000 bp) were obtained from all picked colonies for each construct. However, colonies 3 and 4 from *pDrive-AtGH3.17*-transformed *E.coli* population did not return a clear amplicon. The electropherogram of *pDrive-AtGH3.3* (Figure 11A)

illustrated several additional bands around 1000 bp. The first positive colony was selected for further experiments for all three constructs.

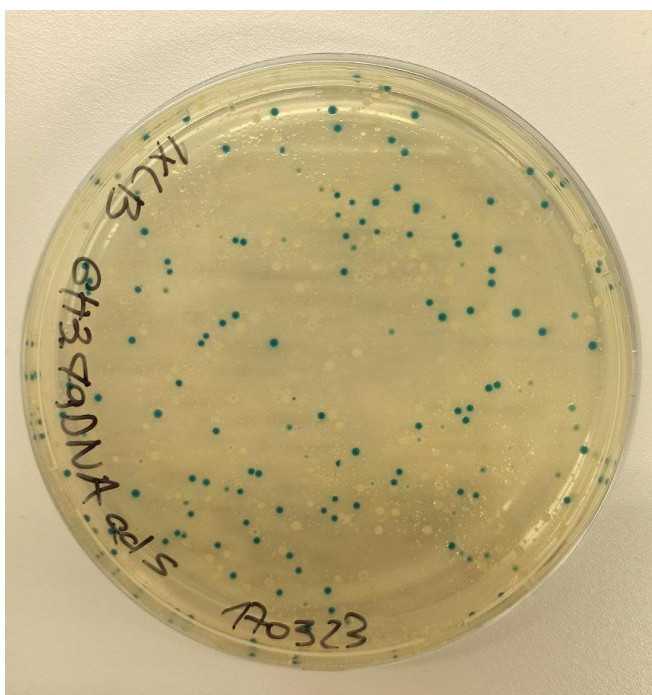


Figure 10. The example of blue-white screening for transformed TOP10 competent cells carrying *pDrive-AtGH3.4*. The colonies carrying recombinant protein appear white.

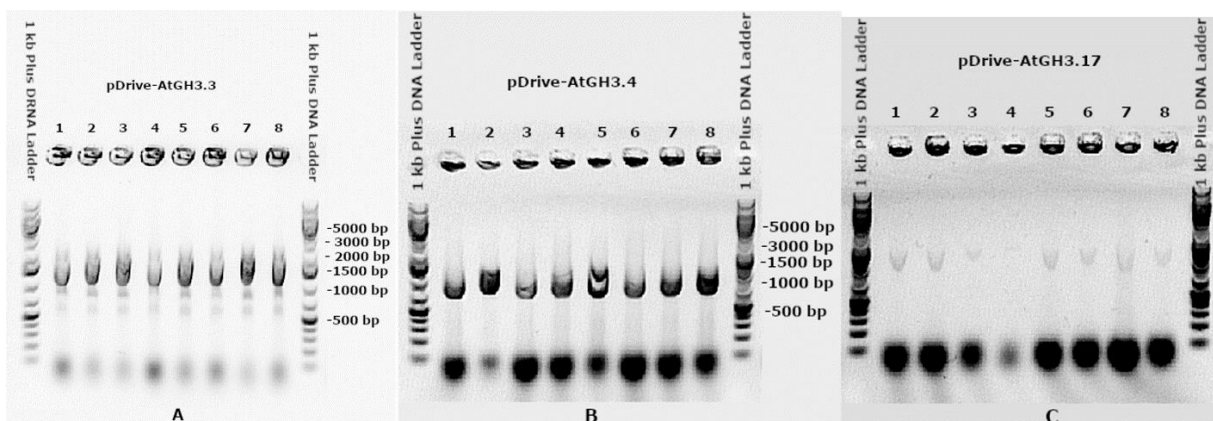


Figure 11. Agarose gel electropherograms of *pDrive-AtGH3.3* (A), *pDrive-AtGH3.4* (B), and *pDrive-AtGH3.17* (C) PCR amplicons. In each electropherogram, the 1 kb Plus DNA Ladder (marker) was loaded in the first and last lanes. Lanes 2-9 show colony-PCR products obtained from transformed TOP 10 *E.coli* colonies grown on LB medium.

The DNA concentration and purity of plasmids containing the gene inserts was quantified to ensure optimal sequencing results (Table 9). The *pDrive-AtGH3.3* plasmid exhibited the highest concentration at 409,2 ng/ $\mu$ l, whereas the lowest concentration was observed for *pDrive-AtGH3.17* at 253,3 ng/ $\mu$ l. In terms of purity, all three vectors displayed an average  $A_{260/280}$  ratio of 1,85, indicating their high purity. The *pDrive-AtGH3.3* plasmid also demonstrated the best  $A_{260/230}$  purity ratio.



Table 9. Recombinant *pDrive* vectors' concentrations and purity

Name	Concentration (ng/μl)	A260/280	A260/230
pDrive-AtGH3.3	409,2	1,85	2,20
pDrive-AtGH3.4	342,5	1,87	2,32
pDrive-AtGH3.17	253,3	1,84	2,19

Sequencing results confirmed the presence of the target gene sequences within the recombinant plasmids without nucleotide alterations, thereby validating the efficiency of TA cloning (Attachment 5-7). Figure 12 illustrates sequencing results of the *GH3.4* coding region, inserted to the *pDrive* vector.

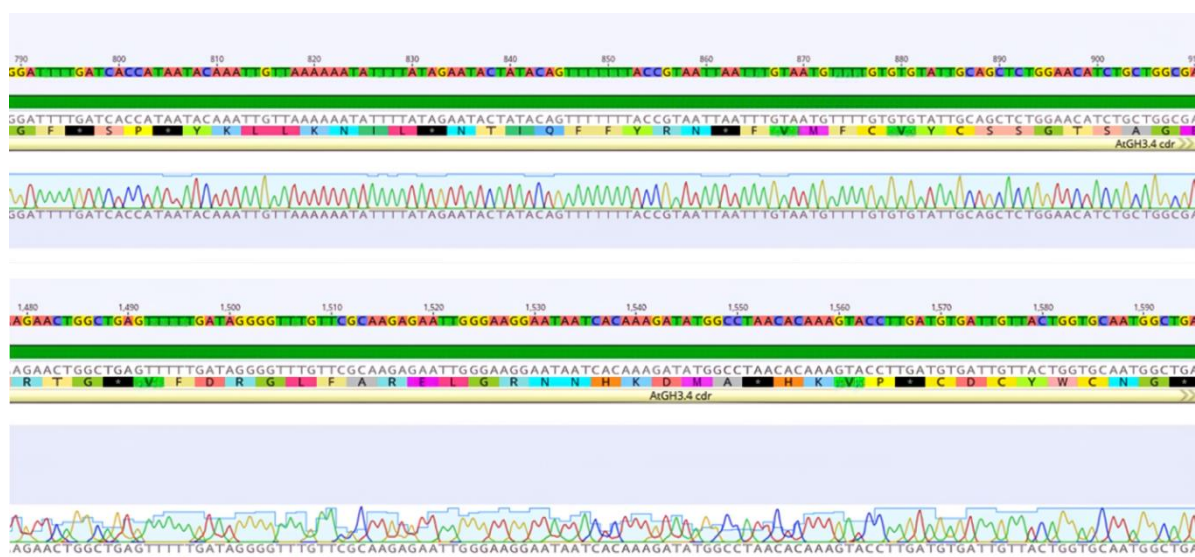


Figure 12. Representation of the *GH3.4* gene sequence inserted into *pDrive* vector, created in Geneious Prime.

## 5.4 Green Gate cloning

Green Gate cloning is a technique that allows assembling several DNA elements into a single destination vector. Six entry vectors (*pGGA-pNAtGH3s*, *pGGB003*, *pDrive-AtGH3s*, *pGGD001*, *pGGE001*, *pGGF005*) were used in a reaction with the destination vector *pGGZ003* carrying spectinomycin resistance to generate the final assembly vector (Attachments 8-9, Figure 13).

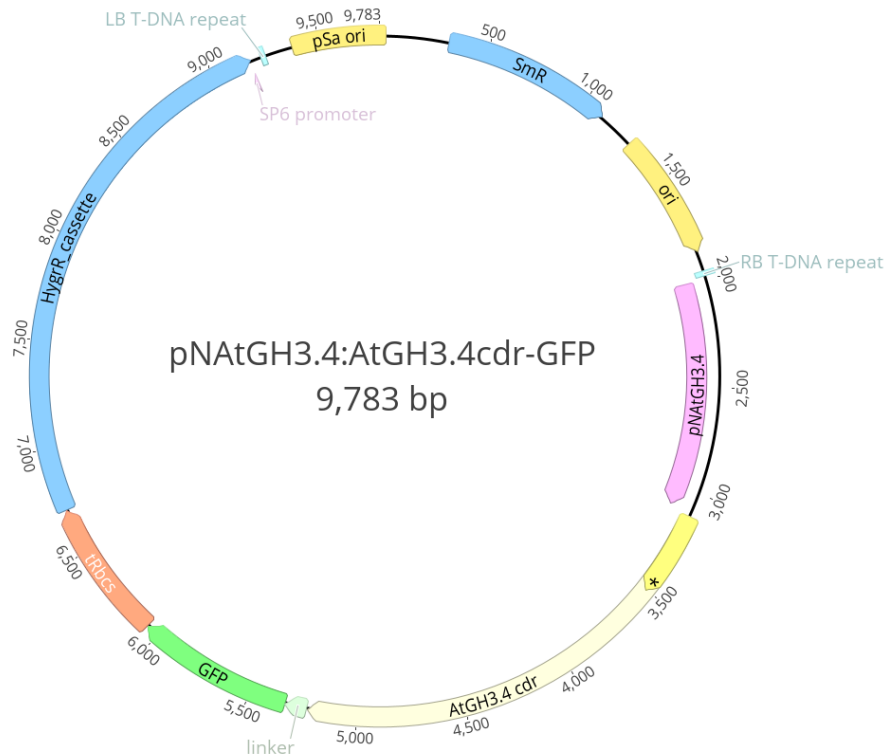


Figure 13. Representation of *pNAtGH3.4:AtGH3.4cdr-GFP* vector, created in *Geneious Prime*. The plasmid is 9,783 bp and carries origin of replication ORI, native promoter *pNAtGH3.4*, *AtGH3.4* inserted gene sequence, GFP sequence with a linker, *tRbcs* (terminator), *HygrR* (hygromycin resistance cassette), *pSa ori* (origin of replication for bacteria), *SmR* (spectinomycin resistance cassette)

To confirm the correct assembly of the plant transformation vector, colony PCR was carried out. Eight colonies from each construct (*pNAtGH3.3:AtGH3.3cdr-GFP*, *pNAtGH3.4:AtGH3.4cdr-GFP*, *pNAtGH3.17:AtGH3.17cdr-GFP*) were selected. The results of the colony PCR were visualized by electrophoresis (Figure 14). The expected amplicon size was approximately 2000 bp for all three constructs, and amplicons of this size indicated possible successful assembly. Figure 14 shows that each colony from each construct returned a band of expected size, confirming the efficiency of the Green Gate cloning method. For further experiments the first positive colony of each construct was inoculated, plasmid was isolated and sent for sequence validation.

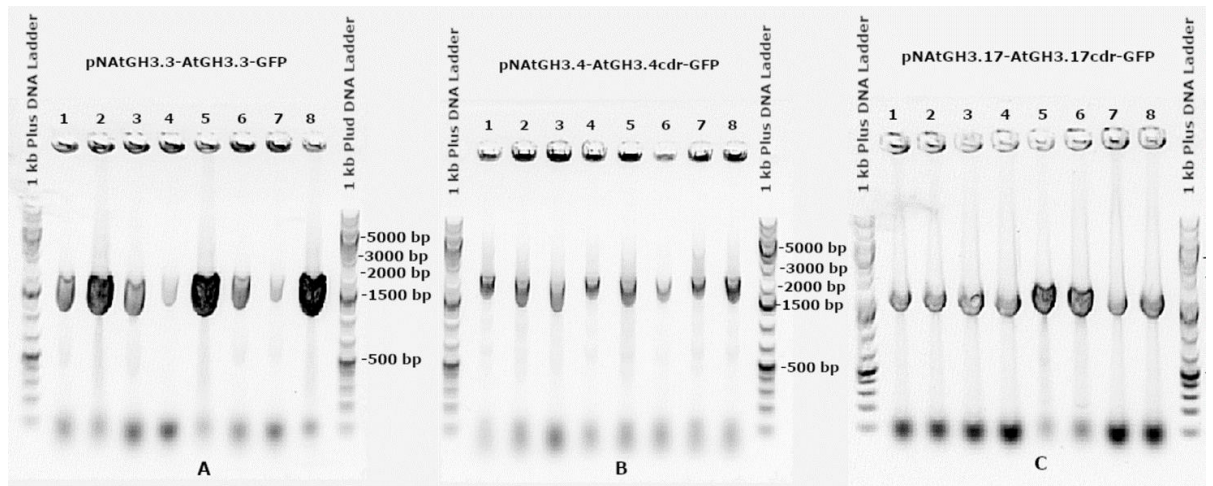


Figure 14. Agarose gel electropherograms of *pNAtGH3.3:AtGH3.3cdr-GFP* (A), *pNAtGH3.4:AtGH3.4cdr-GFP* (B), and *pNAtGH3.17:AtGH3.17cdr-GFP* (C) PCR amplicons. In each electropherogram, the 1 kb Plus DNA Ladder (marker) was loaded in the first and last lanes. Lanes 2-9 exhibit colony-PCR products obtained from transformed TOP 10 *E.coli* colonies grown on LB medium.

Concentrations and purity of recombinant vectors *pNAtGH3.3:AtGH3.3cdr-GFP*, *pNAtGH3.4:AtGH3.4cdr-GFP*, and *pNAtGH3.17:AtGH3.17cdr-GFP* were validated to ensure optimal sequencing results (Table 10). The *pNAtGH3.3:AtGH3.3cdr-GFP* vector had the highest concentration at 927,6 ng/μl, while the *pNAtGH3.4:AtGH3.4cdr-GFP* vector had the lowest concentration at 475,7 ng/μl. The purity ratios for all three constructs showed minimal deviation, with an average  $A_{260/280}$  value of 1,85, and an average  $A_{260/230}$  value of 2,3.

Table 10. Final Green Gate recombinant *pGGZ003* vectors' concentrations and purity

Name	Concentration (ng/μl)	$A_{260/280}$	$A_{260/230}$
<i>pNAtGH3.3:AtGH3.3cdr-GFP</i>	927,6	1,87	2,34
<i>pNAtGH3.4:AtGH3.4cdr-GFP</i>	475,7	1,90	2,40
<i>pNAtGH3.17:AtGH3.17cdr-GFP</i>	502,0	1,77	2,10

Sequencing results confirmed the correctness of the assembly of all modules and the sequence (Attachments 10-21). Figure 15 illustrates sequencing results of the *pNAtGH3.4:AtGH3.4cdr-GFP* vector, highlighting the correct assembling of the *GH3.4* coding region and GFP coding sequence.

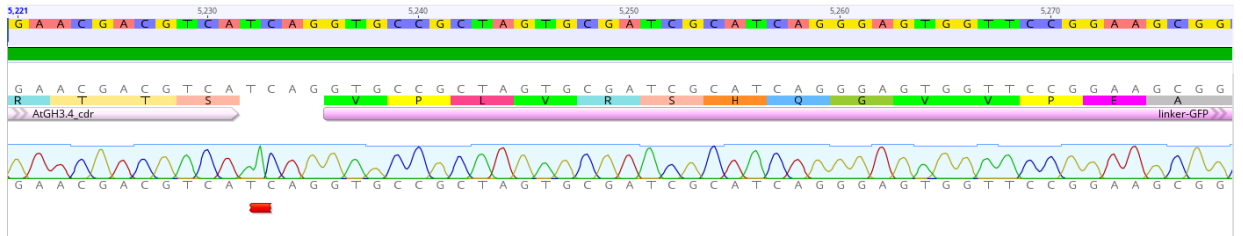


Figure 15. Representation of the GH3.4 gene sequence, assembled to a GFP sequence via the GFP linker, created in Geneious Prime.

## 5.5 Selection of transformants

Selection of transformed seeds was performed on  $\frac{1}{2}$  MS plates supplemented with 30  $\mu\text{g/ml}$  hygromycin B. After a ten-day growth period, the identification of hygromycin-resistant seedlings was based on cotyledon size and vigor. Seedlings exhibiting robust growth and larger cotyledons were considered as transgenic. Larger seedlings were observed in *pNA<sub>t</sub>GH3.4:AtGH3.4cdr-GFP* plant population. These seedlings were transferred to the soil for continued growth and development (Figure 16). In contrast, no transformants were detected for the plants transformed with *pNA<sub>t</sub>GH3.3:AtGH3.3cdr-GFP* and *pNA<sub>t</sub>GH3.17:AtGH3.17cdr-GFP*. The seeds collected from this initial screening give rise to the first generation of transformants T<sub>1</sub>.



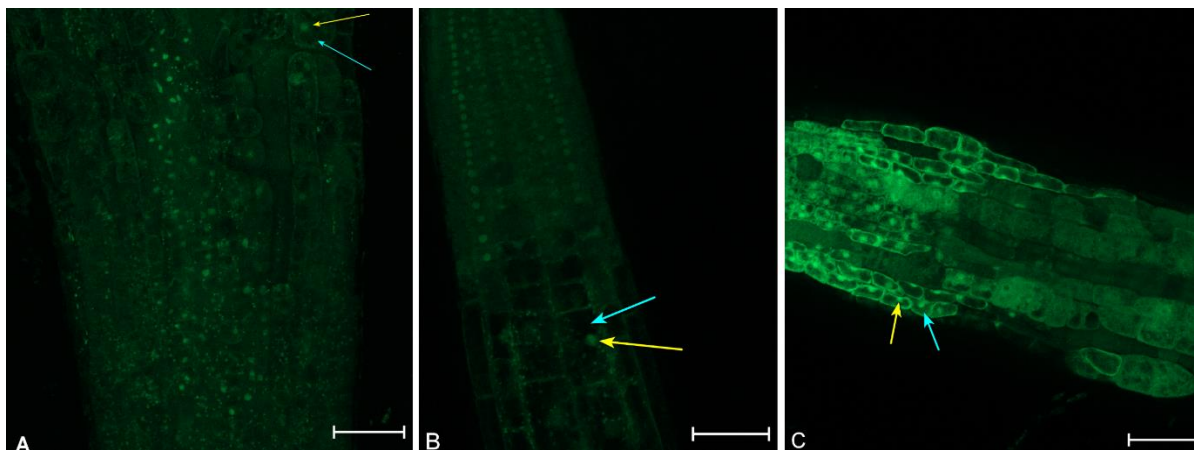
Figure 16. Selection of T<sub>0</sub> transformants on  $\frac{1}{2}$  MS round plates supplemented with 30  $\mu\text{g/ml}$  hygromycin B. Transformant harboring *pNA<sub>t</sub>GH3.4:AtGH3.4cdr-GFP* construct is highlighted by an orange circle. The transgenic seedling displayed a different morphology to non-transformant seedlings, such as larger size, hypocotyl, and cotyledon growth.

## 5.6 Confocal imaging

Confocal microscopy was used to visualize and compare the fluorescence signals from the negative control Col-0, a transgenic line expressing the *pNA<sub>t</sub>GH3.4:AtGH3.4cdr-GFP*, and the positive control *UBQ10::AtGH3.3-GFP* (Figure 17). All images were obtained using uniform imaging parameters to ensure comparability across the samples.

The GFP fluorescence signal was detected in the positive control, confirming the successful expression of the transgene and validating the reliability of the imaging setup. In contrast, the negative control samples did not show any GFP fluorescence signal, indicating the absence of the transgene and the specificity of the observed signal.

Confocal imaging of the T<sub>1</sub> generation of the *pNAtGH3.4:AtGH3.4cdr-GFP* transgenic line revealed a fluorescence profile similar to the negative control, meaning that the GFP fluorescence was not detected in the transgenic line.



*Figure 17. Confocal images from negative Col-0 control (A), pNAtGH3.4:AtGH3.4cdr-GFP (B), and positive control UBQ10-GH3.3-GFP (C) in 10-day seedlings of A.thaliana. Observed with standard settings at 20 times magnification. The yellow arrow (↑) indicates nucleus, blue arrow (↑) indicates cytosol. Scale bar is 50 μm.*

## 6. Discussion

Auxin is an essential plant hormone, whose metabolism is tightly regulated by several mechanisms, such as biosynthesis, degradation, and inactivation. This thesis focused on three *Arabidopsis* proteins belonging to the GRETCHEN HAGEN 3 family that directly play a role in the conjugation of auxin to amino acids, which leads to temporary auxin inactivation<sup>87</sup>. Since the first *GH3* gene was isolated from soybean in 1984<sup>88</sup>, not all the genes from this family have been fully characterized. This diploma thesis contributed to the creation of three plant transformation vectors containing the coding regions of three *Arabidopsis* genes *GH3.3*, *GH3.4*, and *GH3.17*. These genes were placed under their corresponding native promoters, followed by a C-terminal GFP tag, plant terminator, and plant resistance cassette. Those vectors were used to transform *A.thaliana* Col-0 plants, leading to a selection of transformed progeny for the *pNAtGH3.4:AtGH3.4cdr-GFP* line. The transgenic line underwent confocal microscopy analysis, providing data that might contribute to understanding the native localization of the GH3.4 protein in *Arabidopsis*.

In the first part of this thesis, the experiments were performed to prepare plant transformation vectors. The first step was to extract the gDNA from *Arabidopsis* leaves using gDNA extraction buffer. Although the extracted DNA had sufficient concentrations (231,6 ng/μl and 114,4 ng/μl), the purity of extracted DNA was low and DNA amplification of selected gene region did not lead to a result. According to P. Desjardins<sup>89</sup>, pure nucleic acids usually have a 260/280 ratio of around 2.0 and 260/230 typically ranges from 1.8 to 2.2. The sufficient purity of extracted DNA was accomplished by using the E.Z.N.A.® Plant DNA Kit (Table 8). DNA extracted by this method was used in downstream applications.

Onwards, extracted gDNA was used as a template for PCR amplification of the coding regions of *GH3.3*, *GH3.4* and *GH3.17* genes. The genes of interest were amplified using overhanging primers that contained *BsaI* recognition sites (Figure 5). Additionally, for reverse primers, the stop codon was removed, to ensure GH3-GFP fusion proteins in frame translation. PCR products were checked *via* agarose gel electrophoresis, which visualized bands corresponding to the expected sizes of the DNA amplicons (Figure 6). Amplified DNA was used for the *BsaI*/T4 ligation cloning with a *pGGC000* vector. This entry vector advantageously had T7 and SP6 bacteriophage promoters and *lac* operon for the blue-white screening<sup>57</sup> (Attachments 1-2, Figure 7). The prepared vectors were first inserted into TOP 10 *E.coli*, and transformation was verified by colony PCR and sequencing. The amplicons were expected to be roughly 2000 bp. On the agarose gel, bands of the expected size were observed in all three cases (Figure 8). Besides bands of expected sizes, additional bands

around 5000 bp and 500 bp were also observed on electropherograms. Those bands could appear due to incomplete DNA amplification cycles, which can be the result of shorter DNA fragments, such as primer dimers, heteroduplexes, chimeras or hairpin structures<sup>90</sup>. Another possible reason of unspecific bands is DNA thermal damage<sup>91</sup>. There are three main negative effects of thermal damage: A+G depurination, the oxidation of guanine to 8-oxoG, and cytosine deamination to uracil. In addition to causing incorrect nucleotide incorporation during replication, high temperatures increase DNA degradation, resulting in lower yield<sup>91</sup>. Sanger sequencing confirmed that the transformation was unsuccessful. During the second repetition of the same experiment, troubleshooting steps were undertaken. This included optimizing the concentration of the gDNA amplicon, using unopened buffers, and preparing fresh solutions (dNTPs). Additionally, a different competent cell strain for transformation was tested; TOP 10 was used for the first trial, while NEB5 $\alpha$  for the second attempt. The troubleshooting steps did not have an effect on the outcome of the cloning. The exact reason for the unsuccessful outcome remained unclear. The most frequent reasons of unsuccessful cloning are damaged template or a template with low purity<sup>92</sup>, irrelevantly chosen polymerases and restriction endonucleases<sup>93</sup>, long sequence of the cloning gene and repeated sequences<sup>94</sup>.

Following the first unsuccessful cloning attempt, a different strategy was adopted. A TA cloning with a *pDrive* vector was tested. This vector also had T7 and SP6 bacteriophage promoters and *lac* operon, but contained kanamycin and ampicillin resistance cassettes (Attachments 3-4, Figure 9). Taq-polymerase amplified coding regions of three genes with T-overhangs were inserted into *pDrive* vectors with complementary A-overhangs. The ligation products were then transformed into the TOP 10 competent cells, followed by blue-white screening. Screening divided all bacterial colonies into two groups: white - recombinant ones and blue – non-recombinant ones (Figure 10). White colonies were picked for colony PCR. Results of the colony PCR were visualized by agarose gel electrophoresis, and amplicons were observed as bands around 2000 bp for each vector (Figure 11). Additional bands of unexpected size were considered impurities or false primers' aligning<sup>95</sup>. Sanger sequencing confirmed the successful transformation (Attachments 5-7, Figure 12).

The subsequent step involved GreenGate cloning method, which was chosen by efficiency in saving time and effort, since both digestion and ligation steps carry out simultaneously in a single-tube reaction<sup>57</sup>. Comparing to conventional cloning methods, such as traditional restriction enzyme cloning, Gateway cloning or TOPO cloning, GreenGate allows to assemble six modules at the same time with no need to purify insert DNA fragments and vector<sup>57</sup>. GreenGate was successfully used in gene expression analysis<sup>96</sup>,

protein subcellular localization<sup>97</sup>, and generating transgenic lines<sup>98</sup>. The *pDrive* vectors containing the coding regions of either *GH3.3*, *GH3.4* or *GH3.17* were used as alternative module C and assembled with *pGGA-pNAtGH3s* (native promoter for each gene), *pGGB003*, *pGGD001* (GFP tag), *pGGE001*, and *pGGF005* into the *pGGZ003* destination vector using *BsaI/T4* ligation cloning<sup>57</sup> (Attachments 8-9, Figure 13). TOP 10 competent cells were transformed with the final assembly vectors *pNAtGH3.3:AtGH3.3cdr-GFP*, *pNAtGH3.4:AtGH3.4cdr-GFP*, and *pNAtGH3.17:AtGH3.17cdr-GFP*, and subsequently subjected to colony PCR. Electrophoresis visualized the colony PCR results and confirmed the presence of the expected bands of 2000 bp for each vector (Figure 14). Three final vectors were sent for sequencing, which confirmed the correct assembly and sequence of *pNAtGH3.3:AtGH3.3cdr-GFP*, *pNAtGH3.4:AtGH3.4cdr-GFP*, *pNAtGH3.17:AtGH3.17cdr-GFP* (Attachments 10-21, Figure 15).

Three final vectors were transformed to the *Agrobacterium* electrocompetent cells. Plant transformation mediated by *Agrobacterium* through the floral-dip method is a widely used plant transformation technique, characterized by its simplicity, high-efficiency with reduced time and non-destructivity for plants<sup>99</sup>. Thus, transformation with the generated constructs of Col-0 *Arabidopsis* plants with unopened floral buds (4-6 weeks) was performed by floral dip. Seeds resulting from this transformation belonged to the T<sub>0</sub> generation and underwent a selection process, based on hygromycin-B antibiotic resistance. The genetically modified seedlings distinguished from non-transgenic seedlings by their larger size, hypocotyl and cotyledon growth, and overall resilience<sup>100</sup>(Figure 16). Transformation on *Arabidopsis* with the the *pNAtGH3.4:AtGH3.4cdr-GFP* vector was successful, T<sub>0</sub> transgenic seedlings were selected and cultivated until the subsequent T<sub>1</sub> generation of seeds was collected. *Arabidopsis* plants with inserted *pNAtGH3.3:AtGH3.3cdr-GFP* and *pNAtGH3.17:AtGH3.17cdr-GFP* vectors did not contain any recombinant seedlings, so the transformation process was repeated. Despite several floral dip repetitions, these lines failed to produce transgenic progeny. Study of the S. De Buck<sup>101</sup> states that floral dip transformation yields an average 4-6 recombinant T-DNA copies *per* transformant. Additionally, transgene after transformation can undergo different unexpected modifications, leading to gene silencing. The most well-studied silencing is homology-dependent gene silencing (HDGS)<sup>102</sup>. This phenomenon suggests that homology between the transgene, and either the native gene or another transgene leads to gene silencing. All of the above together with bad plant condition before transformation, such as weak or stressed plants, can be considered as the reason of the lack of the transgenic lines for *pNAtGH3.3:AtGH3.3cdr-GFP* and *pNAtGH3.17:AtGH3.17cdr-GFP* vectors.



Inspection of the GFP signal in the generated transgenic line by confocal laser scanning microscopy represented the last step of this thesis. Detection was performed with 10-day *Arabidopsis* seedlings of T<sub>1</sub> generation expressing *pNAtGH3.4:AtGH3.4cdr-GFP*. As positive control, *UBQ10::AtGH3.3-GFP* stable transgenic line was used, and as negative control *Arabidopsis* Col-0 was used (Figure 17). Highly intense GFP signal in roots was observed in the positive control, confirming the overexpression of the fusion protein due to constitutive promoter. Constitutive promoters ensure stable gene expression consistently across different tissues and developmental stages. They are especially beneficial in fluorescent microscopy of transgenes, as they provide prolonged expression of the fluorescently-tagged proteins<sup>103</sup>. Alongside, negative control didn't show any specific fluorescence signal besides autofluorescence, which is consistent with the expected result because the plants did not contain any GFP tag. Autofluorescence is the natural ability of certain biomolecules to emit fluorescent light when excited by specific wavelengths of light. Chlorophyll and multiple cell wall components can exhibit autofluorescence after excitation by ultraviolet, blue or green light<sup>104</sup>. The T<sub>1</sub> generation of plants transformed with the *pNAtGH3.4:AtGH3.4cdr-GFP* vector did not show any fluorescence signal besides autofluorescence, similar to the negative control, suggesting a lack of GFP signal in the transformed plant line. Generally, GFP signal may not be observed under a native promoter due to low protein expression levels, inappropriate observation timing and protein localization, unexpected protein post-translational modifications, or limitations of detection methods<sup>105</sup>. According to the *Arabidopsis* gene expression map (*Arabidopsis* eFP Browser)<sup>106</sup>, the expression levels of the GH3.4 protein significantly vary across different stages of seed development. The main purpose of this diploma thesis was to inspect the localization of GH3s proteins, which was done on 10-day seedlings. At this developmental stage, GH3.4 protein expression in the root is lower compared to other stages. This could explain the low GFP signal detection by confocal microscopy, even though the seeds harbored a recombinant vector with a GFP tag. However, the *pNAtGH3.4:AtGH3.4cdr-GFP* transgenic line might have an important role in studying the key pathways of *Arabidopsis* and its response under different conditions. Changes in hormone levels during stress (surplus/the lack of water, chemicals, etc.) trigger the expression of other proteins in the plant that should help it survive the adverse conditions. Recent studies on ARFs group<sup>107</sup> of proteins and CLE9 peptide<sup>108</sup> show that they change their expression levels as a response to gradient concentration of abscisic acid, hydrogen peroxide, nitric oxide, and salt (NaCl) treatments. Using the tagged GH3.4 protein, it is possible to track its expression levels under

different conditions, which might extend the understanding of IAA metabolism and the possible interaction with other plant hormones.

## 7. Conclusion

In the theoretical part of the diploma thesis, in-depth literature review was conducted on auxins, particularly on biosynthesis, degradation, and inactivation mechanisms mediated by GH3s enzymes. The latter part of the theoretical section mainly explored cloning strategies and confocal laser microscopy.

The experimental section consisted of several steps. The initial step involved extracting gDNA from *Arabidopsis* using the E.Z.N.A.® Plant DNA Kit and a gDNA extraction buffer. It was experimentally demonstrated that the E.Z.N.A.® Plant DNA Kit yielded better results compared to the gDNA extraction buffer. The main goal of the subsequent step was to incorporate three *Arabidopsis* genes (*GH3.3*, *GH3.4*, *GH3.17*) into the *pGGC000* Green Gate module using *BsaI/T4* ligation.

After an unsuccessful transformation attempt, the cloning method was changed to TA cloning with a *pDrive* vector. This change led to the creation of three Green Gate entry vectors: *pDrive-AtGH3.3cdr*, *pDrive-AtGH3.4cdr*, and *pDrive-AtGH3.17cdr*. Subsequently, those vectors served as modules for the creation of final GreenGate assembly vector with a GFP tag: *pNAtGH3.3:AtGH3.3cdr-GFP*, *pNAtGH3.4:AtGH3.4cdr-GFP*, *pNAtGH3.17:AtGH3.17cdr-GFP*. The *Arabidopsis* transformation was achieved through the *Agrobacterium*-mediated floral dip method, with *Agrobacterium* carrying the corresponding vectors. The experiment succeeded in creation of a *pNAtGH3.4:AtGH3.4cdr-GFP Arabidopsis* transgenic line. No transgenic lines were obtained with the two other vectors.

Ten-day-old seedlings from the T<sub>1</sub> generation of *Arabidopsis* expressing *pNAtGH3.4:AtGH3.4cdr-GFP*, along with the positive control *Arabidopsis* expressing *UBQ10::AtGH3.3-GFP* and the negative control *Arabidopsis Col-0*, were used for GFP signal inspection by laser confocal microscopy. The transgenic line expressing *pNAtGH3.4:AtGH3.4cdr-GFP* did not exhibit any fluorescence, and its lack of signal was identical to that of the negative control. However, this line may be used for further studies related to IAA metabolism, such as for investigating changes in GH3.4 expression in response to specific hormones or alternated biotic and abiotic conditions.

Two additional vectors were designed to be used for plant transformation, and further protein localization driven by native promoter.

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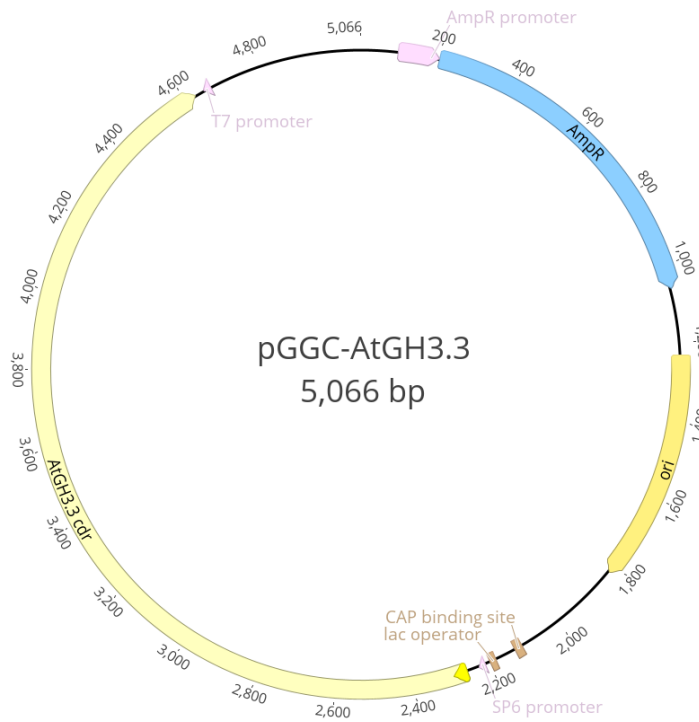
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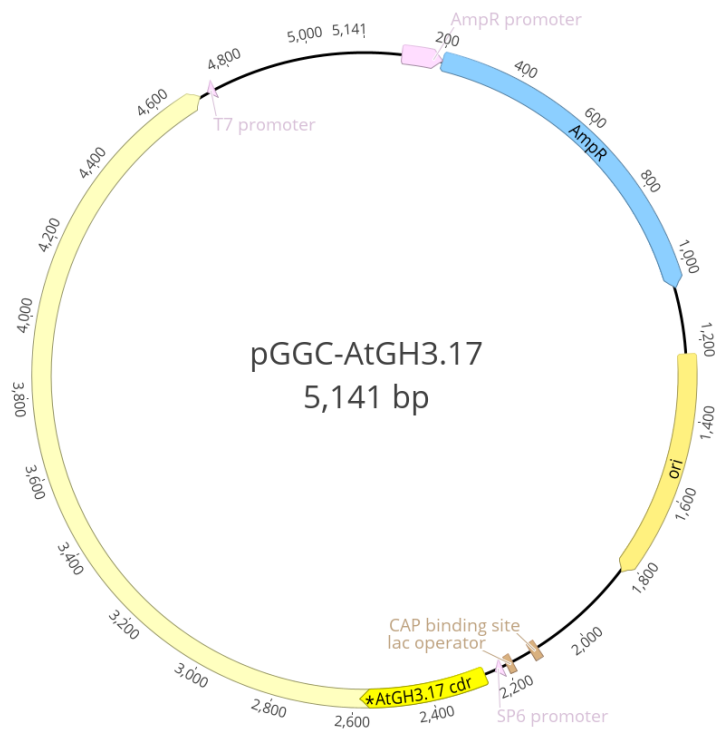
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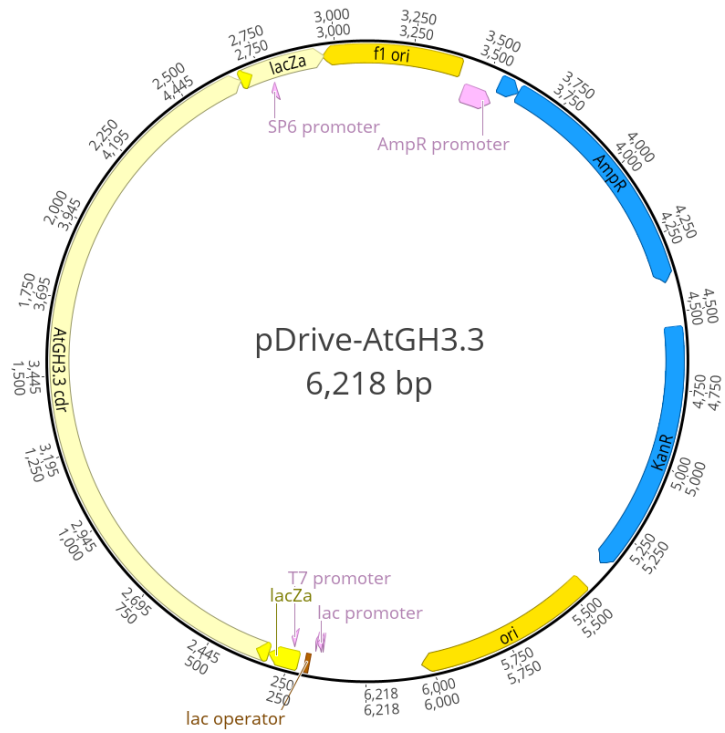
## Attachments



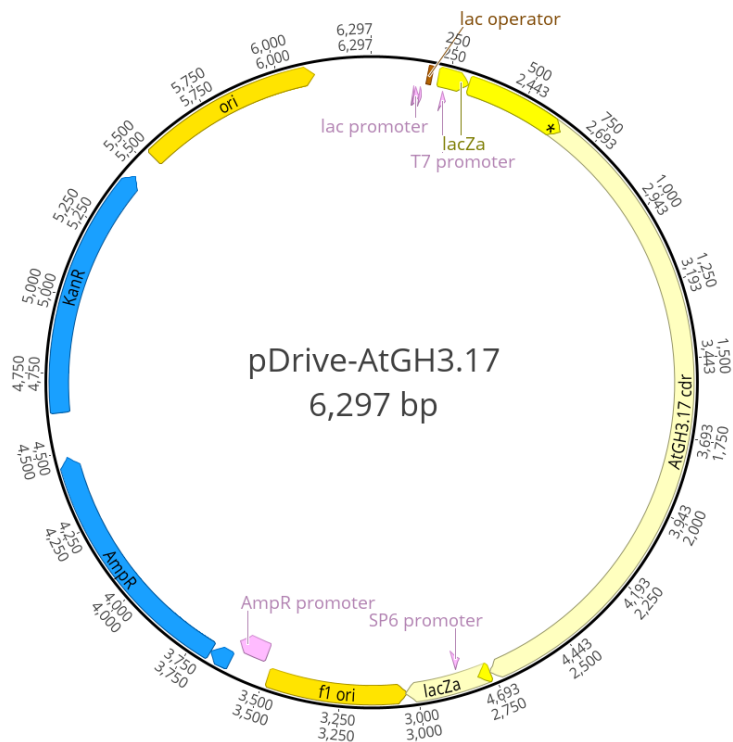
*Attachment 1. Representation of pGGC00 plasmid with inserted AtGH3.3 gene, created in Geneious Prime. The plasmid is 5,066 bp and carries the origin of replication ori, lac operator (required for lactose metabolism), CAP binding site (positive regulator of lac operon), SP6 promoter, AtGH3.3 inserted gene sequence, T7 promoter, and ampicillin resistance cassette*



*Attachment 2. Representation of pGGC00 plasmid with inserted AtGH3.17 gene, created in Geneious Prime. The plasmid is 5,141 bp and carries the origin of replication ori, lac operator (required for lactose metabolism), CAP binding site (positive regulator of lac operon), SP6 promoter, AtGH3.17 inserted gene sequence, T7 promoter, and ampicillin resistance cassette.*



Attachment 3. Representation of pDrive vector with inserted AtGH3.3 gene, created in Geneious Prime. The plasmid is 6,218 bp and carries the origin of replication ori, f1 ori, lac operator with LacZa (required for lactose metabolism), CAP binding site (positive regulator of lac operon), SP6 promoter, AtGH3.3 inserted gene sequence, T7 promoter, ampicillin, and kanamycin resistance cassette.



Attachment 4. Representation of pDrive vector with inserted AtGH3.17 gene, created in Geneious Prime. The plasmid is 6,297 bp and carries the origin of replication ori, f1 ori, lac operator with LacZa (is required for lactose metabolism), CAP binding site (positive regulator of lac operon), SP6 promoter, AtGH3.17 inserted gene sequence, T7 promoter, ampicillin, and kanamycin resistance cassette.

pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	ATACGACTCACTATAGGGAAAGCTCGGTACCACGCATGCTGCAGACGCGTTACGTATCGG ----- -----	300
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	ATCCAGAATTCGTGAT-----CGTTGATTCAGCTCTGCG -----GTTGATTCAGCTCTGCG -----	334 17
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	ATCTCCGATGATGCACTCACCGTCCACTAAGGACGTGAAGGCTCTAAGGTTCAATTGAGGA ATCTCCGATGATGCACTCACCGTCCACTAAGGACGTGAAGGCTCTAAGGTTCAATTGAGGA -----	394 77
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	GATGACACGTAACGTCGATTTTCGTTCCAGAAGAAAGTGATTAGAGAGATACTTAGTCGTAA GATGACACGTAACGTCGATTTTCGTTCCAGAAGAAAGTGATTAGAGAGATACTTAGTCGTAA -----	454 137
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	CTCGGACACTGAGTACCTGAAACGGTTTGGTCTCAAGGGATTCACTGACCGTAAACATT CTCGGACACTGAGTACCTGAAACGGTTTGGTCTCAAGGGATTCACTGACCGTAAACATT -----	514 197
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	TAAGACCAAAGTTCGGTGGTTATCTACGATGATCTTAAACCGGAGATTCAACGTATTGC TAAGACCAAAGTTCGGTGGTTATCTACGATGATCTTAAACCGGAGATTCAACGTATTGC -----	574 257
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	CAATGGTGACCGGTCAATGATCTTGTCTTCTTACCCCATCACAGAGTTCCTCACAAAGGTA CAATGGTGACCGGTCAATGATCTTGTCTTCTTACCCCATCACAGAGTTCCTCACAAAGGTA -----	634 317
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	TATACTTAACCCAAAATGTATATTATGTTATCTTAGACTCTTAGACAGAGTTAATCATCT TATACTTAACCCAAAATGTATATTATGTTATCTTAGACTCTTAGACAGAGTTAATCATCT -----	694 377
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	ATAATTGTGGATTAATCTTGTTTTGTATCCAGCTCTGGGACATCAGCTGGTGAAGGAAG ATAATTGTGGATTAATCTTGTTTTGTATCCAGCTCTGGGACATCAGCTGGTGAAGGAAG -----	754 437
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	TTGATGCCAACCAATTGATGAAGACATGGACCGACGTCAGCTTTTATACAGCTTCTCATG TTGATGCCAACCAATTGATGAAGACATGGACCGACGTCAGCTTTTATACAGCTTCTCATG -----	814 497
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	CCTGTGATGAATCTGTAAGTTACAGTTATTTAATTTGCAATTAACCTCTATTTTGTAGTA CCTGTGATGAATCTGTAAGTTACAGTTATTTAATTTGCAATTAACCTCTATTTTGTAGTA -----	874 557
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	ATATCTACGTGCCAAGATTAATACGTTAAAATTGGAAAAATTATGAATTAATCTAATTTA ATATCTACGTGCCAAGATTAATACGTTAAAATTGGAAAAATTATGAATTAATCTAATTTA -----	934 617
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	TATTAATAATTTTAGCAAAAATATTTTAAATCTGAACTTTGGACTCTACATTCTAAATTTA TATTAATAATTTTAGCAAAAATATTTTAAATCTGAACTTTGGACTCTACATTCTAAATTTA -----	994 677
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	AAACGTGAAGACTAAATGCTGAGGTTTAAACCATAAATCAGAAACCTTAAACCTAATTC AAACGTGAAGACTAAATGCTGAGGTTTAAACCATAAATCAGAAACCTTAAACCTAATTC -----	1054 737
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	TTGAAATTTAAAAAATTAACACTAAAATATAAACCAAATCATATATATTTACTTAATTTTC TTGAAATTTAAAAAATTAACACTAAAATATAAACCAAATCATATATATTTACTTAATTTTC -----	1114 797
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	AAAAGATCTAATTAACATGTGTTTTGCTTTAGTTTCATATTTTGTGAGATTTTTTTTGTCA AAAAGATCTAATTAACATGTGTTTTGCTTTA----- -----	1174 857
pDrive-AtGH3.3 3_1_1.ab1 3_2_1.ab1 (reversed)	ATTACGTACATCCTTTAAAATGTTATTTCACTATCGCAATAACGTAACATTTTTTTTTTC ----- -----	1234 917

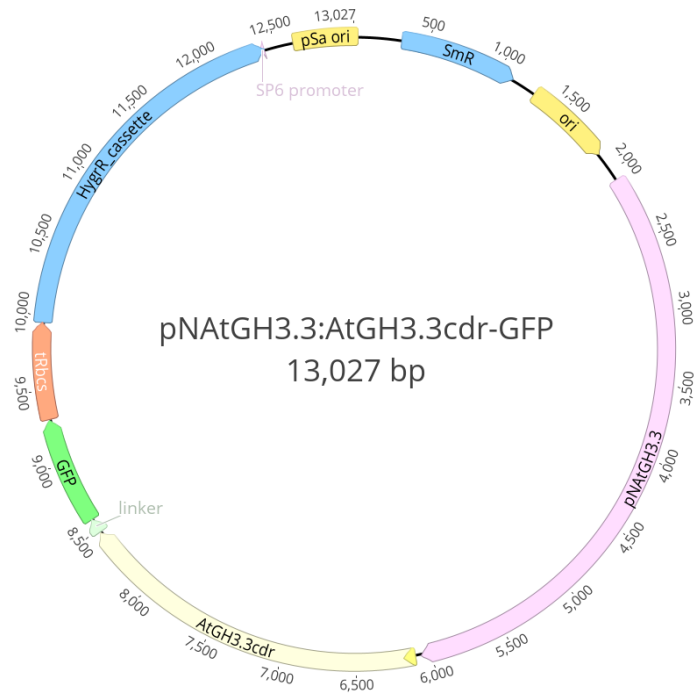
Attachment 5. Sequence alignment of pDrive-AtGH3.3cdr with a fragment of the GH3.3 gene resulting from the sequencing and confirming the correctness of the inserted sequence.

Consensus	TCGGATCCAGAATTCGTGATGCTGTTGATTGCTTCTTCAATCTGGGATGGCTTCACCGA	356
pDrive-AtGH3.4	TCGGATCCAGAATTCGTGATGCTGTTGATTGCTTCTTCAATCTGGGATGGCTTCACCGA	356
AtGH3.4_1	-----ATTCGCTTCTTCAATCTGGGATGGCTTCACCGA	33
AtGH3.4_2	-----	
Consensus	CGACATCTGAGACAGAGGTGAAGGCTCTCAAGTTCATTGAGGAGATTACTCGGAACCTG	416
pDrive-AtGH3.4	CGACATCTGAGACAGAGGTGAAGGCTCTCAAGTTCATTGAGGAGATTACTCGGAACCTG	416
AtGH3.4_1	CGACATCTGAGACAGAGGTGAAGGCTCTCAAGTTCATTGAGGAGATTACTCGGAACCTG	93
AtGH3.4_2	-----	
Consensus	ACTCGTTCAAGAAAAGTTCCTGGAGAGATACTTAGTCGTAACCTCGAACACGGAATATC	476
pDrive-AtGH3.4	ACTCGTTCAAGAAAAGTTCCTGGAGAGATACTTAGTCGTAACCTCGAACACGGAATATC	476
AtGH3.4_1	ACTCGTTCAAGAAAAGTTCCTGGAGAGATACTTAGTCGTAACCTCGAACACGGAATATC	153
AtGH3.4_2	-----	
Consensus	TGAAACGGTTCGATCTTAATGGTGCCGTTGATAGGAAATCGTTCAAGAGCAAAGTCCGG	536
pDrive-AtGH3.4	TGAAACGGTTCGATCTTAATGGTGCCGTTGATAGGAAATCGTTCAAGAGCAAAGTCCGG	536
AtGH3.4_1	TGAAACGGTTCGATCTTAATGGTGCCGTTGATAGGAAATCGTTCAAGAGCAAAGTCCGG	213
AtGH3.4_2	-----	
Consensus	TGGTAATCTACGAAGATTTGAAGACGGATATCAACGTATATCCAACGGTGATCGTTCTC	596
pDrive-AtGH3.4	TGGTAATCTACGAAGATTTGAAGACGGATATCAACGTATATCCAACGGTGATCGTTCTC	596
AtGH3.4_1	TGGTAATCTACGAAGATTTGAAGACGGATATCAACGTATATCCAACGGTGATCGTTCTC	273
AtGH3.4_2	-----	
Consensus	CGATCTTGTCTTCTCATCCCATCACCAGTTTTCTCACAAGGTATGTTACTTAAGTCAACC	656
pDrive-AtGH3.4	CGATCTTGTCTTCTCATCCCATCACCAGTTTTCTCACAAGGTATGTTACTTAAGTCAACC	656
AtGH3.4_1	CGATCTTGTCTTCTCATCCCATCACCAGTTTTCTCACAAGGTATGTTACTTAAGTCAACC	333
AtGH3.4_2	-----	
Consensus	AACTATATCAATGATTAATATCGTTTTCTTTGTTTTTACAACCTTATCAAGGAGTACCAA	716
pDrive-AtGH3.4	AACTATATCAATGATTAATATCGTTTTCTTTGTTTTTACAACCTTATCAAGGAGTACCAA	716
AtGH3.4_1	AACTATATCAATGATTAATATCGTTTTCTTTGTTTTTACAACCTTATCAAGGAGTACCAA	393
AtGH3.4_2	-----	
Consensus	TCCCCTTGTACTAGGTCACCATCCTTTTAGTAATGACTAAAACGTTGTTATAAAAAAATT	776
pDrive-AtGH3.4	TCCCCTTGTACTAGGTCACCATCCTTTTAGTAATGACTAAAACGTTGTTATAAAAAAATT	776
AtGH3.4_1	TCCCCTTGTACTAGGTCACCATCCTTTTAGTAATGACTAAAACGTTGTTATAAAAAAATT	453
AtGH3.4_2	-----	
Consensus	CAATGACGGGATTTTGATCACCATAATACAAATTGTTAAAAAATATTTTATAGAATACTA	836
pDrive-AtGH3.4	CAATGACGGGATTTTGATCACCATAATACAAATTGTTAAAAAATATTTTATAGAATACTA	836
AtGH3.4_1	CAATGACGGGATTTTGATCACCATAATACAAATTGTTAAAAAATATTTTATAGAATACTA	513
AtGH3.4_2	-----	
Consensus	TACAGTTTTTTTACCGTAATTAATTTGTAATGTTTTGTGTGATTGCAGCTCTGGAACAT	896
pDrive-AtGH3.4	TACAGTTTTTTTACCGTAATTAATTTGTAATGTTTTGTGTGATTGCAGCTCTGGAACAT	896
AtGH3.4_1	TACAGTTTTTTTACCGTAATTAATTTGTAATGTTTTGTGTGATTGCAGCTCTGGAACAT	573
AtGH3.4_2	-----	
Consensus	CTGCTGGCGAGAGGAAATTAATGCCGACAATTGAAGAAGACATAAACCGACGTCAGCTTT	956
pDrive-AtGH3.4	CTGCTGGCGAGAGGAAATTAATGCCGACAATTGAAGAAGACATAAACCGACGTCAGCTTT	956
AtGH3.4_1	CTGCTGGCGAGAGGAAATTAATGCCGACAATTGAAGAAGACATAAACCGACGTCAGCTTT	633
AtGH3.4_2	-----	
Consensus	TAGGCAATCTTCTCATGCCTGTGATGAATCTGTTAGTATATTACTTTTAACATTATTTAA	1016
pDrive-AtGH3.4	TAGGCAATCTTCTCATGCCTGTGATGAATCTGTTAGTATATTACTTTTAACATTATTTAA	1016
AtGH3.4_1	TAGGCAATCTTCTCATGCCTGTGATGAATCTGTTAGTATATTACTTTTAACATTATTTAA	693
AtGH3.4_2	-----	
Consensus	CAAAATTGTAACGATTTATAAAATTAACCAAAAATGTTTAACTACAGCTACGTGCCG	1076
pDrive-AtGH3.4	CAAAATTGTAACGATTTATAAAATTAACCAAAAATGTTTAACTACAGCTACGTGCCG	1076
AtGH3.4_1	CAAAATTGTAACGATTTATAAAATTAACCAAAAATGTTTAACTACAGCTACGTGCCG	753
AtGH3.4_2	-----	
Consensus	GGATTAGACAAAGGCAAAGGTTTATACTTCTTATTTGTGAAGTCGGAGTCTACGACATCA	1136
pDrive-AtGH3.4	GGATTAGACAAAGGCAAAGGTTTATACTTCTTATTTGTGAAGTCGGAGTCTACGACATCA	1136
AtGH3.4_1	GGATTAG-----	813
AtGH3.4_2	-----	

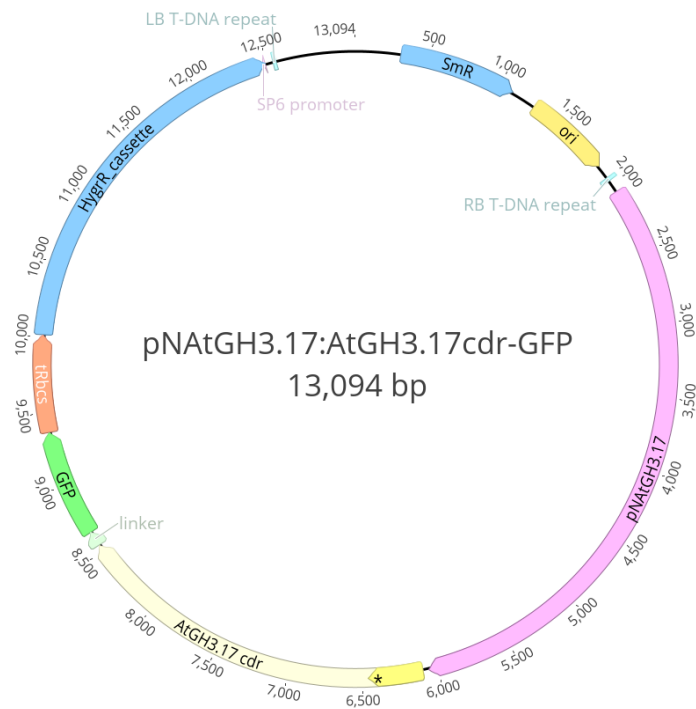
Attachment 6. Sequence alignment of pDrive-AtGH3.4cdr with a fragment of the GH3.4 gene resulting from the sequencing and confirming the correctness of the inserted sequence.

Consensus	ATCCAGAATTCGTGAT-----ATACCAAGTTACGACCCAAA	336
pDrive-AtGH3.17	ATCCAGAATTCGTGAT-----ATACCAAGTTACGACCCAAA	336
17_1_1.ab1	-----	
seq2	-----	
Consensus	TGATACAGAGGCTGGTCTCAAGCTTCTCGAGGATCTGACAACAAATGCAGAGGCTATCCA	396
pDrive-AtGH3.17	TGATACAGAGGCTGGTCTCAAGCTTCTCGAGGATCTGACAACAAATGCAGAGGCTATCCA	396
17_1_1.ab1	-----CTGGTCTCAAGCTTCTCGAGGATCTGACAACAAATGCAGAGGCTATCCA	49
seq2	-----	
Consensus	ACAACAAGTTCTTACCACAAATACTCTCTCAAAACTCTGGAACCTCAATATCTCCGAGCATT	456
pDrive-AtGH3.17	ACAACAAGTTCTTACCACAAATACTCTCTCAAAACTCTGGAACCTCAATATCTCCGAGCATT	456
17_1_1.ab1	ACAACAAGTTCTTACCACAAATACTCTCTCAAAACTCTGGAACCTCAATATCTCCGAGCATT	109
seq2	-----	
Consensus	TCTGGACGGAGAAGCCGACAAGAATCAACAAAGCTTCAAAAACAAAGTCCCTGTGGTGAA	516
pDrive-AtGH3.17	TCTGGACGGAGAAGCCGACAAGAATCAACAAAGCTTCAAAAACAAAGTCCCTGTGGTGAA	516
17_1_1.ab1	TCTGGACGGAGAAGCCGACAAGAATCAACAAAGCTTCAAAAACAAAGTCCCTGTGGTGAA	169
seq2	-----	
Consensus	TTATGACGACGTAAGCCTTTTCATTCAACGAATCGCTGATGGAGAATCATCTGATATCGT	576
pDrive-AtGH3.17	TTATGACGACGTAAGCCTTTTCATTCAACGAATCGCTGATGGAGAATCATCTGATATCGT	576
17_1_1.ab1	TTATGACGACGTAAGCCTTTTCATTCAACGAATCGCTGATGGAGAATCATCTGATATCGT	229
seq2	-----	
Consensus	CTCTGCTCAGCCCATCACAGAACTCCTCACTAGGTAACATATTTTTTACCACAAATTCTA	636
pDrive-AtGH3.17	CTCTGCTCAGCCCATCACAGAACTCCTCACTAGGTAACATATTTTTTACCACAAATTCTA	636
17_1_1.ab1	CTCTGCTCAGCCCATCACAGAACTCCTCACTAGGTAACATATTTTTTACCACAAATTCTA	289
seq2	-----	
Consensus	CAAAAACTATCGTTATATACTGTTCTCTTAAGAATTTTCGTTATACTTATTAAGTAATTC	696
pDrive-AtGH3.17	CAAAAACTATCGTTATATACTGTTCTCTTAAGAATTTTCGTTATACTTATTAAGTAATTC	696
17_1_1.ab1	CAAAAACTATCGTTATATACTGTTCTCTTAAGAATTTTCGTTATACTTATTAAGTAATTC	349
seq2	-----	
Consensus	TTTGAGTTAATATTGGATGCAGTTTCGGGACTTCTGCAGGAAAAGCCGAAGTTGATGCCTT	756
pDrive-AtGH3.17	TTTGAGTTAATATTGGATGCAGTTTCGGGACTTCTGCAGGAAAAGCCGAAGTTGATGCCTT	756
17_1_1.ab1	TTTGAGTTAATATTGGATGCAGTTTCGGGACTTCTGCAGGAAAAGCCGAAGTTGATGCCTT	409
seq2	-----	
Consensus	CTACAGCTGAAGAATTGGAAAAGGAAAGACATTTTTCTACAGCATGCTTGTGCCTATCATGA	816
pDrive-AtGH3.17	CTACAGCTGAAGAATTGGAAAAGGAAAGACATTTTTCTACAGCATGCTTGTGCCTATCATGA	816
17_1_1.ab1	CTACAGCTGAAGAATTGGAAAAGGAAAGACATTTTTCTACAGCATGCTTGTGCCTATCATGA	469
seq2	-----	
Consensus	ACAAGTAATAAATGAGTTTTTATTACTTCAATCTTAATTTACGTAATTAATTTTTTTGTT	876
pDrive-AtGH3.17	ACAAGTAATAAATGAGTTTTTATTACTTCAATCTTAATTTACGTAATTAATTTTTTTGTT	876
17_1_1.ab1	ACAAGTAATAAATGAGTTTTTATTACTTCAATCTTAATTTACGTAATTAATTTTTTTGTT	529
seq2	-----	
Consensus	TTGTGCATTAATATCTCTTAAATTCATTCTCATTCCAAAATAAAAAAAAAAAGAGAAATA	936
pDrive-AtGH3.17	TTGTGCATTAATATCTCTTAAATTCATTCTCATTCCAAAATAAAAAAAAAAAGAGAAATA	936
17_1_1.ab1	TTGTGCATTAATATCTCTTAAATTCATTCTCATTCCAAAATAAAAAAAAAAAGAGAAATA	589
seq2	-----	
Consensus	CAGAAATATTTCTTAAGAAAATAAAAAACACAATAGTATATAATAATTTTTGTAGCATCC	996
pDrive-AtGH3.17	CAGAAATATTTCTTAAGAAAATAAAAAACACAATAGTATATAATAATTTTTGTAGCATCC	996
17_1_1.ab1	CAGAAATATTTCTTAAGAAAATAAAAAACACAATAGTATATAATAATTTTTGTAGCATCC	649
seq2	-----	
Consensus	AACAGAAAACAATATACGTAACAGCTCTCTTATTGGGCTTAAAACATTTAGGAAATATAT	1056
pDrive-AtGH3.17	AACAGAAAACAATATACGTAACAGCTCTCTTATTGGGCTTAAAACATTTAGGAAATATAT	1056
17_1_1.ab1	AACAGAAAACAATATACGTAACAGCTCTCTTATTGGGCTTAAAACATTTAGGAAATATAT	709
seq2	-----	
Consensus	TTGGCTTCTCTTTGCTACAAAACACCTTGCAGATTGAATATGTAGGATCCTAGTTACACT	1116
pDrive-AtGH3.17	TTGGCTTCTCTTTGCTACAAAACACCTTGCAGATTGAATATGTAGGATCCTAGTTACACT	1116
17_1_1.ab1	TTGGCTTCTCTTTGCTACAAAACACCTTGCAGATTGAATATGTAGGATCCTAGTTACACT	769
seq2	-----	
Consensus	CATGAGAGGGCAAAAATAAATCAATTATATCTGTTTTCTTTAGTCAAAAGATTAACACAG	1176
pDrive-AtGH3.17	CATGAGAGGGCAAAAATAAATCAATTATATCTGTTTTCTTTAGTCAAAAGATTAACACAG	1176
17_1_1.ab1	CATGAGAGGGCAAAAATAAATCAATTATATCTGTTTTCTTTAGTCAAAAGATTAACACAG	829
seq2	-----	
Consensus	TGAAGGAATTTTTTTTTGATAGATTGGATTGTGTTACATATTTGAAATTCAGATATGT	1236
pDrive-AtGH3.17	TGAAGGAATTTTTTTTTGATAGATTGGATTGTGTTACATATTTGAAATTCAGATATGT	1236
17_1_1.ab1	TGAAGGAATTTTTTTTTG-----	889
seq2	-----	

Attachment 7. Sequence alignment of pDrive-AtGH3.17cdr with a fragment of the GH3.17 gene resulting from the sequencing and confirming the correctness of the inserted sequence.



Attachment 8. Representation of pNAtGH3.3:AtGH3.3cdr-GFP vector, created in Geneious Prime. The plasmid is 13,027 bp and carries origin of replication ori, native promoter pNAtGH3.3, AtGH3.3 gene, GFP sequence with a linker, tRbcs (terminator), HygrR (hygromycin resistance cassette), pSa ori (origin of replication for bacteria), SmR (spectinomycin resistance cassette)



Attachment 9. Representation of pNAtGH3.17:AtGH3.17cdr-GFP vector, created in Geneious Prime. The plasmid is 13,094 bp and carries origin of replication ori, native promoter pNAtGH3.17, AtGH3.17 gene, GFP sequence with a linker, tRbcs (terminator), HygrR (hygromycin resistance cassette), pSa ori (origin of replication for bacteria), SmR (spectinomycin resistance cassette)



Consensus	CAACGTGTACACGAGCCCTAACGAAGCCATCCTTTGTCCAGACTCATCCCAAAGCATGTA	7320
pNAAtGH3.3:AtGH3.3cdr-GFP	CAACGTGTACACGAGCCCTAACGAAGCCATCCTTTGTCCAGACTCATCCCAAAGCATGTA	7320
AtGH3.3_1	-----	5308
AtGH3.3_2	-----	4117
AtGH3.3_3	-----	3218
AtGH3.3_4	CAACGTGTACACGAGCCCTAACGAAGCCATCCTTTGTCCAGACTCATCCCAAAGCATGTA	398
AtGH3.3_5	-----AGACTCATCCCAAAGCATGTA	21
AtGH3.3_6	-----	
AtGH3.3_7	-----	
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	CACGCAGATGCTTTGTGGTCTCCTTATGCGTCACGAAATCCTCCGTCTCGGCGCCGTCTT	7380
pNAAtGH3.3:AtGH3.3cdr-GFP	CACGCAGATGCTTTGTGGTCTCCTTATGCGTCACGAAATCCTCCGTCTCGGCGCCGTCTT	7380
AtGH3.3_1	-----	5368
AtGH3.3_2	-----	4177
AtGH3.3_3	-----	3278
AtGH3.3_4	CACGCAGATGCTTTGTGGTCTCCTTATGCGTCACGAAATCCTCCGTCTCGGCGCCGTCTT	458
AtGH3.3_5	CACGCAGATGCTTTGTGGTCTCCTTATGCGTCACGAAATCCTCCGTCTCGGCGCCGTCTT	81
AtGH3.3_6	-----	
AtGH3.3_7	-----	
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	CGCTTCTGGTCTCCTCCGTGCCATTGSAATTCCTTCAAACCAATTGSAAGAACTCGCCGA	7440
pNAAtGH3.3:AtGH3.3cdr-GFP	CGCTTCTGGTCTCCTCCGTGCCATTGSAATTCCTTCAAACCAATTGSAAGAACTCGCCGA	7440
AtGH3.3_1	-----	5428
AtGH3.3_2	-----	4237
AtGH3.3_3	-----	3338
AtGH3.3_4	CGCTTCTGGTCTCCTCCGTGCCATTGSAATTCCTTCAAACCAATTGSAAGAACTCGCCGA	518
AtGH3.3_5	CGCTTCTGGTCTCCTCCGTGCCATTGSAATTCCTTCAAACCAATTGSAAGAACTCGCCGA	141
AtGH3.3_6	-----	
AtGH3.3_7	-----	
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	CGATATCTCCACCGBTACCTTAAAGTTCAAGAATCTCTGACCCGGCCATTAAAGAGAGCAT	7500
pNAAtGH3.3:AtGH3.3cdr-GFP	CGATATCTCCACCGBTACCTTAAAGTTCAAGAATCTCTGACCCGGCCATTAAAGAGAGCAT	7500
AtGH3.3_1	-----	5488
AtGH3.3_2	-----	4297
AtGH3.3_3	-----	3398
AtGH3.3_4	CGATATCTCCACCGBTACCTTAAAGTTCAAGAATCTCTGACCCGGCCATTAAAGAGAGCAT	578
AtGH3.3_5	CGATATCTCCACCGBTACCTTAAAGTTCAAGAATCTCTGACCCGGCCATTAAAGAGAGCAT	201
AtGH3.3_6	-----	
AtGH3.3_7	-----	
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	GTCCAAGATCTTGACCAAACCGGACCAAGAACTGGCTGATTTATAAATCTCGGTATGTGG	7560
pNAAtGH3.3:AtGH3.3cdr-GFP	GTCCAAGATCTTGACCAAACCGGACCAAGAACTGGCTGATTTATAAATCTCGGTATGTGG	7560
AtGH3.3_1	-----	5548
AtGH3.3_2	-----	4357
AtGH3.3_3	-----	3458
AtGH3.3_4	GTCCAAGATCTTGACCAAACCGGACCAAGAACTGGCTGATTTATAAATCTCGGTATGTGG	638
AtGH3.3_5	GTCCAAGATCTTGACCAAACCGGACCAAGAACTGGCTGATTTATAAATCTCGGTATGTGG	261
AtGH3.3_6	-----	
AtGH3.3_7	-----	
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	TCAAGACAATAGTTGGGAAGGTATTATTACTAAGATTTGGCCTAACACTAAGTACCTTGA	7620
pNAAtGH3.3:AtGH3.3cdr-GFP	TCAAGACAATAGTTGGGAAGGTATTATTACTAAGATTTGGCCTAACACTAAGTACCTTGA	7620
AtGH3.3_1	-----	5608
AtGH3.3_2	-----	4417
AtGH3.3_3	-----	3518
AtGH3.3_4	TCAAGACAATAGTTGGGAAGGTATTATTACTAAGATTTGGCCTAACACTAAGTACCTTGA	698
AtGH3.3_5	TCAAGACAATAGTTGGGAAGGTATTATTACTAAGATTTGGCCTAACACTAAGTACCTTGA	321
AtGH3.3_6	-----	
AtGH3.3_7	-----	
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	CGTCATCGTTACTGSAAGCCATGGCTCAGTATATCCCGATGCTTGAGTACTATAAGCGGCGG	7680
pNAAtGH3.3:AtGH3.3cdr-GFP	CGTCATCGTTACTGSAAGCCATGGCTCAGTATATCCCGATGCTTGAGTACTATAAGCGGCGG	7680
AtGH3.3_1	-----	5668
AtGH3.3_2	-----	4477
AtGH3.3_3	-----	3578
AtGH3.3_4	CGTCATCGTTACTGSAAGCCATGGCTCAGTATATCCCGATGCTTGAGTACTATAAGCGGCGG	758
AtGH3.3_5	CGTCATCGTTACTGSAAGCCATGGCTCAGTATATCCCGATGCTTGAGTACTATAAGCGGCGG	381
AtGH3.3_6	-----	

Attachment 10. Sequence alignment of pNAAtGH3.3:AtGH3.3cdr-GFP with a fragment of AtGH3.3 coding region resulting from the sequencing and confirming the correctness of assembly (part 1).

Consensus	GTTACCGATGGCTTGCACGATGTATGCATCGTCCGAGAGTTACTTTGGGATCAACTTGAA	7740
:NAAtGH3.3:AtGH3.3cdr-GFP	GTTACCGATGGCTTGCACGATGTATGCATCGTCCGAGAGTTACTTTGGGATCAACTTGAA	7740
ltGH3.3_1	-----	5728
ltGH3.3_2	-----	4537
ltGH3.3_3	-----	3638
ltGH3.3_4	GTTACCGATGGCTTGCACGATGTATGCATCGTCCGAGAGTTACTTTGGGATCAACTTGAA	818
ltGH3.3_5	GTTACCGATGGCTTGCACGATGTATGCATCGTCCGAGAGTTACTTTGGGATCAACTTGAA	441
ltGH3.3_6	-----	
ltGH3.3_7	-----	
ltGH3.3_8	-----	
ltGH3.3_9	-----	
ltGH3.3_10	-----	
Consensus	ACCAATGTGTA AACCTTCTGAGGTTTCTTATACCATTATGCCAAACATGGCATACTTCGA	7800
:NAAtGH3.3:AtGH3.3cdr-GFP	ACCAATGTGTA AACCTTCTGAGGTTTCTTATACCATTATGCCAAACATGGCATACTTCGA	7800
ltGH3.3_1	-----	5788
ltGH3.3_2	-----	4597
ltGH3.3_3	-----	3698
ltGH3.3_4	ACCAATGTGTA AACCTTCTGAGGTTTCTTATACCATTATGCCAAACATGGCATACTTCGA	878
ltGH3.3_5	ACCAATGTGTA AACCTTCTGAGGTTTCTTATACCATTATGCCAAACATGGCATACTTCGA	501
ltGH3.3_6	-----	
ltGH3.3_7	-----	
ltGH3.3_8	-----	
ltGH3.3_9	-----	
ltGH3.3_10	-----	
Consensus	GTTTCTCCCTCATCATGAAGTCCCAACCGAAAAATCCGAACCTTGTGGAGCTAGCTGATGT	7860
:NAAtGH3.3:AtGH3.3cdr-GFP	GTTTCTCCCTCATCATGAAGTCCCAACCGAAAAATCCGAACCTTGTGGAGCTAGCTGATGT	7860
ltGH3.3_1	-----	5848
ltGH3.3_2	-----	4657
ltGH3.3_3	-----	3758
ltGH3.3_4	GTTTCTCCCTCATCATGAAGTCCCAACCGAAAAATCCGAACCTTGTGGAGCTAGCTGATGT	938
ltGH3.3_5	GTTTCTCCCTCATCATGAAGTCCCAACCGAAAAATCCGAACCTTGTGGAGCTAGCTGATGT	561
ltGH3.3_6	-----	
ltGH3.3_7	-----	
ltGH3.3_8	-----	
ltGH3.3_9	-----	
ltGH3.3_10	-----	
Consensus	CGAGGTCGGGAAAGAGTACGAGCTTGTGATCACAACCTATGCTGGGCTTAACCGTTATAG	7920
:NAAtGH3.3:AtGH3.3cdr-GFP	CGAGGTCGGGAAAGAGTACGAGCTTGTGATCACAACCTATGCTGGGCTTAACCGTTATAG	7920
ltGH3.3_1	-----	5908
ltGH3.3_2	-----	4717
ltGH3.3_3	-----	3818
ltGH3.3_4	CGAGGTCG-----	998
ltGH3.3_5	CGAGGTCGGGAAAGAGTACGAGCTTGTGATCACAACCTATGCTGGGCTTAACCGTTATAG	621
ltGH3.3_6	-----	
ltGH3.3_7	-----	
ltGH3.3_8	-----	
ltGH3.3_9	-----	
ltGH3.3_10	-----	

Attachment 11. Sequence alignment of pNAAtGH3.3:AtGH3.3cdr-GFP with a fragment of AtGH3.3 coding region resulting from the sequencing and confirming the correctness of assembly (part 2).

Consensus	AATGGTGAGCAAGGGCGAGGAGCTGTTACCCG--GGGTGGTGCCCATCCTGGTCGAGCTG	8638
pNAtGH3.3:AtGH3.3cdr-GFP	AATGGTGAGCAAGGGCGAGGAGCTGTTACCCG--GGGTGGTGCCCATCCTGGTCGAGCTG	8634
AtGH3.3_1	-----	6628
AtGH3.3_2	-----	5437
AtGH3.3_3	-----	4538
AtGH3.3_4	-----	1718
AtGH3.3_5	-----	1341
AtGH3.3_6	AATGGTGAGCAAGGGCGAGGAGCTGTTACCCG--GGGTGGTGCCCATCCTGGTCGAGCTG	389
AtGH3.3_7	-----TCGAGCTG	8
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	GACGGCGACGTA AACGGCCACAAGTT CAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACC	8698
pNAtGH3.3:AtGH3.3cdr-GFP	GACGGCGACGTA AACGGCCACAAGTT CAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACC	8694
AtGH3.3_1	-----	6688
AtGH3.3_2	-----	5497
AtGH3.3_3	-----	4598
AtGH3.3_4	-----	1778
AtGH3.3_5	-----	1401
AtGH3.3_6	GACGGCGACGTA AACGGCCACAAGTT CAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACC	449
AtGH3.3_7	GACGGCGACGTA AACGGCCACAAGTT CAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACC	68
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	TACGGCAAGCTGACCCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTCCCTGGCCC	8758
pNAtGH3.3:AtGH3.3cdr-GFP	TACGGCAAGCTGACCCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTCCCTGGCCC	8754
AtGH3.3_1	-----	6748
AtGH3.3_2	-----	5557
AtGH3.3_3	-----	4658
AtGH3.3_4	-----	1838
AtGH3.3_5	-----	1461
AtGH3.3_6	TACGGCAAGCTGACCCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTCCCTGGCCC	509
AtGH3.3_7	TACGGCAAGCTGACCCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTCCCTGGCCC	128
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	ACCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATG	8818
pNAtGH3.3:AtGH3.3cdr-GFP	ACCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATG	8814
AtGH3.3_1	-----	6808
AtGH3.3_2	-----	5617
AtGH3.3_3	-----	4718
AtGH3.3_4	-----	1898
AtGH3.3_5	-----	1521
AtGH3.3_6	ACCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATG	569
AtGH3.3_7	ACCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATG	188
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	AAGCAGCAGGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATC	8878
pNAtGH3.3:AtGH3.3cdr-GFP	AAGCAGCAGGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATC	8874
AtGH3.3_1	-----	6868
AtGH3.3_2	-----	5677
AtGH3.3_3	-----	4778
AtGH3.3_4	-----	1958
AtGH3.3_5	-----	1581
AtGH3.3_6	AAGCAGCAGGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATC	629
AtGH3.3_7	AAGCAGCAGGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATC	248
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	TTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACC	8938
pNAtGH3.3:AtGH3.3cdr-GFP	TTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACC	8934
AtGH3.3_1	-----	6928
AtGH3.3_2	-----	5737
AtGH3.3_3	-----	4838
AtGH3.3_4	-----	2018
AtGH3.3_5	-----	1641
AtGH3.3_6	TTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACC	689
AtGH3.3_7	TTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACC	308
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	

Attachment 12. Sequence alignment of pNAtGH3.3:AtGH3.3cdr-GFP with a fragment of GFP coding region resulting from the sequencing and confirming the correctness of assembly (part 1).

Consensus	CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGG	8998
pNAtGH3.3:AtGH3.3cdr-GFP	CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGG	8994
AtGH3.3_1	-----	6988
AtGH3.3_2	-----	5797
AtGH3.3_3	-----	4898
AtGH3.3_4	-----	2078
AtGH3.3_5	-----	1701
AtGH3.3_6	CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGG	749
AtGH3.3_7	CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGG	368
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	CACAAGCTGGAGTACAACCTACAACAGCCACAACGTCTATATCATG6CCGACAAGCAGAAG	9058
pNAtGH3.3:AtGH3.3cdr-GFP	CACAAGCTGGAGTACAACCTACAACAGCCACAACGTCTATATCATG6CCGACAAGCAGAAG	9054
AtGH3.3_1	-----	7048
AtGH3.3_2	-----	5857
AtGH3.3_3	-----	4958
AtGH3.3_4	-----	2138
AtGH3.3_5	-----	1761
AtGH3.3_6	CACAAGCTGGAGTACAACCTACAACAGCCACAACGTCTATATCATG6CCGACAAGCAGAAG	809
AtGH3.3_7	CACAAGCTGGAGTACAACCTACAACAGCCACAACGTCTATATCATG6CCGACAAGCAGAAG	428
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	AACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTC	9118
pNAtGH3.3:AtGH3.3cdr-GFP	AACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTC	9114
AtGH3.3_1	-----	7108
AtGH3.3_2	-----	5917
AtGH3.3_3	-----	5018
AtGH3.3_4	-----	2198
AtGH3.3_5	-----	1821
AtGH3.3_6	AACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTC	869
AtGH3.3_7	AACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTC	488
AtGH3.3_8	-----	
AtGH3.3_9	-----	
AtGH3.3_10	-----	
Consensus	GCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAAC	9178
pNAtGH3.3:AtGH3.3cdr-GFP	GCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAAC	9174
AtGH3.3_1	-----	7168
AtGH3.3_2	-----	5977
AtGH3.3_3	-----	5078
AtGH3.3_4	-----	2258
AtGH3.3_5	-----	1881
AtGH3.3_6	GCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAAC	929
AtGH3.3_7	GCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAAC	548
AtGH3.3_8	-----GGCAGCAGCCCCGTGCTGCTGCCCGACAAC	30
AtGH3.3_9	-----	
AtGH3.3_10	-----	

Attachment 13. Sequence alignment of pNAtGH3.3:AtGH3.3cdr-GFP with a fragment of GFP coding region resulting from the sequencing and confirming the correctness of assembly (part 2).

Consensus	AGTCCTAAGGAAGCCATCCTCTGCTGTGACTCGTCTCAAAGCATGTATACGCAAATGCTA	4877
lP_AtGH3_4:AtGH3_4cds:GFP:HygR	AGTCCTAAGGAAGCCATCCTCTGCTGTGACTCGTCTCAAAGCATGTATACGCAAATGCTA	4877
ltGH3_4_1	-----	2008
ltGH3_4_2	AGTCCTAAGGAAGCCATCCTCTGCTGTGACTCGTCTCAAAGCATGTATACGCAAATGCTA	497
ltGH3_4_3	-----	
ltGH3_4_4	-----	
ltGH3_4_5	-----	
ltGH3_4_6	-----	
ltGH3_4_7	-----	
ltGH3_4_8	-----	
Consensus	TGTGGTCTCTTAATGCGCCATGAAGTTAACC GACTCGGTGCGGTGTTTCCTTCTGGTCTC	4137
lP_AtGH3_4:AtGH3_4cds:GFP:HygR	TGTGGTCTCTTAATGCGCCATGAAGTTAACC GACTCGGTGCGGTGTTTCCTTCTGGTCTC	4137
ltGH3_4_1	-----	2068
ltGH3_4_2	TGTGGTCTCTTAATGCGCCATGAAGTTAACC GACTCGGTGCGGTGTTTCCTTCTGGTCTC	557
ltGH3_4_3	-----	39
ltGH3_4_4	-----	
ltGH3_4_5	-----	
ltGH3_4_6	-----	
ltGH3_4_7	-----	
ltGH3_4_8	-----	
Consensus	CTCCGTGCCATAAGCTTCTCCAGAACAATTGGAAGGAACTTCTCAGGATATCTCAACC	4197
lP_AtGH3_4:AtGH3_4cds:GFP:HygR	CTCCGTGCCATAAGCTTCTCCAGAACAATTGGAAGGAACTTCTCAGGATATCTCAACC	4197
ltGH3_4_1	-----	2128
ltGH3_4_2	CTCCGTGCCATAAGCTTCTCCAGAACAATTGGAAGGAACTTCTCAGGATATCTCAACC	617
ltGH3_4_3	CTCCGTGCCATAAGCTTCTCCAGAACAATTGGAAGGAACTTCTCAGGATATCTCAACC	99
ltGH3_4_4	-----	
ltGH3_4_5	-----	
ltGH3_4_6	-----	
ltGH3_4_7	-----	
ltGH3_4_8	-----	
Consensus	GGGACCCCTAAGTTCTAAAAATCTTTGATCATGCGATTAAAACTCGAATGTCGAATATTTTG	4257
lP_AtGH3_4:AtGH3_4cds:GFP:HygR	GGGACCCCTAAGTTCTAAAAATCTTTGATCATGCGATTAAAACTCGAATGTCGAATATTTTG	4257
ltGH3_4_1	-----	2188
ltGH3_4_2	GGGACCCCTAAGTTCTAAAAATCTTTGATCATGCGATTAAAACTCGAATGTCGAATATTTTG	677
ltGH3_4_3	GGGACCCCTAAGTTCTAAAAATCTTTGATCATGCGATTAAAACTCGAATGTCGAATATTTTG	159
ltGH3_4_4	-----	
ltGH3_4_5	-----	
ltGH3_4_6	-----	
ltGH3_4_7	-----	
ltGH3_4_8	-----	
Consensus	AACAAACCTGATCAAGAACTGGCTGAGTTTTTGATAGGGGTTTGTTCGCAAGAGAATTGG	4317
lP_AtGH3_4:AtGH3_4cds:GFP:HygR	AACAAACCTGATCAAGAACTGGCTGAGTTTTTGATAGGGGTTTGTTCGCAAGAGAATTGG	4317
ltGH3_4_1	-----	2248
ltGH3_4_2	AACAAACCTGATCAAGAACTGGCTGAGTTTTTGATAGGGGTTTGTTCGCAAGAGAATTGG	737
ltGH3_4_3	AACAAACCTGATCAAGAACTGGCTGAGTTTTTGATAGGGGTTTGTTCGCAAGAGAATTGG	219
ltGH3_4_4	-----	
ltGH3_4_5	-----	
ltGH3_4_6	-----	
ltGH3_4_7	-----	
ltGH3_4_8	-----	
Consensus	GAAGGAATAATCACAAAGATATGGCCTAACACAAAGTACCTTGATGTGATTGTTACTGGT	4377
lP_AtGH3_4:AtGH3_4cds:GFP:HygR	GAAGGAATAATCACAAAGATATGGCCTAACACAAAGTACCTTGATGTGATTGTTACTGGT	4377
ltGH3_4_1	-----	2308
ltGH3_4_2	GAAGGAATAATCACAAAGATATGGCCTAACACAAAGTACCTTGATGTGATTGTTACTGGT	797
ltGH3_4_3	GAAGGAATAATCACAAAGATATGGCCTAACACAAAGTACCTTGATGTGATTGTTACTGGT	279
ltGH3_4_4	-----	
ltGH3_4_5	-----	
ltGH3_4_6	-----	
ltGH3_4_7	-----	
ltGH3_4_8	-----	
Consensus	GCAATGGCTGAGTATATCCCAATGTTGGAGTACTATAGCGGTGGGTTACCAATGGCAAGC	4437
lP_AtGH3_4:AtGH3_4cds:GFP:HygR	GCAATGGCTGAGTATATCCCAATGTTGGAGTACTATAGCGGTGGGTTACCAATGGCAAGC	4437
ltGH3_4_1	-----	2368
ltGH3_4_2	GCAATGGCTGAGTATATCCCAATGTTGGAGTACTATAGCGGTGGGTTACCAATGGCAAGC	857
ltGH3_4_3	GCAATGGCTGAGTATATCCCAATGTTGGAGTACTATAGCGGTGGGTTACCAATGGCAAGC	339
ltGH3_4_4	-----	
ltGH3_4_5	-----	
ltGH3_4_6	-----	
ltGH3_4_7	-----	
ltGH3_4_8	-----	
Consensus	ATGATTTATGCTTCATCCGAAAGTTACTTCGGGATTAACCTAAATCCGATGTGTAACCC	4497
lP_AtGH3_4:AtGH3_4cds:GFP:HygR	ATGATTTATGCTTCATCCGAAAGTTACTTCGGGATTAACCTAAATCCGATGTGTAACCC	4497
ltGH3_4_1	-----	2428
ltGH3_4_2	ATGATTTATGCTTCATCCGAAAGTTACTTCGGGATTAACCTAAATCCGATGTGTAACCC	917
ltGH3_4_3	ATGATTTATGCTTCATCCGAAAGTTACTTCGGGATTAACCTAAATCCGATGTGTAACCC	399
ltGH3_4_4	-----	
ltGH3_4_5	-----	
ltGH3_4_6	-----	
ltGH3_4_7	-----	
ltGH3_4_8	-----	

Attachment 14. Sequence alignment of pNA<sub>l</sub>GH3.4:AtGH3.4cdr-GFP with a fragment of AtGH3.4 coding region resulting from the sequencing and confirming the correctness of assembly (part 1).

Consensus	TCGGAGGTTTCTTACACAATCTTCCCCAACATGGCCTACTTCGAATTCCTCCACATAAT	4557
NP_AtGH3.4:AtGH3.4cfs:GFP:HygR	TCGGAGGTTTCTTACACAATCTTCCCCAACATGGCCTACTTCGAATTCCTCCACATAAT	4557
AtGH3.4_1	-----	2488
AtGH3.4_2	TCGGAGGTTTCTTACACAATCTTCCCCAACATGGCCTACTTCGAATTCCTCC	977
AtGH3.4_3	TCGGAGGTTTCTTACACAATCTTCCCCAACATGGCCTACTTCGAATTCCTCCACATAAT	459
AtGH3.4_4	-----	
AtGH3.4_5	-----	
AtGH3.4_6	-----	
AtGH3.4_7	-----	
AtGH3.4_8	-----	
Consensus	CACGATGGAGATGGAGGAGTAGAAGCAACCTCACTTGTGGAGCTAGCTGATGTTGAGGTT	4617
NP_AtGH3.4:AtGH3.4cfs:GFP:HygR	CACGATGGAGATGGAGGAGTAGAAGCAACCTCACTTGTGGAGCTAGCTGATGTTGAGGTT	4617
AtGH3.4_1	-----	2548
AtGH3.4_2	-----	1037
AtGH3.4_3	CACGATGGAGATGGAGGAGTAGAAGCAACCTCACTTGTGGAGCTAGCTGATGTTGAGGTT	519
AtGH3.4_4	-----	
AtGH3.4_5	-----	
AtGH3.4_6	-----	
AtGH3.4_7	-----	
AtGH3.4_8	-----	
Consensus	GGAAAGGAGTATGAACCTGTGATCACGACCTACGCGGGCTCTACCGTTACAGAGTTGGC	4677
NP_AtGH3.4:AtGH3.4cfs:GFP:HygR	GGAAAGGAGTATGAACCTGTGATCACGACCTACGCGGGCTCTACCGTTACAGAGTTGGC	4677
AtGH3.4_1	-----	2688
AtGH3.4_2	-----	1097
AtGH3.4_3	GGAAAGGAGTATGAACCTGTGATCACGACCTACGCGGGCTCTACCGTTACAGAGTTGGC	579
AtGH3.4_4	-----	
AtGH3.4_5	-----	
AtGH3.4_6	-----	
AtGH3.4_7	-----	
AtGH3.4_8	-----	
Consensus	GACATTCTTCGTGTACGCGGGTTTCATAATCCGCTCCACAGTCAAATTCATACGGAGA	4737
NP_AtGH3.4:AtGH3.4cfs:GFP:HygR	GACATTCTTCGTGTACGCGGGTTTCATAATCCGCTCCACAGTCAAATTCATACGGAGA	4737
AtGH3.4_1	-----	2668
AtGH3.4_2	-----	1157
AtGH3.4_3	GACATTCTTCGTGTACGCGGGTTTCATAATCCGCTCCACAGTCAAATTCATACGGAGA	639
AtGH3.4_4	-----	
AtGH3.4_5	-----	
AtGH3.4_6	-----	
AtGH3.4_7	-----	
AtGH3.4_8	-----	
Consensus	GAGAATGTTTTGCTAAGCATTGAATCTGATAAAAACAGACGAGGCTGATTTACAAAAGGCA	4797
NP_AtGH3.4:AtGH3.4cfs:GFP:HygR	GAGAATGTTTTGCTAAGCATTGAATCTGATAAAAACAGACGAGGCTGATTTACAAAAGGCA	4797
AtGH3.4_1	-----	2728
AtGH3.4_2	-----	1217
AtGH3.4_3	GAGAATGTTTTGCTAAGCATTGAATCTGATAAAAACAGACGAGGCTGATTTACAAAAGGCA	699
AtGH3.4_4	-----	
AtGH3.4_5	-----	
AtGH3.4_6	-----	
AtGH3.4_7	-----	
AtGH3.4_8	-----	
Consensus	GTGGAGAATGCGTCGAGGTTGCTTGCAGAGCAAGGAACACGTGTGATCGAGTATACGAGC	4857
NP_AtGH3.4:AtGH3.4cfs:GFP:HygR	GTGGAGAATGCGTCGAGGTTGCTTGCAGAGCAAGGAACACGTGTGATCGAGTATACGAGC	4857
AtGH3.4_1	-----	2788
AtGH3.4_2	-----	1277
AtGH3.4_3	GTGGAGAATGCGTCGAGGTTGCTTGCAGAGCAAGGAACACGTGTGATCGAGTATACGAGC	759
AtGH3.4_4	-----	
AtGH3.4_5	-----	
AtGH3.4_6	-----	
AtGH3.4_7	-----	
AtGH3.4_8	-----	
Consensus	TACGCAGATACGAAGACTATACCTGGTCACTACGTAATCTACTGGGAGCTACTTAGTAGA	4917
NP_AtGH3.4:AtGH3.4cfs:GFP:HygR	TACGCAGATACGAAGACTATACCTGGTCACTACGTAATCTACTGGGAGCTACTTAGTAGA	4917
AtGH3.4_1	-----	2848
AtGH3.4_2	-----	1337
AtGH3.4_3	TACGCAGATACGAAGACTATACCTG-----	819
AtGH3.4_4	-----	
AtGH3.4_5	-----	
AtGH3.4_6	-----	
AtGH3.4_7	-----	
AtGH3.4_8	-----	

Attachment 15. Sequence alignment of pNAtGH3.4:AtGH3.4cdr-GFP with a fragment of AtGH3.4 coding region resulting from the sequencing and confirming the correctness of assembly (part 2).

onsensus	TGAGCAAGGGCGAGGAGCTGTTACCAGGGGTGGTGCATCCTGGTCGAGCTGACGGCG	5397
P_AtGH3.4:AtGH3.4cdfs:GFP:HygR	TGAGCAAGGGCGAGGAGCTGTTACCAGGGGTGGTGCATCCTGGTCGAGCTGACGGCG	5397
tGH3.4_1	-----	3328
tGH3.4_2	-----	1817
tGH3.4_3	-----	1299
tGH3.4_4	TGAGCAAGGGCGAGGAGCTGTTACCAGGGGTGGTGCATCCTGGTCGAGCTGACGGCG	399
tGH3.4_5	-----	
tGH3.4_6	-----	
tGH3.4_7	-----	
tGH3.4_8	-----	
onsensus	ACGTAAACGGCCACAAGTTCAAGCTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCA	5457
P_AtGH3.4:AtGH3.4cdfs:GFP:HygR	ACGTAAACGGCCACAAGTTCAAGCTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCA	5457
tGH3.4_1	-----	3388
tGH3.4_2	-----	1877
tGH3.4_3	-----	1359
tGH3.4_4	ACGTAAACGGCCACAAGTTCAAGCTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCA	459
tGH3.4_5	-CGTAAACGGCCACAAGTTCAAGCTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCA	59
tGH3.4_6	-----	
tGH3.4_7	-----	
tGH3.4_8	-----	
onsensus	AGCTGACCCTGAAGTTTCATCTGCACCACCGCAAGCTGCCCTGGCCACCCTCG	5517
P_AtGH3.4:AtGH3.4cdfs:GFP:HygR	AGCTGACCCTGAAGTTTCATCTGCACCACCGCAAGCTGCCCTGGCCACCCTCG	5517
tGH3.4_1	-----	3448
tGH3.4_2	-----	1937
tGH3.4_3	-----	1419
tGH3.4_4	AGCTGACCCTGAAGTTTCATCTGCACCACCGCAAGCTGCCCTGGCCACCCTCG	519
tGH3.4_5	AGCTGACCCTGAAGTTTCATCTGCACCACCGCAAGCTGCCCTGGCCACCCTCG	119
tGH3.4_6	-----	
tGH3.4_7	-----	
tGH3.4_8	-----	
onsensus	TGACCACCTTCACCTACGGCGTGCAGTGTTCAGCCGCTACCCCGACCACATGAAGCAGC	5577
P_AtGH3.4:AtGH3.4cdfs:GFP:HygR	TGACCACCTTCACCTACGGCGTGCAGTGTTCAGCCGCTACCCCGACCACATGAAGCAGC	5577
tGH3.4_1	-----	3588
tGH3.4_2	-----	1997
tGH3.4_3	-----	1479
tGH3.4_4	TGACCACCTTCACCTACGGCGTGCAGTGTTCAGCCGCTACCCCGACCACATGAAGCAGC	579
tGH3.4_5	TGACCACCTTCACCTACGGCGTGCAGTGTTCAGCCGCTACCCCGACCACATGAAGCAGC	179
tGH3.4_6	-----	
tGH3.4_7	-----	
tGH3.4_8	-----	
onsensus	ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCA	5637
P_AtGH3.4:AtGH3.4cdfs:GFP:HygR	ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCA	5637
tGH3.4_1	-----	3568
tGH3.4_2	-----	2057
tGH3.4_3	-----	1539
tGH3.4_4	ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCA	639
tGH3.4_5	ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCA	239
tGH3.4_6	-----	
tGH3.4_7	-----	
tGH3.4_8	-----	
onsensus	AGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTTCGAGGGCGACACCCTGGTGA	5697
P_AtGH3.4:AtGH3.4cdfs:GFP:HygR	AGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTTCGAGGGCGACACCCTGGTGA	5697
tGH3.4_1	-----	3628
tGH3.4_2	-----	2117
tGH3.4_3	-----	1599
tGH3.4_4	AGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTTCGAGGGCGACACCCTGGTGA	699
tGH3.4_5	AGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTTCGAGGGCGACACCCTGGTGA	299
tGH3.4_6	-----	
tGH3.4_7	-----	
tGH3.4_8	-----	
onsensus	ACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGC	5757
P_AtGH3.4:AtGH3.4cdfs:GFP:HygR	ACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGC	5757
tGH3.4_1	-----	3688
tGH3.4_2	-----	2177
tGH3.4_3	-----	1659
tGH3.4_4	ACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGC	759
tGH3.4_5	ACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGC	359
tGH3.4_6	-----	
tGH3.4_7	-----	
tGH3.4_8	-----	
onsensus	TGGAGTACAACCTACAACAGCCACAACGTCTATATCATGSCCGACAAGCAGAAGAACGGCA	5817
P_AtGH3.4:AtGH3.4cdfs:GFP:HygR	TGGAGTACAACCTACAACAGCCACAACGTCTATATCATGSCCGACAAGCAGAAGAACGGCA	5817
tGH3.4_1	-----	3748
tGH3.4_2	-----	2237
tGH3.4_3	-----	1719
tGH3.4_4	TGGAGTACAACCTACAACAGCCACAACGTCTATATCATGSCCGACAAGCAGAAGAACGGCA	819
tGH3.4_5	TGGAGTACAACCTACAACAGCCACAACGTCTATATCATGSCCGACAAGCAGAAGAACGGCA	419
tGH3.4_6	-----	
tGH3.4_7	-----	
tGH3.4_8	-----	

Attachment 16. Sequence alignment of pNAtGH3.4:AtGH3.4cdr-GFP with a fragment of GFP coding region resulting from the sequencing and confirming the correctness of assembly (part 1).

Consensus	TCAAGGTGAACCTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACC	5877
NP_AtGH3.4:AtGH3.4cds:GFP:HygR	TCAAGGTGAACCTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACC	5877
AtGH3.4_1	-----	3888
AtGH3.4_2	-----	2297
AtGH3.4_3	-----	1779
AtGH3.4_4	TCAAGGTGAACCTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACC	879
AtGH3.4_5	TCAAGGTGAACCTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACC	479
AtGH3.4_6	-----	
AtGH3.4_7	-----	
AtGH3.4_8	-----	
Consensus	ACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACC	5937
NP_AtGH3.4:AtGH3.4cds:GFP:HygR	ACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACC	5937
AtGH3.4_1	-----	3868
AtGH3.4_2	-----	2357
AtGH3.4_3	-----	1839
AtGH3.4_4	ACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACC	939
AtGH3.4_5	ACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACC	539
AtGH3.4_6	-----CCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACC	43
AtGH3.4_7	-----	
AtGH3.4_8	-----	
Consensus	TGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGCTCCTGC	5997
NP_AtGH3.4:AtGH3.4cds:GFP:HygR	TGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGCTCCTGC	5997
AtGH3.4_1	-----	3928
AtGH3.4_2	-----	2417
AtGH3.4_3	-----	1899
AtGH3.4_4	TGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGCTCCTGC	999
AtGH3.4_5	TGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGCTCCTGC	599
AtGH3.4_6	TGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGCTCCTGC	183
AtGH3.4_7	-----	
AtGH3.4_8	-----	
Consensus	TGGAGTTCGTGACCGCCGCCGGGATCACTCACGGCATGGACGAGCTGTACAAGTAGCTGC	6057
NP_AtGH3.4:AtGH3.4cds:GFP:HygR	TGGAGTTCGTGACCGCCGCCGGGATCACTCACGGCATGGACGAGCTGTACAAGTAGCTGC	6057
AtGH3.4_1	-----	3988
AtGH3.4_2	-----	2477
AtGH3.4_3	-----	1959
AtGH3.4_4	TG-----	1059
AtGH3.4_5	TGGAGTTCGTGACCGCCGCCGGGATCACTCACGGCATGGACGAGCTGTACAAGTAGCTGC	659
AtGH3.4_6	TGGAGTTCGTGACCGCCGCCGGGATCACTCACGGCATGGACGAGCTGTACAAGTAGCTGC	163
AtGH3.4_7	-----	
AtGH3.4_8	-----	
Consensus	AAAGCTTTCGTTTCGTATCATCGGTTTCGACAACGTTTCGTCAAGTTCAATGCATCAGTTTC	6117
NP_AtGH3.4:AtGH3.4cds:GFP:HygR	AAAGCTTTCGTTTCGTATCATCGGTTTCGACAACGTTTCGTCAAGTTCAATGCATCAGTTTC	6117
AtGH3.4_1	-----	4848
AtGH3.4_2	-----	2537
AtGH3.4_3	-----	2019
AtGH3.4_4	-----	1119
AtGH3.4_5	AAAGCTTTCGTTTCGTATCATCGGTTTCGACAACGTTTCGTCAAGTTCAATGCATCAGTTTC	719
AtGH3.4_6	AAAGCTTTCGTTTCGTATCATCGGTTTCGACAACGTTTCGTCAAGTTCAATGCATCAGTTTC	223
AtGH3.4_7	-----	
AtGH3.4_8	-----	

Attachment 17. Sequence alignment of pNAtGH3.4:AtGH3.4cdr-GFP with a fragment of GFP coding region resulting from the sequencing and confirming the correctness of assembly (part 2).



Consensus	TTCAGAAACAGACCATTCAACAAGTACAACGCTACACTAGCCCTGACACAGACCATTCTT	7200
pNAtGH3.17: AtGH3.17cdr-GFP	TTCAGAAACAGACCATTCAACAAGTACAACGCTACACTAGCCCTGACACAGACCATTCTT	7200
AtGH3.17_1	-----	5193
AtGH3.17_2	-----	4840
AtGH3.17_3	-----	3984
AtGH3.17_4	TTCAGAAACAGACCATTCAACAAGTACAACGCTACACTAGCCCTGACACAGACCATTCTT	237
AtGH3.17_5	-----CCTGACACAGACCATTCTT	18
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	TGTCAAGACAGCAAGCAGAGCATGTACTGTCAGCTTCTCTGCGGTCTAGTACAGCGATCT	7260
pNAtGH3.17: AtGH3.17cdr-GFP	TGTCAAGACAGCAAGCAGAGCATGTACTGTCAGCTTCTCTGCGGTCTAGTACAGCGATCT	7260
AtGH3.17_1	-----	5253
AtGH3.17_2	-----	4100
AtGH3.17_3	-----	3964
AtGH3.17_4	TGTCAAGACAGCAAGCAGAGCATGTACTGTCAGCTTCTCTGCGGTCTAGTACAGCGATCT	297
AtGH3.17_5	TGTCAAGACAGCAAGCAGAGCATGTACTGTCAGCTTCTCTGCGGTCTAGTACAGCGATCT	78
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	CATGTCCTAAGAGTCGGAGCTGTCTTTCCTCTGCCTTTCTTCGAGCAGTCAAGTCTTG	7320
pNAtGH3.17: AtGH3.17cdr-GFP	CATGTCCTAAGAGTCGGAGCTGTCTTTCCTCTGCCTTTCTTCGAGCAGTCAAGTCTTG	7320
AtGH3.17_1	-----	5313
AtGH3.17_2	-----	4160
AtGH3.17_3	-----	4824
AtGH3.17_4	CATGTCCTAAGAGTCGGAGCTGTCTTTCCTCTGCCTTTCTTCGAGCAGTCAAGTCTTG	357
AtGH3.17_5	CATGTCCTAAGAGTCGGAGCTGTCTTTCCTCTGCCTTTCTTCGAGCAGTCAAGTCTTG	138
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	GAGGATCATTACAAAGAGCTTTGCGCTGACATTAGAACCAGTACTGTCACTAGCTGGATC	7380
pNAtGH3.17: AtGH3.17cdr-GFP	GAGGATCATTACAAAGAGCTTTGCGCTGACATTAGAACCAGTACTGTCACTAGCTGGATC	7380
AtGH3.17_1	-----	5373
AtGH3.17_2	-----	4220
AtGH3.17_3	-----	4884
AtGH3.17_4	GAGGATCATTACAAAGAGCTTTGCGCTGACATTAGAACCAGTACTGTCACTAGCTGGATC	417
AtGH3.17_5	GAGGATCATTACAAAGAGCTTTGCGCTGACATTAGAACCAGTACTGTCACTAGCTGGATC	198
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	ACTGACTCATCCTGCAGAGACTCGGTCTTGTGCGATCCTTAATGGCCCAAATCAAGAATTG	7440
pNAtGH3.17: AtGH3.17cdr-GFP	ACTGACTCATCCTGCAGAGACTCGGTCTTGTGCGATCCTTAATGGCCCAAATCAAGAATTG	7440
AtGH3.17_1	-----	5433
AtGH3.17_2	-----	4280
AtGH3.17_3	-----	4144
AtGH3.17_4	ACTGACTCATCCTGCAGAGACTCGGTCTTGTGCGATCCTTAATGGCCCAAATCAAGAATTG	477
AtGH3.17_5	ACTGACTCATCCTGCAGAGACTCGGTCTTGTGCGATCCTTAATGGCCCAAATCAAGAATTG	258
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	GCTGATGAAATTGAGAGTGAAGTGCCTGAAAAGTCTGSGAAGGAATCTTGAGGAGGATA	7500
pNAtGH3.17: AtGH3.17cdr-GFP	GCTGATGAAATTGAGAGTGAAGTGCCTGAAAAGTCTGSGAAGGAATCTTGAGGAGGATA	7500
AtGH3.17_1	-----	5493
AtGH3.17_2	-----	4340
AtGH3.17_3	-----	4204
AtGH3.17_4	GCTGATGAAATTGAGAGTGAAGTGCCTGAAAAGTCTGSGAAGGAATCTTGAGGAGGATA	537
AtGH3.17_5	GCTGATGAAATTGAGAGTGAAGTGCCTGAAAAGTCTGSGAAGGAATCTTGAGGAGGATA	318
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	TGGCCTAAGGCTAAATATGTTGAGGTGATTGTGACTGGTTCGATGGCTCAATACATTCCG	7560
pNAtGH3.17: AtGH3.17cdr-GFP	TGGCCTAAGGCTAAATATGTTGAGGTGATTGTGACTGGTTCGATGGCTCAATACATTCCG	7560
AtGH3.17_1	-----	5553
AtGH3.17_2	-----	4400
AtGH3.17_3	-----	4264
AtGH3.17_4	TGGCCTAAGGCTAAATATGTTGAGGTGATTGTGACTGGTTCGATGGCTCAATACATTCCG	597
AtGH3.17_5	TGGCCTAAGGCTAAATATGTTGAGGTGATTGTGACTGGTTCGATGGCTCAATACATTCCG	378
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	ACACTAGAGTTTTATAGCGGAGGTTTACCCTGGTTTCAACGATGTATGCTTCTCTGAG	7620
pNAtGH3.17: AtGH3.17cdr-GFP	ACACTAGAGTTTTATAGCGGAGGTTTACCCTGGTTTCAACGATGTATGCTTCTCTGAG	7620
AtGH3.17_1	-----	5613
AtGH3.17_2	-----	4460
AtGH3.17_3	-----	4324
AtGH3.17_4	ACACTAGAGTTTTATAGCGGAGGTTTACCCTGGTTTCAACGATGTATGCTTCTCTGAG	657
AtGH3.17_5	ACACTAGAGTTTTATAGCGGAGGTTTACCCTGGTTTCAACGATGTATGCTTCTCTGAG	438
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	

Attachment 18. Sequence alignment of pNAtGH3.17:AtGH3.17cdr-GFP with a fragment of AtGH3.17 coding region resulting from the sequencing and confirming the correctness of assembly (part 1).

Consensus	TGTTACTTTGGTATCAACCTTAATCCGTTGTGTGATCCTGCCGATGTTTCCTACACGCTT	7680
pNAtGH3.17:AtGH3.17cdr-GFP	TGTTACTTTGGTATCAACCTTAATCCGTTGTGTGATCCTGCCGATGTTTCCTACACGCTT	7680
AtGH3.17_1	-----	5673
AtGH3.17_2	-----	4520
AtGH3.17_3	-----	4384
AtGH3.17_4	TGTTACTTTGGTATCAACCTTAATCCGTTGTGTGATCCTGCCGATGTTTCCTACACGCTT	717
AtGH3.17_5	TGTTACTTTGGTATCAACCTTAATCCGTTGTGTGATCCTGCCGATGTTTCCTACACGCTT	498
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	CTTCCTAACATGGCTTACTTCGAGTTCCTGCCCGTCGACGACAAATCCCACGAAGAGATT	7740
pNAtGH3.17:AtGH3.17cdr-GFP	CTTCCTAACATGGCTTACTTCGAGTTCCTGCCCGTCGACGACAAATCCCACGAAGAGATT	7740
AtGH3.17_1	-----	5733
AtGH3.17_2	-----	4580
AtGH3.17_3	-----	4444
AtGH3.17_4	CTTCCTAACATGGCTTACTTCGAGTTCCTGCCCGTCGACGACAAATCCCACGAAGAGATT	777
AtGH3.17_5	CTTCCTAACATGGCTTACTTCGAGTTCCTGCCCGTCGACGACAAATCCCACGAAGAGATT	558
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	CACTTTGCAACTCACTCCAACACCGATGATGATGATGATGCTCTCAAGGAAGATCTCATC	7800
pNAtGH3.17:AtGH3.17cdr-GFP	CACTTTGCAACTCACTCCAACACCGATGATGATGATGATGCTCTCAAGGAAGATCTCATC	7800
AtGH3.17_1	-----	5793
AtGH3.17_2	-----	4640
AtGH3.17_3	-----	4504
AtGH3.17_4	CACTTTGCAACTCACTCCAACACCGATGATGATGATGATGCTCTCAAGGAAGATCTCATC	837
AtGH3.17_5	CACTTTGCAACTCACTCCAACACCGATGATGATGATGATGCTCTCAAGGAAGATCTCATC	618
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	GTCAATCTTGTTAATGTGCGAAGTCGGTCAATACTACGAAATCGTCATCACTACATTCA	7860
pNAtGH3.17:AtGH3.17cdr-GFP	GTCAATCTTGTTAATGTGCGAAGTCGGTCAATACTACGAAATCGTCATCACTACATTCA	7860
AtGH3.17_1	-----	5853
AtGH3.17_2	-----	4700
AtGH3.17_3	-----	4564
AtGH3.17_4	GTCAATCTTGTTAATGTGCGAAGTCGGTCAATACTACGAAATCGTCATCACTACATTCA	897
AtGH3.17_5	GTCAATCTTGTTAATGTGCGAAGTCGGTCAATACTACGAAATCGTCATCACTACATTCA	678
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	GGTCAGTGACTCTCTATACGAACACAATCTAGGTCTGATGTGGATTCTAAAACGGATAG	7920
pNAtGH3.17:AtGH3.17cdr-GFP	GGTCAGTGACTCTCTATACGAACACAATCTAGGTCTGATGTGGATTCTAAAACGGATAG	7920
AtGH3.17_1	-----	5913
AtGH3.17_2	-----	4760
AtGH3.17_3	-----	4624
AtGH3.17_4	GGTCAGTGACTCTCTATACGAACACAATCTAGGTCTGATGTGGATTCTAAAACGGATAG	957
AtGH3.17_5	GGTCAGTGACTCTCTATACGAACACAATCTAGGTCTGATGTGGATTCTAAAACGGATAG	738
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	TAACCTGTAGCACAAGTGACCTGTTACATATGTTTTGTCAATAGGTTGTACAGATACA	7980
pNAtGH3.17:AtGH3.17cdr-GFP	TAACCTGTAGCACAAGTGACCTGTTACATATGTTTTGTCAATAGGTTGTACAGATACA	7980
AtGH3.17_1	-----	5973
AtGH3.17_2	-----	4820
AtGH3.17_3	-----	4684
AtGH3.17_4	TAACCTGTAGCACAAGTGACCTGTTACATAT-----	1017
AtGH3.17_5	TAACCTGTAGCACAAGTGACCTGTTACATATGTTTTGTCAATAGGTTGTACAGATACA	798
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	GAGTAGGCGATATTCTAAAAGTGACGGGTTCCACAACAAAGCGCCTCAATTCCGTTTCG	8040
pNAtGH3.17:AtGH3.17cdr-GFP	GAGTAGGCGATATTCTAAAAGTGACGGGTTCCACAACAAAGCGCCTCAATTCCGTTTCG	8040
AtGH3.17_1	-----	6033
AtGH3.17_2	-----	4880
AtGH3.17_3	-----	4744
AtGH3.17_4	-----	1077
AtGH3.17_5	GAGTAGGCGATATTCTAAAAGTGACGGGTTCCACAACAAAGCGCCTCAATTCCGTTTCG	858
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	

Attachment 19. Sequence alignment of pNAtGH3.17:AtGH3.17cdr-GFP with a fragment of AtGH3.17 coding region resulting from the sequencing and confirming the correctness of assembly (part 2).

Consensus	ATCGGCGGCCGCTGCAATGGTGAGCAAGGGCGAGGAGCTGTTCAACGGGGTGGTGCCCAT	8780
pNAtGH3.17:AtGH3.17cdr-GFP	ATCGGCGGCCGCTGCAATGGTGAGCAAGGGCGAGGAGCTGTTCAACGGGGTGGTGCCCAT	8780
AtGH3.17_1	-----	6693
AtGH3.17_2	-----	5540
AtGH3.17_3	-----	5484
AtGH3.17_4	-----	1737
AtGH3.17_5	-----	1518
AtGH3.17_6	-----	
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	CCTGGTCGAGCTGGACGGCGACGTAACGGCCACAAGTTCAAGCGTGTCCGGCGAGGGCGA	8760
pNAtGH3.17:AtGH3.17cdr-GFP	CCTGGTCGAGCTGGACGGCGACGTAACGGCCACAAGTTCAAGCGTGTCCGGCGAGGGCGA	8760
AtGH3.17_1	-----	6753
AtGH3.17_2	-----	5680
AtGH3.17_3	-----	5464
AtGH3.17_4	-----	1797
AtGH3.17_5	-----	1578
AtGH3.17_6	-----TCGAGCTGGACGGCGACGTAACGGCCACAAGTTCAAGCGTGTCCGGCGAGGGCGA	55
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	GGGCGATGCCACCTACGGCAAGCTGACCCGAAAGTTCACTGCACCACCGGCAAGCTGCC	8820
pNAtGH3.17:AtGH3.17cdr-GFP	GGGCGATGCCACCTACGGCAAGCTGACCCGAAAGTTCACTGCACCACCGGCAAGCTGCC	8820
AtGH3.17_1	-----	6813
AtGH3.17_2	-----	5660
AtGH3.17_3	-----	5524
AtGH3.17_4	-----	1857
AtGH3.17_5	-----	1638
AtGH3.17_6	GGGCGATGCCACCTACGGCAAGCTGACCCGAAAGTTCACTGCACCACCGGCAAGCTGCC	115
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	CGTGCCCTGGCCACCCTCGTGACCACCTTCACTACGGCGTGCAGTGCTTCAGCCGCTA	8880
pNAtGH3.17:AtGH3.17cdr-GFP	CGTGCCCTGGCCACCCTCGTGACCACCTTCACTACGGCGTGCAGTGCTTCAGCCGCTA	8880
AtGH3.17_1	-----	6873
AtGH3.17_2	-----	5720
AtGH3.17_3	-----	5584
AtGH3.17_4	-----	1917
AtGH3.17_5	-----	1698
AtGH3.17_6	CGTGCCCTGGCCACCCTCGTGACCACCTTCACTACGGCGTGCAGTGCTTCAGCCGCTA	175
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	CCCCGACCACATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCA	8940
pNAtGH3.17:AtGH3.17cdr-GFP	CCCCGACCACATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCA	8940
AtGH3.17_1	-----	6933
AtGH3.17_2	-----	5780
AtGH3.17_3	-----	5644
AtGH3.17_4	-----	1977
AtGH3.17_5	-----	1758
AtGH3.17_6	CCCCGACCACATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCA	235
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	GGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAAGTT	9880
pNAtGH3.17:AtGH3.17cdr-GFP	GGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAAGTT	9880
AtGH3.17_1	-----	6993
AtGH3.17_2	-----	5840
AtGH3.17_3	-----	5784
AtGH3.17_4	-----	2837
AtGH3.17_5	-----	1818
AtGH3.17_6	GGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAAGTT	295
AtGH3.17_7	-----	
AtGH3.17_8	-----	

Attachment 20. Sequence alignment of pNAtGH3.17:AtGH3.17cdr-GFP with a fragment of GFP coding region resulting from the sequencing and confirming the correctness of assembly (part 1).

Consensus	CGAGGGCGACACCCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGG	9060
pNAtGH3.17: AtGH3.17cdr-GFP	CGAGGGCGACACCCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGG	9060
AtGH3.17_1	-----	7053
AtGH3.17_2	-----	5900
AtGH3.17_3	-----	5764
AtGH3.17_4	-----	2097
AtGH3.17_5	-----	1878
AtGH3.17_6	CGAGGGCGACACCCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGG	355
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	CAACATCCTGGGGCACAAGCTGGAGTACAACACCAAGCCACAACGTCTATATCATGGC	9120
pNAtGH3.17: AtGH3.17cdr-GFP	CAACATCCTGGGGCACAAGCTGGAGTACAACACCAAGCCACAACGTCTATATCATGGC	9120
AtGH3.17_1	-----	7113
AtGH3.17_2	-----	5960
AtGH3.17_3	-----	5824
AtGH3.17_4	-----	2157
AtGH3.17_5	-----	1938
AtGH3.17_6	CAACATCCTGGGGCACAAGCTGGAGTACAACACCAAGCCACAACGTCTATATCATGGC	415
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	CGACAAGCAGAAAGAACGGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAGGACGG	9180
pNAtGH3.17: AtGH3.17cdr-GFP	CGACAAGCAGAAAGAACGGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAGGACGG	9180
AtGH3.17_1	-----	7173
AtGH3.17_2	-----	6020
AtGH3.17_3	-----	5884
AtGH3.17_4	-----	2217
AtGH3.17_5	-----	1998
AtGH3.17_6	CGACAAGCAGAAAGAACGGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAGGACGG	475
AtGH3.17_7	-----	
AtGH3.17_8	-----	
Consensus	CAGCGTGCAGCTCGCCGACCACTACCAGCAGAAACACCCCATCGGCGACGGCCCCGTGCT	9240
pNAtGH3.17: AtGH3.17cdr-GFP	CAGCGTGCAGCTCGCCGACCACTACCAGCAGAAACACCCCATCGGCGACGGCCCCGTGCT	9240
AtGH3.17_1	-----	7233
AtGH3.17_2	-----	6000
AtGH3.17_3	-----	5944
AtGH3.17_4	-----	2277
AtGH3.17_5	-----	2058
AtGH3.17_6	CAGCGTGCAGCTCGCCGACCACTACCAGCAGAAACACCCCATCGGCGACGGCCCCGTGCT	535
AtGH3.17_7	-----GGCGACGGCCCCGTGCT	17
AtGH3.17_8	-----	
Consensus	GCTGCCCGACAACCACTACCTGAGCACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAA	9300
pNAtGH3.17: AtGH3.17cdr-GFP	GCTGCCCGACAACCACTACCTGAGCACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAA	9300
AtGH3.17_1	-----	7293
AtGH3.17_2	-----	6140
AtGH3.17_3	-----	6004
AtGH3.17_4	-----	2337
AtGH3.17_5	-----	2118
AtGH3.17_6	GCTGCCCGACAACCACTACCTGAGCACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAA	595
AtGH3.17_7	GCTGCCCGACAACCACTACCTGAGCACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAA	77
AtGH3.17_8	-----	

Attachment 21. . Sequence alignment of pNAtGH3.17:AtGH3.17cdr-GFP with a fragment of GFP coding region resulting from the sequencing and confirming the correctness of assembly (part 2).