University of São Paulo
"Luiz de Queiroz" College of Agriculture

Sugarcane *thi1* homologues: a molecular and functional study

Andréia Prata Vieira

Thesis presented to obtain the degree of Doctor in Science. Program: International Plant Cell and Molecular Biology

Piracicaba
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Bachelor and Licentiate in Biological Sciences

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EPIGRAPHE

“You know what you gotta do when life gets you down?

Just keep swimming...”

Dory – “Finding Nemo”

TABLE OF CONTENTS

ABSTRACT

Sugarcane *thi1* homologues: a molecular and functional study

Thiazole biosynthetic protein (THI1) is involved in the synthesis of the thiazole ring, a thiamine (vitamin B1) component. Thiamine is an essential co-factor in several carbohydrate and amino acid metabolic pathways. Prokaryotes and a few eukaryotes, such as fungi and plants, are able to synthesize thiamine *de novo*. These organisms contain the genes that encode the corresponding enzymes (such as THI1) that perform this metabolic function. THI1 actually functions as a reagent rather than as a conventional catalytic enzyme, as the THI1 polypeptide itself serves as the sulfide donor for thiazole formation. This gene also plays a role in organelle DNA damage tolerance. *Arabidopsis thaliana* has only one copy of the *thi1* gene (*At-thi1*). Transcripts derived from *At-thi1* are targeted simultaneously to chloroplasts and mitochondria by differential usage of two in-frame initiation codons. The *tz-201 A. thaliana thi1* mutant has been shown to accumulate more sucrose in its tissues than wild-type plants. This suggests that a better understanding of *thi1* genes and the role they play in cellular sucrose accumulation may be relevant for improving commercially important crops such as sugarcane. Sugarcane (*Saccharum* spp.) is a C4 photosynthesis monocot. Unlike *A. thaliana*, sugarcane has at least two *thi1* copies (*sc-thi1.1* and *sc-thi1.2*), as do the other C4 grasses. This thesis concerns the molecular and functional analyses of sugarcane *thi1* (*sc-thi1*) gene homologues. The identified alleles related to *sc-thi1.2* have some differences in sequence and seems to be diverging into two subgroups (*sc-thi1.2a* and *sc-thi1.2b*), based on phylogenetic analyses. Expression analysis showed that each *sc-thi1* copy is expressed differentially in individual tissues and in developing stages levels. Subcellular analysis showed that *sc-thi1.1* and *sc-thi1.2*b have the same cellular distribution pattern, distinct from the observed for *sc-thi1.2a*. *Sc-thi1.1* and *sc-thi1.2*b were also able to partially complement thiamine auxotrophy in a yeast mutant deficient in thiamine biosynthesis. A similar complementation assay is not possible in the *A. thaliana tz-201* mutant owing to low transformation efficiencies. Thus, *Physcomitrella patens* was chosen to generate *thi1* mutant lines for future functional complementation studies. *P. patens* is a moss used as a plant model, with a small size, short life cycle and a haploid dominant phase. Despite its simplicity, it has six *thi1* homologues copies. Homologous Recombination was used to generate *P. patens thi1* mutants. In each case, a target *thi1* gene was disrupted by replacing its coding region with an antibiotic resistance gene cassette. Single mutants were obtained for all six *thi1* gene copies. All the knockout lines were able to survive and grow with only minor effects on morphology and physiology. Deletion of one of the *thi1* gene copies (*PpThi1*.20F) drastically affected protoplast survival and regeneration, suggesting a role for this gene in early (polar) cell division and differentiation. The experimental design, which permits recycling of the selectable marker cassettes, provides a research platform for the construction of double, triple, quadruple or quintuple mutants in the future. The individual mutants line generated in this work, as well as the possible multiple mutants, will be useful for *thi1* functional complementation experiments and for discerning the specific functions of individual *thi1* gene family members.

Keywords: *Thi1* gene; Thiamine; Evolution; Genomic characterization; Sugarcane; *Physcomitrella patens*; Functional complementation

RESUMO

Homólogos a *thi1* em cana-de-açúcar: estudo molecular e funcional

THI1 (proteína da biossíntese de tiazol) está envolvida na síntese do anel de tiazol, um componente de tiamina (vitamina B1). A tiamina é um cofator essencial em várias vias metabólicas de carboidratos e aminoácidos. Somente procariontes e alguns eucariontes, como fungos e plantas, são capazes de sintetizar a tiamina *de novo*. A proteína THI1 atua mais como um reagente do que como uma enzima catalítica convencional, pois usa a si mesmo como doador de sulfeto para a formação do anel de tiazol. Este gene também está envolvido na tolerância ao dano no DNA das organelas. *A. thaliana* apresenta apenas uma cópia do gene *thi1*. Seu transcrito primário é direcionado simultaneamente aos cloroplastos e mitocôndrias através do uso diferencial de dois códons de iniciação, presentes no mesmo quadro aberto de leitura. Além disso, o mutante *tz-201* de *A. thaliana* acumula mais sacarose em seus tecidos do que a planta selvagem. Isso sugere que um melhor entendimento do gene *thi1* e seu papel no acúmulo de sacarose podem ser importantes para o melhoramento comercial de cultivares, como cana-de-açúcar. Cana-de-açúcar (*Saccharum* spp.) é uma monocotiledônea de metabolismo fotossintético C4. Diferentemente do observado em *A. thaliana*, a cana-de-açúcar possui pelo menos duas cópias (*sc-thi1.1* e *sc-thi1.2*) homólogas a *thi1*, como observado também para outras gramíneas C4. Nesta tese são discutidas análises moleculares e funcionais dos homólogos do gene *thi1* (*sc-thi1*) de cana-de-açúcar. Os alelos identificados como relativos a *sc-thi1.2* apresentam algumas diferenças em suas sequências e, baseado em análises filogenéticas, parecem estar divergindo em dois subgrupos (*sc-thi1.2a* e *sc-thi1.2b*). As análises de expressão mostraram que cada cópia de *sc-thi1* é diferencialmente expressa em diferentes tecidos e estágios de desenvolvimento. A análise de localização subcelular mostrou *sc-thi1.1* e *sc-thi1.2*b apresentam o mesmo padrão de distribuição, distinto do observado para *sc-thi1.2a*. *Sc-thi1.1* e *sc-thi1.2*b também foram capazes de complementar parcialmente a auxotrofia para tiamina em leveduras mutantes, deficientes na via de biossíntese de tiamina. Um teste similar de complementação funcional mutante *tz-201* de *A. thaliana* não é possível no devido à baixa eficiência de transformação. Assim, *Physcomitrella patens* foi escolhida para gerar linhagens mutantes de *thi1* para futuros estudos de complementação funcional. *P. patens* é um musgo usado como planta modelo, apresenta tamanho pequeno, um ciclo de vida curto e uma fase dominante haploide. Apesar de sua simplicidade, possui seis cópias homólogas a *thi1*. A técnica de Recombinação Homóloga foi escolhida para gerar os mutantes *thi1* de *P. patens*. Em cada mutante, uma das cópias de *thi1* foi interrompida, substituindo sua região codificante por um cassete de gene de resistência. Mutantes individuais foram obtidos para as seis cópias do gene *thi1*. As linhagens *knockouts* foram capazes de sobreviver e crescer apenas com alguns pequenos efeitos em sua morfologia e fisiologia. A deleção de uma das cópias de *thi1* (*PpThi1*.20F) afetou drasticamente a sobrevivência e regeneração dos protoplastos, sugerindo um papel deste cópia gênica no inicio da divisão e diferenciação celular. O desenho experimento utilizado para a geração destes mutantes permite a reciclagem dos cassetes de seleção, fornecendo uma plataforma para a construção de duplos, triplos, quádruplos, quíntuplos e sêxtuplos mutantes no futuro. Os mutantes individuais para cada cópia de *thi1* gerados nesse trabalho, bem como os possíveis mutantes múltiplos, serão úteis para experimentos de complementação funcional e o discernimento de funções específicas de diferentes membros da família gênica *thi1*.

Palavras-chave: Gene *thi1*; Tiamina; Evolução; Caracterização genômica; Cana-de-açúcar; *Physcomitrella patens*; Complementação funcional

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

ABA – Abscisic acid
ADK – Adenosine kinase
ADT – Adenosine diphospho-5-(b-ethyl)-4-methylthiazole-2-carboxylic acid
AHZ – 2-carboxylate-4-methyl-5-β-(ethyl adenosine 5'-diphosphate) thiazole
AIR – 5-aminoimidazole ribonucleotide
ANFAEVA – *Associação Nacional dos Fabricantes de Veículos Automotores* (National Association of Motor Vehicle Manufacturers)
At – *Arabidopsis thaliana*
BAC – Bacterial artificial chromosome
Bd – *Brachypodium distachyon*
BLAST – Basic Local Alignment Search Tool
CaMV 35S – Cauliflower Mosaic Virus 35S promoter
cDNA – Complementary DNA
CDS – Coding DNA Sequence
CEFAP – *Centro de Facilidade de Apoio a Pesquisa da Universidade de São Paulo* (Facility Center for Research Support of University of São Paulo)
CI – Consistency Index
cpDNA – Chloroplast DNA
CPK33 – Calcium-dependent protein kinase 33
CTP – Chloroplast transited peptide
DAB –– Days after blend
DAI – Days after inoculation
DHA – Dehydroalanine
DNA – Deoxyribonucleic acid
DSBs – DNA double-strand breaks
ER – Endoplasmic reticulum
EST – Expressed sequence tag
FAD – Flavin adenine dinucleotide
FAO - Food and Agriculture Organization of the United Nations
FAOSTAT – Food and Agriculture Organization of the United Nations: Statistics
FPKM – Fragments Per Kilobase Million
GaTE Lab – Genomic and transposable element Laboratory
GFP – Green fluorescence protein
GUS – β-glucuronidase
HAD – Haloacid dehalogenase phosphatase
HET-P –– Thiazole ring or 4-methyl-5-b-hydroxyethylthiazole phosphate
HMP-P – 4-amino-2-methyl-5-hydroxymethylpyrimidine monophosphate
HMP-PP – Pyrimidine ring or 4-amino-2-methyl-5-hydroxymethylpyrimidine diphosphate
HR –– Homologous recombination
KO – Knockouts

LAFIECO – *Laboratório de Fisiologia Ecológica de Plantas* (Laboratory of Ecological Plant Physiology)
LD – Light-Dark
LL – Light- Light
MMR – Mismatch repair
MPS – Mitochondrial pre-sequence
mRNA – Messenger RNA
MYA – Million years ago
NAD – Nicotinamide adenine dinucleotide
NAD(P) – Nicotinamide adenine dinucleotide phosphate
PCR – Polymerase chain reaction
PEG – Polyethylene glycol
Pi – Inorganic phosphate
Pp – *Physcomitrella patens*
qRT-PCR – Real time quantitative PCR
RENAVAM – *Registro Nacional de Veículos Automotores* (National Registry of Motor Vehicles)
RI – Retention Index
RNA – Ribonucleic acid
RNA-seq – RNA sequencing
ROS – Reactive oxygen species
RPKM – Reads Per Kilobase Million
SAS – Sugarcane Assembled Sequences
sb – *Sorghum bicolor*
sc – Sugarcane
si – *Setaria italica*
SUCEST – Sugarcane expressed sequence tag
TDPK -– ThDP kinase or Thiamine diphosphokinase
TGR -– Targeted gene replacement
TH1 – Biosynthetic bifunctional enzyme (HMP-P kinase/ThMP pyrophosphorylase)
ThDP (or ThPP or TPP) – Thiamine diphosphate or thiamine pyrophosphate ThMP pyrophosphorylase
THI1 -– Thiazole biosynthetic protein (plants)
$thi1_{as}$ – *thi1* antisense
$thi1_{s}$ – *thi1* sense
THI4 – Thiazole biosynthetic protein (yeast)
THIC – HMP-P synthase
ThMP – Thiamine monophosphate
ThMPase – ThMP phosphatase
ThTP – Thiamine triphosphate
USP – University of São Paulo
UTR – Untranslated region
WT – Wild-type
zm – *Zea mays*

LIST OF SYMBOLS

°C – Degrees Celsius

bp – Base pairs

cm – Centimeter

Gb – Gigabase

Kb – Kilobase

L – Liters

Mbp – Megabase pair

mg – Milligram

ml – Milliliter

mm – Millimeter

mM – Millimolar

nm – Nanometer

µl – Microliter

µm – Micrometer

1. INTRODUCTION

1.1. Thiamine

Thiamine was the first of the water-soluble vitamins to be identified, leading to the discovery of more such trace compounds essential for survival and to the notion of vitamin. It was also the first vitamin B to be described, hence the name B_1. It was isolated from rice polishings as the "anti-beriberi factor" in 1926 by Jansen and Donath, 300 years after the Dutch physicist Jacobus Bonitus described the beriberi (meaning "sheep") disease on Java. He wrote in 1630 [(Friedrich, 1988)]:

"A certain troublesome affliction which attacks men is called by the inhabitants [of Java] beriberi. I believe those whom this disease attacks with their knees shaking and legs raised up, walk like sheep. It is a kind of paralysis or rather tremor: for it penetrates the motion and sensation of the hands and feet, indeed, sometimes the whole body…"

In the 19th century, beriberi was widespread in eastern and Southeast Asia, and it was especially a problem for sailors on long voyages. The first insight into the real cause of beriberi came in the 1880s in the Japanese Navy, when a correlation between the sailors' diet and beriberi was noted by a Japanese naval surgeon, Kanehiro Takaki. After changing their diet, introducing meat and dry milk, the incidence of beriberi plummeted sharply. Despite this compelling connection, most of the medical community continued to believe that beriberi was the result of a microbial infection or a toxin produced by a microorganism (Fattal-Valevski, 2011).

In 1886 a Dutch medical officer, Dr. Christian Eijkman, discovered that chickens fed with white (polished or milled) rice developed a beriberi-like condition (polyneuritis), whereas red (partially polished) rice, unhusked rice (padi) and rice hulls prevented and even cured the disease. In 1901, another Dutch physician, Grijens, theorized that natural foodstuffs contained an unknown factor, absent in polished rice, which prevents the development of the disease (Berdanier and Adkins, 1998).

In 1906, Frederick Gowland Hopkins, a biochemist at Cambridge University, expressed the belief that there were unsuspected "dietetic factors" besides protein, carbohydrates, fat, and minerals that were vital for health (Semba and Bloem, 2008). In 1911 a young chemist in London, Dr. Casimir Funk, crystallized an amine substance from rice bran. He thought he had isolated the dietary factor involved in beriberi and coined the name vitamin (from "vital amine) for it (Funk, 1911). A year later, he presented the idea that beriberi, pellagra and scurvy were all nutritional

deficiency diseases. Later, in 1929, Eijkman and Hopkins received the Nobel Prize because their observations led to the discovery of vitamins (Semba and Bloem, 2008).

In 1926, Jansen and Donath finally isolated a compound from rice bran and named it aneurine. Unfortunately, those investigators missed the sulfur atom, and their published incorrect formula for aneurine caused confusion for several years. In the 30's, Williams and Cline published the first correct formula and synthesis for the vitamin, and came up with a new name "thiamin". The American Chemical Society added an "e" and "thiamine" is now the accepted term (Fattal-Valevski, 2011).

1.1.1. Thiamine structure and its role in metabolism

The name thiamine comes from the fact that the compound contains both a sulfur group (the thiol group) and nitrogen in its structure. Thiamine is a relatively simple compound of a pyrimidine (4-amino-2-methyl-5-pyrimidyl) ring linked to a thiazole (4-methyl-5-β-hydroxyethylthiazolium) ring by a methylene bridge (Figure 1). It can be phosphorylated on its hydroxyl group to form thiamine monophosphate (ThMP), diphosphate (ThDP), or triphosphate (ThTP) esters (Berdanier and Adkins, 1998).

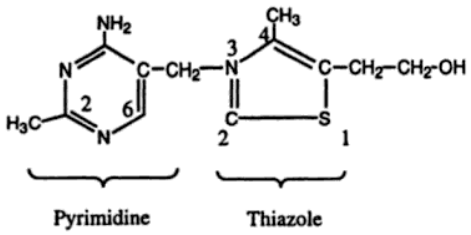

Figure 1. *Structure of thiamine.* Thiamine is composed of a pyrimidine ring linked to a thiazole ring by a methylene bridge (Berdanier and Adkins, 1998).

Thiamine diphosphate (ThDP), also called thiamine pyrophosphate (ThPP) or cocarboxylase, is the active (coenzymic) form of thiamine and it is the most abundant intracellular form of this vitamin in plants (Ajjawi, Tsegaye and Shintani, 2007) and yeast (Schweingruber *et al.*, 1991). It is essential to all living organisms as it serves as enzymatic cofactor in biosynthesis of terpenes, and branched-chain amino acids (valine, leucine and isoleucine) and in a variety of carbohydrate metabolic pathways, including the Calvin cycle, nonoxidative pentose phosphate pathways, the citric acid cycle, and glycolysis (Figure 2). Some of the enzymes for which it serves as cofactors include transketolase, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Belanger *et al.*, 1995). Deficiency of thiamine leads to a reduction in the activity of these enzymes, which is different for each enzyme and shows a strong cell-type dependency.

These activity reductions have been demonstrated using cultured cells, experimental models of thiamine deficiency in rats, and autopsied human tissues (Fattal-Valevski, 2011).

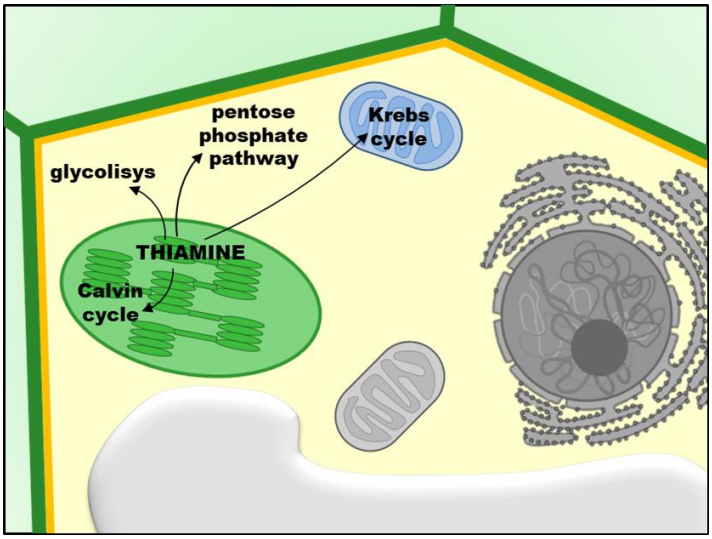

Figure 2. *The role of thiamine in a plant cell.* Plant cell structures are represented by green: chloroplast; blue: mitochondria; yellow: cytoplasm; dark green: cell wall; orange: plasmatic membrane; grey: nucleus; white: vacuole.

It has also been shown that ThDP has a role other than as cofactor, acting in resistance against abiotic and biotic stress (reviewed in Goyer, 2010). Tissues and organs with high cell division activity require thiamine, as Woodward *et al.* (2010) demonstrated in maize.

1.1.2. Bioavailability of thiamine

Though occurring in all living cells, only prokaryotes and a few eukaryotes, such as fungi and plants, are able to *de novo* synthesize thiamine. Human beings and the others higher animals require this cofactor in their diets (Singleton and Martin, 2001).

Thiamine is widely distributed in raw foods, such as cereals, green vegetables, beans, nuts, egg yolk and meats (especially pork and beef). The cereals correspond to the most important food source of thiamine, by virtue of its omnipresence in the diet of humans. However, highly processed foods – as polished rice, refined sugar and unenriched flours – have virtually no thiamine, as this vitamin is found primarily in the aleurone layers and in the embryo (Belanger *et al.*, 1995), parts that are removed during the refinement process. Also, little thiamine is found in foods heated for long time or at high temperature. Processed cereal products (e.g. flour, bread, cereals) have been fortified with thiamine since the early 1940s.

In humans, thiamine uptake is enhanced by thiamine deficiency and is decreased by thyroid hormone, diabetes, alcohol and age. Chronic alcohol consumption is the most common cause of acute thiamine deficiency in affluent societies (Depeint *et al.*, 2006). Thiamine is absorbed primarily in the duodenum and proximal jejunum small intestine by a specific active transport mechanism. At high concentrations, however, thiamine uptake appears to take place through simple passive diffusion (Laforenza *et al.*, 1997). A significant amount of the absorbed thiamine is phosphorylated, mainly to ThPP, thereby trapping the transported thiamine inside the absorptive cell. Intracellular ThPP is dephosphorylated by microsomal phosphatases to produce free thiamine before exit (Ball, 2004).

All cells of the body can accumulate thiamine. However, the human body does not store enough of this vitamin and thus a daily supply is needed. The total thiamine in the body is only about 30 mg, with 40% residing in the muscle and the rest found mainly in the brain, heart, liver and kidney (Depeint *et al.*, 2006). Thiamine excess is excreted in the urine (Berdanier and Adkins, 1998).

The thiamine needs of an individual are influenced by age, energy intake, carbohydrate intake, body weight and pregnancy. The U.S. Food and Nutrition Board (1998) recommend an average dietary allowance of 1.0 mg/day. Fitzpatrick *et al. (*2012) estimated that thiamine content should be increased 3.9-, 5.7- and 3.0-fold in wheat, rice and corn, respectively, to reach the recommended daily allowance if any one of these crops represents 80% of the daily intake of calories.

1.1.3. Thiamine biosynthesis

Thiamine biosynthesis occurs in bacteria, some protozoans, plants, and fungi. Since thiamine was described, many studies have been done to elucidate the biochemical basis for its synthesis in both prokaryotes and eukaryotes. For both kingdoms, the thiamine diphosphate (ThDP) biosynthesis involves the separate syntheses of the thiazole (4-methyl-5-b-hydroxyethylthiazole phosphate, HET-P) and pyrimidine (4-amino-2-methyl-5-hydroxymethylpyrimidine diphosphate, HMP-PP) moieties which are subsequently coupled, with the corresponding release of phosphate (Pi).

In plants, the pyrimidine moiety (HMP-PP) of thiamine is very likely synthesized via a pathway identical to that of bacteria in which the first committed step involves a complex chemical rearrangement of 5-aminoimidazole ribonucleotide (AIR) to 4-amino-2-methyl-5-hydroxymethylpyrimidine monophosphate (HMP-P) which is catalyzed by HMP-P synthase

(THIC). Then, HMP-P is phosphorylated to HMP-PP (the pyrimidine ring) by thiamine biosynthetic bifunctional enzyme (TH1) (which also catalyzes the condensation of HMP-PP and HET-P) (Goyer, 2010) (Figure 3). Indeed, a homologue of bacterial THIC was characterized in *Arabidopsis* (Raschke *et al.*, 2007; Kong *et al.*, 2008), and homologue of bacterial TH1 were characterized in *Brassica napus* (Kim *et al.*, 1998), *Zea mays* (Rapala-Kozik *et al.*, 2007) and *Arabidopsis* (Ajjawi, Tsegaye and Shintani, 2007).

In the case of the thiazole moiety (HET-P), prokaryotes and eukaryotes use different pathways. Thiazole biosynthesis in bacteria is well characterized and involves a complex chain of oxidative condensation reactions that use 1-deoxy-D-xylulose-5-phosphate, glycine (or tyrosine) and a sulfur source (Jurgenson, Begley and Ealick, 2009). Sulfur is proposed to be derived from cysteine and incorporated into the thiazole ring by a series of enzyme-mediated sulfur transfer steps that are encoded by six genes: *ThiS, ThiF, ThiG, ThiI*, and *IscS* (*NifS*) (Hwang *et al.*, 2014).

In eukaryotes, a different pathway leads to the formation of the thiazole moiety and depends on a single gene product, the so-called thiamine thiazole synthase: THI1 in *Arabidopsis thaliana* (Machado *et al.*, 1996) and THI4 in *Saccharomyces cerevisiae* (Praekelt and Meacock, 1992). THI1 catalyzes the formation of an adenylated thiazole product (ADT, adenosine diphospho-5-(b-ethyl)-4-methylthiazole-2-carboxylic acid) from NAD, glycine and a sulfide group (Chatterjee *et al.*, 2007) which is subsequently hydrolyzed to HET-P by a so-far-uncharacterized enzyme (Dong *et al.*, 2016). Chatterjee *et al.* (2011) demonstrated that the THI1 homologue in yeast (THI4) uses itself as a sulfide donor for thiazole formation, modifying itself. In other words, the donor protein functions as a reagent rather than as a conventional catalytic enzyme. The function of the modified THI1 (after sulfide donation) if any, is still unknown.

Thiazole biosynthesis in archaea is poorly understood. Interestingly, most archaea have homologues that cluster to the THI4 family proteins, but lack the conserved cysteine residue of the yeast protein and instead have a histidine residue which is well conserved. Hwang *et al.* (2014), studying *Haloferax volcanii*, proposed a model in which bacterial related enzymes are used for synthesis of the pyrimidine moiety and eukaryotic enzymes are used for formation of the thiazole ring in archaea that present the cysteine residue (halophilic archaea and ammonium oxidizing archaea).

ThMP is then dephosphorylated to thiamine by at least one phosphatase from the haloacid dehalogenase phosphatase (HAD) family (ThMPase (Hasnain *et al.*, 2016)). Both ThMP and thiamine are transported from the chloroplast to the cytosol by unknown enzyme. Thiamine is then pyrophosphorylated to ThDP by ThDP kinase (TDPK) in the cytosol (Ajjawi *et al.*, 2007). ADK add another phosphate to ThDP to form ThTP (Dzeja and Terzic, 2009).

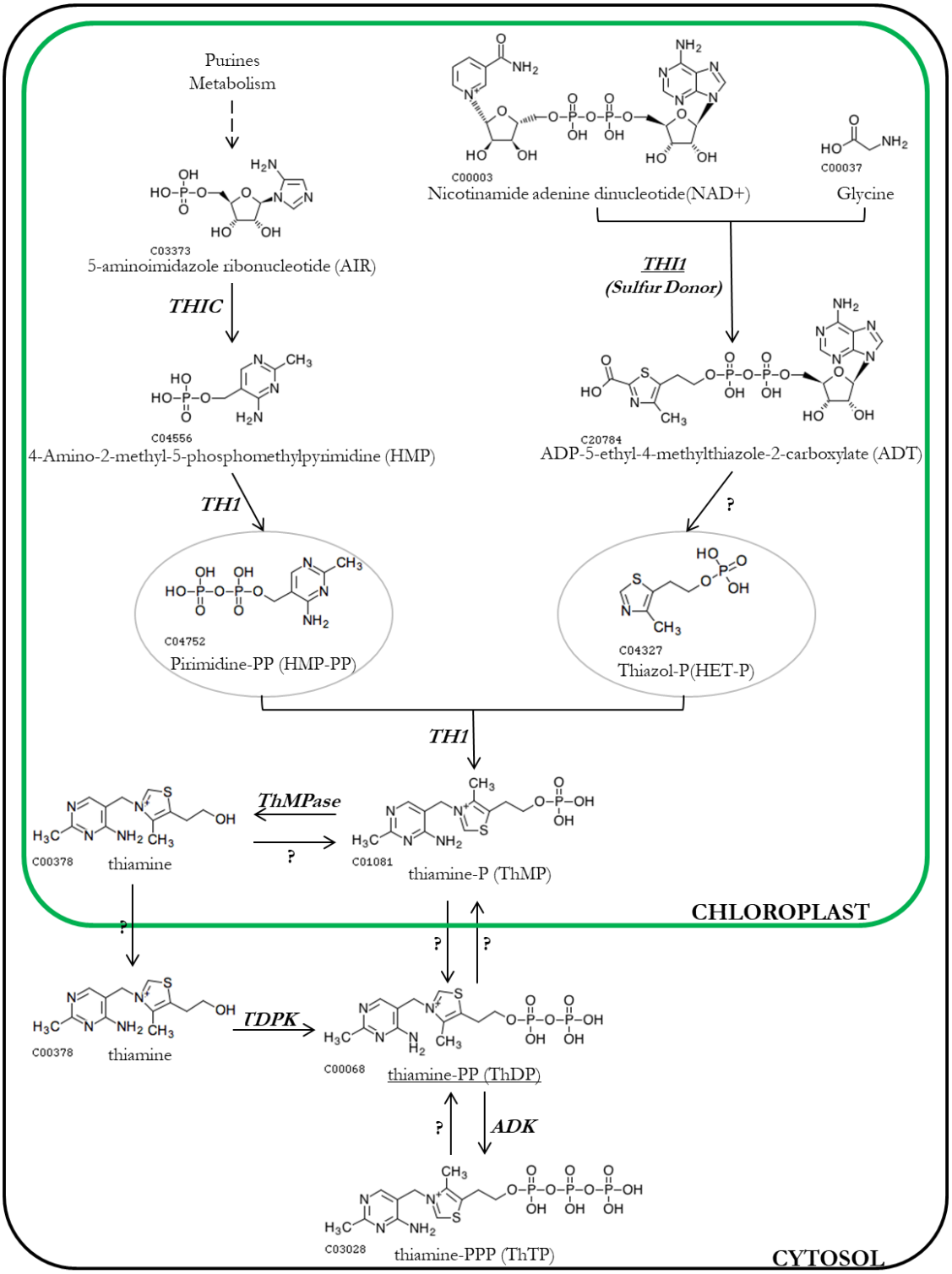

Figure 3. *Thiamine biosynthesis in Plants*. Thiamine-PP (underlined) is the active form of vitamin B_1. The enzymes well described in this route are shown in italics: THIC = HMP-P synthase; THI1 = HET-P synthase; TH1 = HMP-P kinase/ThMP pyrophosphorylase; ThMPase = ThMP phosphatase; TDPK = Thiamine diphosphokinase; ADK = adenosine kinase. The question mark indicates that the gene/enzyme has not been characterized yet. THI1 (underlined) is the objective of this study. It acts as a sulfur donor in the reaction between NAD+ and Glycine to form the precursor molecule of the Thiazole ring (HET-P). (Modified from: (Goyer, 2010; Dong, Stockwell and Goyer, 2015; KEGG website).

1.1.4. Metabolic control of thiamine level

THIC, which synthesizes the pyrimidine moiety, and THI1, which synthesizes the thiazole moiety, are key enzymes in thiamine metabolism, and, as such, are obvious targets for thiamine engineering. Many studies have been done using these two enzymes.

Thiamine biosynthesis is largely regulated by the biological clock. It was demonstrated that both the *thiC* and *thi1* promoters are under the control of the circadian clock (Momoli, 2008; Bocobza *et al.*, 2013, respectively).

The *thiC* gene is feedback regulated by ThDP through a ThDP riboswitch located in its 3'-untranslated region (UTR) that negatively regulates THIC gene expression and protein production when cellular ThDP levels increase. This occurs by causing an alternative splicing that leads to the generation of an unstable transcript, and thus decreasing ThDP biosynthesis (Sudarsan, 2003; Bocobza *et al.*, 2007) (Figure 4). This mechanism seems crucial to prevent thiamine over accumulation. However, *Arabidopsis* plants carrying a deficient riboswitch had only a modest increase of total thiamine levels in leaves and seeds (Bocobza *et al.*, 2013).

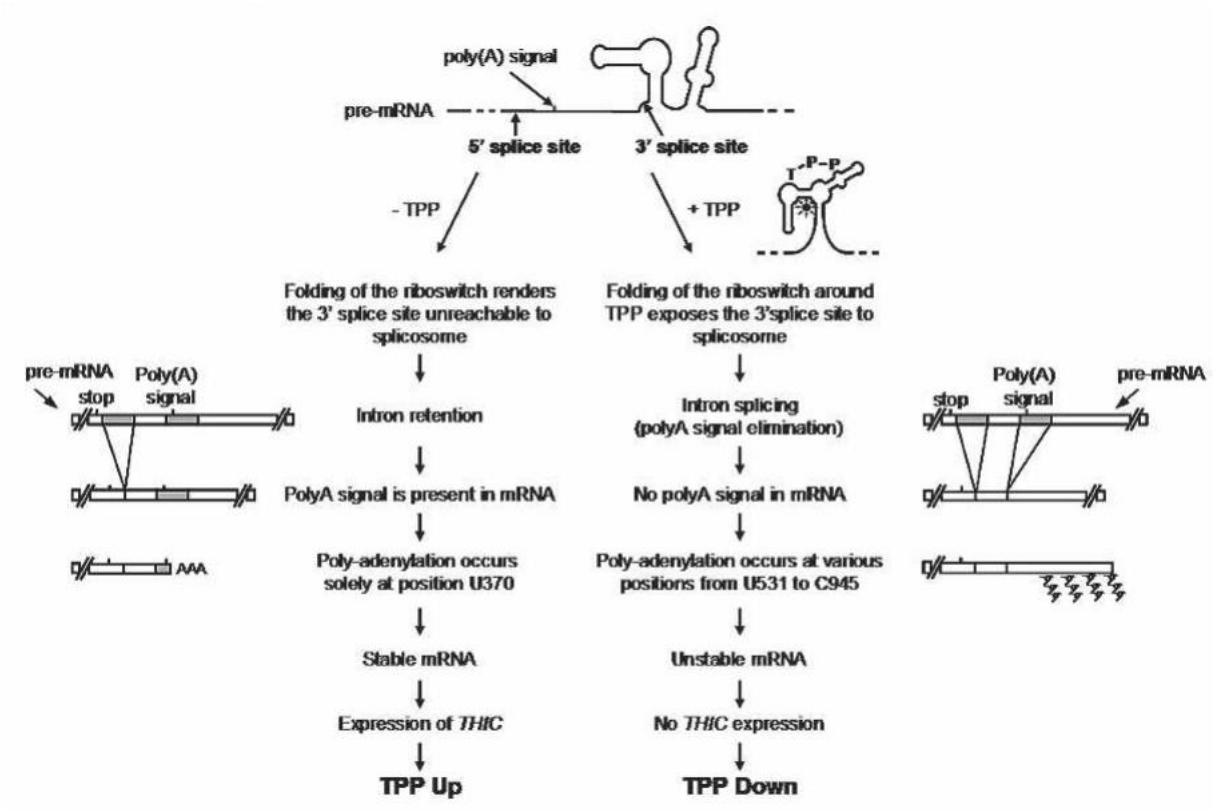

Figure 4. *ThDP (TPP) riboswitch-mediated regulation of the gene expression via alternative splicing in vascular plants.* The 3' splice site is highlighted with an asterisk (reproduced from Bocobza *et al.*, 2007).

While the ThDP riboswitch is present in the *thiC* genes of all plant taxa, the same riboswitch in the *thi1* gene was lost during gymnosperm evolution. However, ancient plants such as *Physcomitrella patens* (bryophyte) contain the ThDP riboswitch sequence in their *thi1* genes. Interestingly, in contrast to *A. thaliana*, *P. patens* have at least four *thi1* genes, of which three possess a ThDP riboswitch (Bocobza *et al.*, 2007).

The expression of *thiC* and *thi1* genes is highly correlated in all *Arabidopsis* organs except during embryo maturation where *thi1* expression is maintained while *thiC* expression declines. Divergent expression of *thiC* and *thi1* is more pronounced in maize, which displays at least six developmental contexts in which metabolically active, non-photosynthetic organs exhibit low expression of one or both branches of the *de novo* thiamine biosynthetic pathway indicating a dependence on inter-cellular transport of thiamine and/or thiamine precursors (Guan *et al.*, 2014).

While overexpression of single-gene THI1 or THIC plants do not accumulate significant amounts of thiamine, plants overexpressing both THIC and THI1 (THI1 × THIC) displayed an increased thiamine content of up to fivefold in unpolished seed, threefold in leaves and twofold in seeds (Dong, Stockwell and Goyer, 2015; Dong *et al.*, 2016).

Although Ahn *et al.* (2005) showed that exogenous application of thiamine leads to enhanced resistance to *Xanthomonas oryzae* pv. *oryzae*, thiamine-accumulating plants by overexpression of THIC and THI1 did not display altered resistance to this pathogen. Moreover, THI1 × THIC plants subjected to various abiotic stresses did not show any visible or biochemical changes compared to the wild type (Dong, Stockwell and Goyer, 2015; Dong *et al.*, 2016).

On the other hand, the increase of thiamine levels in plants subjected to abiotic stress correlates with the accumulation of THI1 mRNA transcripts (Tunc-Ozdemir *et al.*, 2009), and the THI1 promoter was shown to be responsive to stress conditions as shown by THI1 promoter–GUS (β-glucuronidase) fusion experiments performed in *A. thaliana* (Ribeiro *et al.*, 2005). Moreover, transgenic rice plants with repressed expression of OsDR8, a THI1 homologue in rice, had lower levels of thiamine and showed reduced resistance to *X. oryzae* pv. o*ryzae* (Wang *et al.*, 2006). This suggests that, in contrast to THIC, THI1 has another function, in addition to its role in thiamine biosynthesis.

1.2. Thiazole biosynthetic protein (THI1) orthologues

The "Thiazole Biosynthetic Protein" is a conserved protein, with orthologues found in fungi, archaea and plants. The *Schizosaccharomyces pombe* gene *thi4* was the first to be identified (Schweingruber *et al.,* 1991). Homologous genes were identified in other fungi, as *Saccharomyces cerevisiae* (Praekelt and Meacock, 1992) and *Uromyces fabae* (Sohn *et al.*, 2000), as well as in many sequenced archaea, as *Methanococcus jannaschii* (Bult *et al.*, 1996), *Methanobacterium thermoautotrophicum* (Smith *et al.*, 1997), *Archaeoglobus fulgidus* (Klenk *et al.*, 1997), *Pyrococcus horikoshii* (Kawarabayasi *et al.*, 1998) and *Aeropyrum permix* (Kawarabayasi *et al.*, 1999). Just one eubacteria, *Thermotoga marítima*, contains a sequence homologous to *thi4*, probably due to horizontal gene transfer from an archaeon (Nelson *et al.*, 1999).

Homologues were also identified in many plant species. The plant *thi4* homologue was first described in *Zea mays*, and was named *thi1* because it was the first plant protein involved in thiamine biosynthesis to be described (Belanger *et al.*, 1995). Later studies identified *thi4* homologues in several other plants, for example, *At-thi1* from *Arabidopsis thaliana* (Machado *et al.*, 1996), *Ag-thi1* from *Alnus glutinosa* (Ribeiro *et al.*, 1996), *Cs-thi1* from *Citrus sinensis* (Jacob-Wilk *et al.*, 1997) and *OsDR8* from *Oryza sativa* (Wang *et al.*, 2006). Moreover, *thi1* homologues have been identified in all sequenced plants, as will be discussed further in the next Chapter. cDNAs corresponding to *thi1* were also identified in *Saccharum* spp. (Vettore *et al.*, 2003).

1.2.1. THI1 orthologues as a bifunctional protein

Interestingly the first identification of *At-thi1* was not related to the function of the protein in the thiamine biosynthetic pathway. It was actually isolated in a complementation assay screen for genes involved in DNA repair carried out by Machado *et al.* (1996). They confirmed in a complementation assay that the *At-thi1* gene could functionally complement the yeast *thi4* disruption mutant strain by restoring it to thiamine prototrophy. Besides that, the *At-thi1* cDNA shows homology to stress-related genes of *Fusarium* (*sti35*) (Choi *et al.*, 1990). Involvement of THI1 and its orthologues in DNA protection and other stress-related pathways (such as sugar deprivation, high salinity, hypoxia, and oxidative stress) have been proposed (Machado *et al.*, 1996; Ribeiro *et al.*, 2005; Medina-Silva *et al.*, 2006; Ruiz-Roldán *et al.*, 2008; Tunc-Ozdemir *et al.*, 2009). In addition, abscisic acid (ABA) played an important role in up-regulation of thiamine biosynthetic genes THI1 during salt stress (Rapala-Kozik *et al.*, 2012). Li *et al.* (2016)

demonstrated that THI1 interacts with and represses CPK33 kinase activity, a protein essential for ABA-induced stomatal closure in response to drought.

Wang *et al.* (2015) suggested that thiamine could help reduce the generation of ROS (reactive oxygen species) in chloroplasts of *Kandelia candel*. The mechanism of this protection remains unknown. A study in yeast showed that one possibility is that abundant THI4 (THI1 orthologue in yeast) protects the cells by binding free cellular iron, which is known to cause oxidative damage via the generation of ROS (Chatterjee *et al.*, 2011). Thus, THI1 and its orthologues appear to be dual function proteins, with roles in both thiamine biosynthesis and in organellar DNA damage tolerance.

In thiamine biosynthesis of yeast, THI4 is responsible for catalyzing the formation of the thiazole ring from NAD, glycine, and a sulfur donor (Chatterjee *et al.*, 2007) through a process that occurs in the mitochondria in yeast cells (Belanger *et al.*, 1995; Machado *et al.*, 1996). Chatterjee *et al.* (2011) showed that THI4 is a suicide enzyme undergoing only a single turnover reaction in which THI4 Cys205 (a high conserved residue) serves as the sulfur donor, while NAD acts as the source of the carbon chain. Garcia *et al.* (2014) confirmed that At-THI1 acts in the same way. At-THI1 probably loses a sulfur atom from its Cys172 side chain during biosynthesis converting Cys172 to a dehydroalanine (DHA) residue (Figure 5).

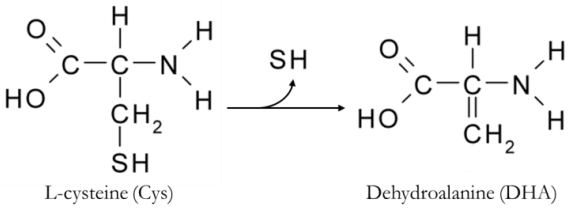

Figure 5. *Probable sulfur loss reaction by the Cys172 of A-THI1 cysteine 172.*

The function of the modified THI1 (after sulfide donation) if any, as well as a mechanism for restoring the THI1, if exists, are still unknown. On possibility is that this exchanges the sulfur for a free cellular iron, as in the mechanism proposed by Chatterjee *et al.* (2011) in yeast.

1.2.2. At-THI1 structure

The At-THI1 predicted polypeptide has structural features characteristic of several protein motifs: amino-terminal chloroplast transit peptide, dinucleotide binding site, DNA binding and bacterial DNA polymerases.

THI1 is one of the few plant enzymes involved in thiamine biosynthesis whose 3D structure is known. The structure was solved by Godoi *et al.* (2006) and reveals that this protein assembles as an octamer arranged as a tetramer of dimers. Also, THI1 combines with a 2-carboxylate-4-methyl-5-β-(ethyl adenosine 5'-diphosphate) thiazole (AHZ) ligand. The same authors also showed that THI1 contains the characteristic GxGxxG motif typical of dinucleotide binding proteins.

The monomeric THI1 protein presents a long α-helix followed by a globular αβ domain with a three-layer (ααβ) sandwich architecture and a topology similar to FAD/NAD(P) binding domain (Godoi *et al.*, 2006) (Figure 6). Nevertheless, several studies suggest that THI1 binds NAD+, whereas FAD binding has not been demonstrated (Chatterjee *et al.*, 2007). THI1 shares some structural similarity with an orthologue from *Bacillus subtilis*, *ThiO*, an FAD-dependent enzyme required for the thiazole biosynthesis, showing a well conserved dinucleotide binding architecture (Godoi *et al.*, 2006).

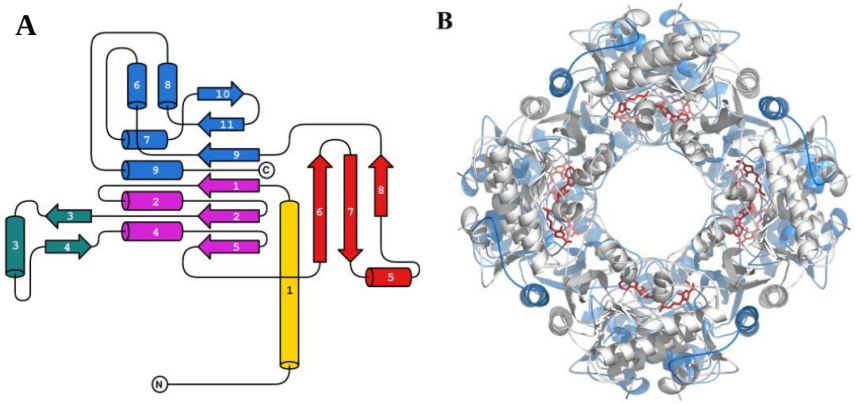

Figure 6. *Structure of THI1.* A) Monomeric structure: topology diagram of THI1 depicting the two halves variation of mononucleotide-binding motifs, colored in magenta and blue. The first α-helix is in yellow, β-subdomain is in green, and the β-meander is colored red. B) Octameric structure: each ring torus layer was distinctly colored (gray and blue). All AHZ molecules are represented in red. (Modified from Godoi *et al.* (2006)).

1.2.3. Subcellular localization of THI1 in *Arabidopsis*

In yeast, the THI4 protein is encoded by the nuclear genome, but bears a mitochondrial transport signal sequence at its N-terminal, which indicates that it is targeted to this organelle (Machado *et al.*, 1997). However, in plants, both thiazole and pyrimidine moieties are synthesized in plastids (Julliard and Douce, 1991; Gerdes *et al.*, 2012).

The At-*thi1* gene is also a single nuclear gene, but At-THI1 protein is targeted to both mitochondrial and chloroplasts by a differential usage of two in-frame translational start codons. The translation initiation at the first AUG directs translocation of THI1 to chloroplasts, which is where thiamine biosynthesis occurs in plants. However, when translation starts from the second AUG, THI1 is directed to the mitochondria (Chabregas *et al.*, 2001, 2003) (Figure 7).

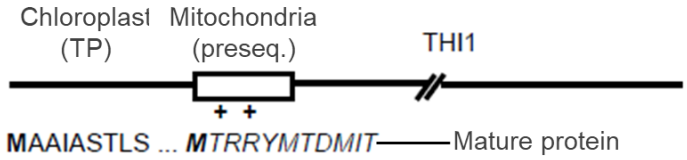

Figure 7. *Schematic representation of the At-THI1 N-terminal region.* Below the line is the consensus sequence (single letter amino acid code) for translational initiation. Both start codons (methionine) are in bold type. (Modified from Chabregas *et al.* (2001)).

Therefore, the dual targeting of HET-P synthase (thiazole) would enable this enzyme to function in protection against DNA damage when targeted to both mitochondria and chloroplast, and to function additionally in thiamine biosynthesis when targeted to chloroplasts. Translation preferentially occurs at the first AUG of this protein, suggesting a likely requirement for THI1 in chloroplasts (Chabregas *et al.*, 2003). One possibility is that THI1 may donate sulfur to other requiring molecules.

Figure 8 summarizes the route of THI1 throughout chloroplast and mitochondria and the role of the *thi1* gene in the thiamine metabolic pathway.

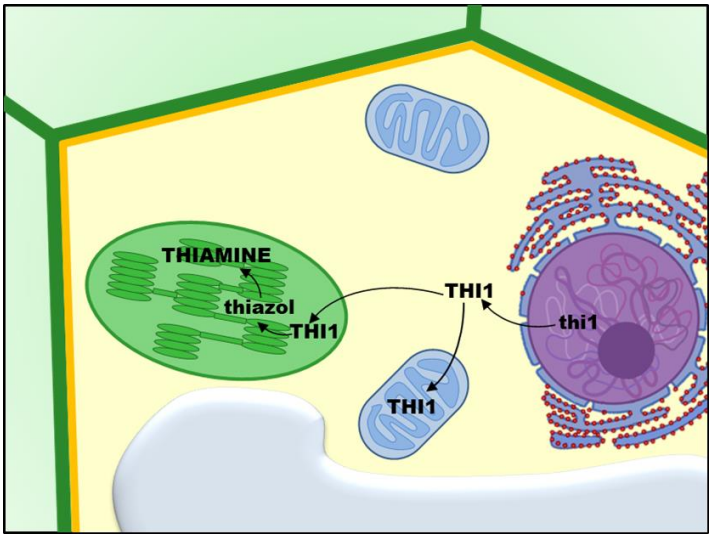

Figure 8. *Dual targeting of THI1 in a plant cell.* The *thi1* gene translates to the THI1 protein (Machado *et al.*, 1996; Ribeiro *et al.*, 1996; Papini-Terzi *et al.*, 2003), which is targeted simultaneously to mitochondria and to chloroplasts, where it acts in the thiamine synthesis (Chabregas *et al.*, 2001). Plant cell structures are represented by purple: nucleus; green: chloroplast; blue: mitochondria; yellow: cytoplasm; dark green: cell wall; orange: plasmatic membrane; white: vacuole.

1.2.4. *At-thi1* promoter and expression

Expression of the plant *At-thi1* mRNA is not affected by thiamine availability but it is associated with cells undergoing particular developmental pathways such as nodule formation and differentiation (Ribeiro *et al.*, 1996; Nagae *et al.*, 2016), ethylene-induced fruit maturation (Jacob-Wilk *et al.*, 1997) and it is affected by light, as transcript levels are reduced in dark conditions (Papini-Terzi *et al.*, 2003).

High expression rates of the *thi1* gene were detected in tissues with intense metabolic activity (such as leaves), as previously described for *thi1* homologues in plants (Belanger *et al.*, 1995; Ribeiro *et al.*, 1996). Maize (*Zea mays*) *thi2*, a paralog of *A. thaliana thi1*, is highly expressed in dividing tissues (shoot apical meristem, immature tassel, and leaf primordium) and regulates organ formation (Woodward *et al.*, 2010).

Papini-Terzi *et al.* (2003) demonstrated that *thi1* mRNA is highly expressed in leaves in contrast to roots, in which only very low amounts of *thi1*-mRNA are detected. This is in spite of the fact that plants used in these analyses were cultivated *in vitro*, and thus their roots were exposed to light. In the same work they suggest that the low levels of *thi1* in roots could be due to lack of plastid maturity and, consequently, readiness for thiamine synthesis, as isolated roots from most plant species cannot grow *in vitro* without thiamine supplementation (Bonner, 1940).

Overexpression and repression of the *At-thi1* gene was obtained by sense (*thi1*$_s$) and antisense (*thi1*$_{as}$) constructs made by Kashiwabara (2003). The overexpression (up to 10-fold, compared to controls) and the suppression of expression (2-fold lower than controls) of *thi1* mRNA by *thi1*$_s$ and *thi1*$_{as}$ lines, respectively, do not trigger substantial physiological alterations. Although the protein levels are altered, since the *thi1*$_s$ produces more RNA, while the *thi1*$_{as}$ produces less RNA, the resulting proteins are nonetheless functional and are sufficient to maintain the vital functions of *thi1*$_{as}$ line (Momoli, 2008).

Ribeiro *et al.* (2005) investigated *thi1* expression, through *thi1* promoter fusion to the β-glucuronidase (GUS) reporter gene *uidA*. They showed that expression derived from the *thi1* promoter is detected early during development and continues throughout the plant's life cycle and is broadly detected in all organs. Moreover, high levels of *thi1* promoter are observed in both shoots and roots during vegetative growth although, in roots, expression is restricted to the vascular system. In shoots, *thi1* promoter activity is comparable to the 'constitutive' CaMV 35S promoter. In the same work, they characterized the *thi1* promoter, showing that a fragment of 306 bp possesses all the essential signals for tissue specificity, as well as for responsiveness to stress conditions such as sugar deprivation, high salinity and hypoxia.

Finally, the *thi1* promoter is under daylight control, whereby the gene is down-regulated in the dark and up-regulated in the presence of light (Ribeiro *et al.*, 2005) which correlates with metabolic activity of pathways in which the ThDP cofactor is needed in the chloroplasts and mitochondria during the day.

Expression analysis (with peaks of expression at 18h, 42h and 66h of culture in light-dark conditions) suggests that *thi1* is not only responsive to light but is also under the control of the circadian clock (Momoli, unpublished data) (Figure 9). Furthermore, the same author showed that *thi1* mRNA levels peak at 3 p.m., while the THI1 protein accumulates at 5 p.m., suggesting that control of *thi1* gene expression is primarily at the transcriptional level. The accumulation at the end of the day suggests that THI1 produced in photosynthetic tissues may be sent to root tissues (Ribeiro *et al.*, 2005).

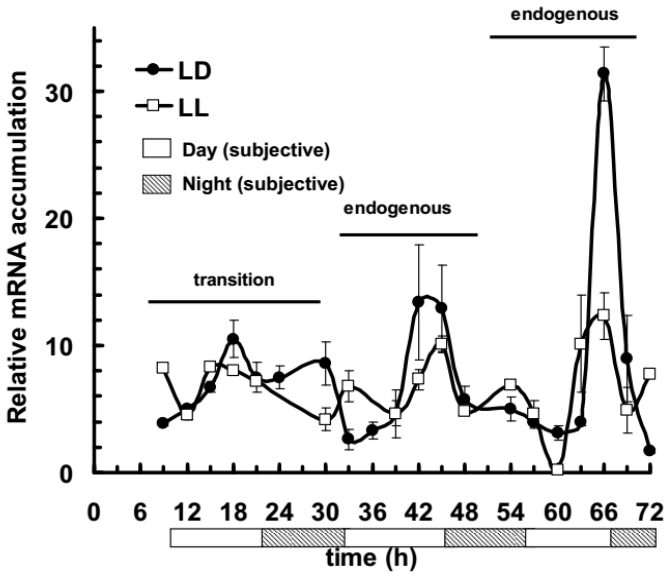

Figure 9. *Transcription of the A. thaliana thi1 gene follows a circadian rhythm. Thi1* mRNA levels quantified by qRT-PCR in wild-type (WT) plants. Open and hatched bars along the horizontal axis represent light and dark to LD condition, and subjective light and subjective dark to LL condition. LD: Plants were submitted to 16 h photoperiod for 72 h. LL: Plants submitted to continuous light (free-running) (Momoli, unpublished data)

1.2.5. *A. thaliana tz-201* mutant

The *At-thi1* gene is located on chromosome V of *A. thaliana* within the *tz* locus (Ribeiro *et al.*, 1996). This gene contains two introns of 123 and 85 bp.

In *A. thaliana*, many thiamine-requiring mutants have been isolated (Langridge, 1955; Feenstra, 1964; Redei, 1965; Li and Rédei, 1969; Koornneef and Hanhart, 1981), and classified into five distinct complementation groups, according to the loci in which the mutations occur. These groups were named *py* (chromosome II), *tz* (V), *th-1* (I), *th-2* (V), *th-3* (IV). The *tz* locus mutant (the *thi1* gene locus), also called *tz*-201 or A140V, has a single polymorphism compared with the *thi1* sequence of wild-type (WT) *A. thaliana:* a C to T transition that leads to a non-synonymous substitution, from Alanine to Valine, at position 140 (Papini-Terzi *et al.*, 2003). This residue is located in a highly conserved region within all *thi1* eukaryotic homologues (Godoi *et al.*, 2006). Garcia *et al.* (2014) showed that this transition disturbs protein stability and influences proper protein folding at the tertiary level. This explains why given almost identical levels of *thi1* mRNA in both WT and *tz-201* mutant, the mutant nonetheless accumulated about half the levels of THI1 protein, compared to WT (Momoli, 2008). (Garcia *et al.*, 2014) also quantified the cysteine content in At-THI1 and *tz-201* mutant, confirming that At-THI1 acts as the thiazole sulfur atom source and showed that the mutant protein contains more sulfur-containing cysteines

than the wild type, indicating that while its function as a sulfur donor is conserved, the rate of sulfur donation might less efficient.

Without thiazole or thiamine supplementation the *tz-201* mutant display green cotyledons, but the true leaves are white and the plants die early during development. Complementation assays in yeast *thi4* mutant confirmed that this mutation hinders thiamine synthesis and, thus, is responsible for the *tz-201* phenotype (Papini-Terzi *et al.*, 2003) (Figure 10).

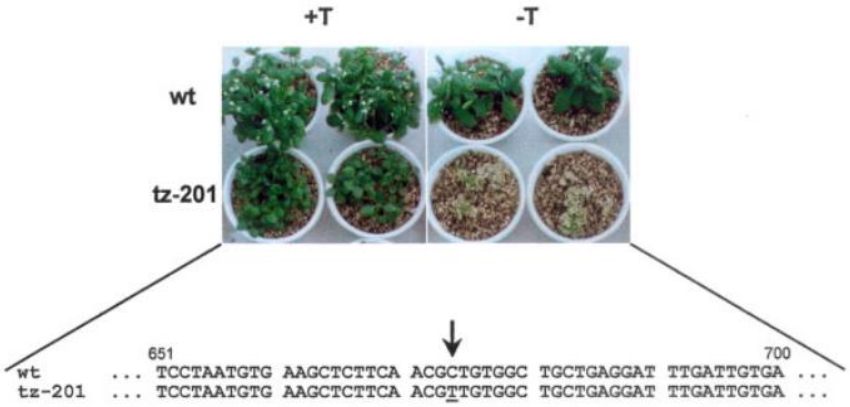

Figure 10. *Comparison of phenotype and genotype between the A. thaliana WT (var. Ler) and the tz-201 mutant.* Plants were grown in medium supplemented (+T) or not (–T) with thiamine. Below, a partial nucleotide sequence of the *thi1* gene showing the altered nucleotide in the mutant line (arrow). (Modified from Papini-Terzi *et al.* (2003)).

Momoli (2008) used a collection of physiological, biochemical and molecular approaches, comparing the WT and the *tz-201* mutant lines to investigate the dual role of *At-thi1*. She demonstrated that seed stock of *tz-201* mutant had a lower germination rate and a higher sensitivity to flooding stress, compared to the WT plants. She also demonstrated that the mutant line is less tolerant to treatment with hydrogen peroxide and speculated that this may indicate that plants with defective THI1 are more susceptible to cpDNA damage.

Interestingly, the mutant line showed a higher root fresh weight, while WT showed a higher shoot fresh weight after 6 weeks. Furthermore, the mutant showed an increase in sucrose and decrease in glucose and fructose in relation to the WT. These results, taken together may indicate that mutant line decreased its photosynthetic rate and directed sucrose to the root, developing this tissue and increasing nutrient uptake, including thiamine (Momoli, 2008).

Lastly, Momoli (2008) also demonstrate that the THI1 defect initially causes a higher production of ROS, leading to enhanced DNA damage. The production of ROS could trigger many of the observed metabolic changes including: up-regulation of antioxidant and detoxification defenses, up-regulation of DNA repair, up-regulation of genes involved in stress response (as phosphoglycerate dehydrogenase) and genes involved in apoptosis (such as enolase), as well as the reduction of glucose levels and accumulation of sucrose.

1.3. Objectives

1.3.1. Motivation and previous results in our laboratory

1.3.1.1. Sugarcane

The *thi1* gene appears to play an important role in sucrose accumulation in sugarcane. As described above, many studies have been carried out to discern both thiazole biosynthesis and the function of *thi1* gene in different plants and other organisms. A refined search in the SUCEST (Sugarcane Expressed Sequence Tag (EST)) program database (Vettore *et al.*, 2003) identified two SAS (Sugarcane Assembled Sequence) as putative sugarcane *At-thi1* homologues. They were named SAS1_*sc-thi1* and SAS2_*sc-thi1*. For the assembly of SAS1_*sc-thi1*, 41 ESTs were used and for the assembly of SAS2_*sc-thi1*, 4 ESTs were used. These ESTs are derived from libraries prepared from different sugarcane tissues: for SAS1_*sc-thi1*, the ESTs are derived from all tissues, but mainly from seeds, whereas for SAS2_*sc-thi1*, the ESTs are derived solely from stem and seed tissues. This suggests a differential expression profile of THI1 in sugarcane.

Initially, all sugarcane ESTs were compared with the THI1 protein form *A. thaliana*. From the ~240,000 ESTs of the library, 48 reads in the SUCEST database have correspondence to At-THI1. One of them, SCRLLV1049411.g, is uniquely associated with SAS1_*sc-thi1*.

Among these 48 ESTs, the ones with the highest scores to each SAS were selected as references. For SAS1_*sc-thi1* the selected EST was SCQGSD1046F12.g, which is 892 bp long. For SAS2_*sc-thi1* the EST SCSGST1070H09.b was selected, which is 657 bp long. Both ESTs have 81% identity with the At-THI1 protein. Between the two reads there is 94% nucleotide identity but there are 30 SNPs within the overlapping region of 500 bases.

The presence of these two isoforms is interesting because it suggests a different type of gene regulation, compared with *A. thaliana* (Figure 11), which has only one copy of the gene (Machado *et al.*, 1996). Interestingly, two *At-thi1* homologues were also identified in *Zea mays* (Belanger *et al.*, 1995), which is a C4 grass, like sugarcane.

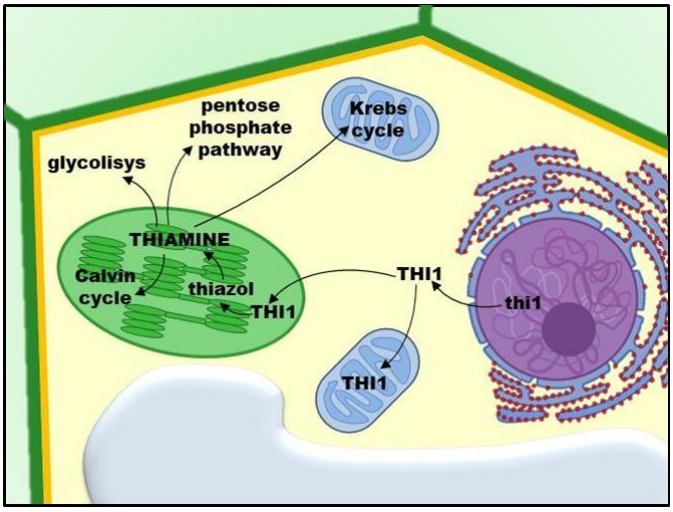

Figure 11. *Summary of the role of thi1 gene on the thiamine metabolic pathways of A. thaliana.* The *thi1* gene translates to the THI1 protein (Machado *et al.*, 1996; Ribeiro *et al.*, 1996; Papini-Terzi *et al.*, 2003) The THI1 is targeted simultaneously to mitochondria – where it has a role in DNA protection – and to chloroplast, where it contributes to thiamine synthesis (Chabregas *et al.*, 2001, 2003). Thiamine acts as a co-factor in many different metabolic pathways, as indicated. Plant cell structures are represented by purple: nucleus; green: chloroplast; blue: mitochondria; yellow: cytoplasm; dark green: cell wall; orange: plasmatic membrane; white: vacuole.

1.3.1.2. Outstanding questions concerning *thi1* function

From the literature review, four outstanding questions remain unanswered. These serve to guide this research into the function of the two isoforms of *thi1* in sugarcane:

- Is each copy targeted to different subcellular compartments? (The work of Chabregas *et al.* (2001) showed that the *A. thaliana* THI1 transcript is targeted to both the mitochondria and the chloroplasts).
- Does each copy have a different function? (The THI1 protein is considered to be bifunctional because it has a role in the biosynthesis of the thiazole moiety of thiamine, probably acting as sulfur donor, as demonstrated recently by Chatterjee *et al.* (2011); it is also involved in DNA damage protection and other stress-related pathways (Machado *et al.*, 1997; Medina-Silva *et al.*, 2006; Momoli, 2008).
- Do both copies have the same function, but are simply expressed at different times and in distinct tissues? (The analysis of the tissue of origin for the EST reads used to cluster SAS1_*sc-thi1* e SAS2_*sc-thi1* showed that the first gene copy is expressed in all tissues but predominantly in seeds, while the second gene copy is expressed solely in stems and seeds).
- Does gene duplication simply reflect the need for additional thiamine or THI1 to cope with the increased demands of C4 photosynthetic metabolism? (This reflecting its role as a cofactor for several enzymes involved in carbon sequestration).

1.3.2. General objective

The objective of this project component was to add knowledge about the sugarcane *thi1* homologues.

1.3.3. Specific objectives

a) Associate the two SUCEST SAS with the corresponding sugarcane genes and genomic regions of this complex polyploid.

b) Evaluate sugarcane allelic diversity.

c) Determine the expression profile of genes homologous to *At-thi1*.

d) Verify possible functional divergences of different *thi1* copies.

e) Generate *thi1* mutants in plant systems for subsequent functional studies.

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2. THIAMINE THIAZOLE ENZYME (THI1): DIVERSITY IN PLANTS, MOLECULAR AND FUNCTIONAL ANALYSES OF TWO SUGARCANE PARALOGS.

ABSTRACT

Sugarcane (*Saccharum* spp.) is a C4 photosynthesis monocot, like corn (*Zea mays*) and sorghum (*Sorghum bicolor*), and is grown for sucrose production, which accumulates in the stem of the plant. It is an important tropical crop, used to produce sugar and ethanol, with the latter being an important biofuel in Brazil. The *thi1* gene was the first reported to be involved in plant thiamine biosynthesis. THI1 protein is known to be involved in the synthesis of the thiazole ring, a thiamine (vitamin B1) component, which is an essential co-factor in several carbohydrate and amino acid metabolic pathways. This research aims to study the genomic structure, evolution and function of *thi1* in sugarcane. While *A. thaliana* and other plants contain only a single copy of *thi1*, we report here that sugarcane has at least two copies (*sc-thi1.1* and *sc-thi1.2*), like other C4 grasses whose genomes have been sequenced. The identified alleles related to *sc-thi1.2* have some differences in sequence and seem to be diverging into two subgroups (*sc-thi1.2a* and *sc-thi1.2b*), based on a phylogenetic analysis. Expression analyses show that *sc-thi1* genes are expressed in different tissues and developmental stages, but in differing levels, although *sc-thi1.1* showed highest expression levels in all cases analyzed. Genetic diversity analysis showed that *thi1* duplication in monocots arose in the ancestral Panicoideae subfamily (between 60-50 MYA) and remains in many modern cultivars of sugarcane. Moreover, subcellular localization assay showed that sc-*thi1.1* and *sc-thi1.2b* are targeted in a pattern that is distinct from that of *sc-thi1.2a*. Finally, complementation studies performed in a yeast THI4 mutant demonstrate that only two of the three *sc-thi1* isoforms are able to restore thiamine auxotrophy, albeit at a low level. As vitamin B1 acts as co-factor in the carbohydrate metabolic pathway, the fundamental biological knowledge about *sc-thi1* is relevant to the sugarcane breeding, for which we seek increased accumulation of sucrose.

Keywords: Sugarcane; *Thi1* gene; Genomic characterization, Evolutionary diversity; Expression analysis; Functional complementation

2.1. Introduction

2.1.1. Economic relevance of sugarcane

The Persians, followed by the Greeks, discovered the famous "reeds that produce honey without bees" in India between the 6th and 4th centuries BC. They adopted and then spread sugarcane agriculture (FAO - Food and Agriculture Organization, 2009).

Sugarcane (*Saccharum* spp.) is an important tropical crop, grown for sucrose, which accumulates in the stalk internodes. Sucrose, extracted and purified in specialized mill factories, is used as raw material in the food industry or is fermented to produce ethanol. Other products derived from sugarcane include molasses, rum, *cachaça* (a traditional alcoholic beverage from Brazil), bagasse (used as biofuel, in the manufacture of pulp and building materials) and fertilizers. Nonetheless, the world demand for sugar is the primary driver of sugarcane agriculture. Sugarcane represents 80% of the world's raw sugar production (European Commission, 2017).

Ethanol is a byproduct of sugar production and can be used as a biofuel alternative to gasoline. It is produced on a large scale by the Brazilian sugarcane industry, as is widely used in their cars. According to RENAVAM (the Brazilian National Registry of Motor Vehicles), in 2017 88.6% of the Brazilian cars are flex-fuel, cars that may be powered by alcohol or gasoline at any mixing rate (ANFAEVA website).

In 2016, The Food and Agriculture Organization (FAOSTAT website) estimates it was cultivated on about 26×10^6 hectares, in more than 90 countries (Figure 12).

Figure 12. *Map of sugarcane growing countries.* Light green plants represent places where sugarcane is cultivated (reproduced from SugarCane.org).

Brazil is the currently largest producer of sugar cane in the world (FAOSTAT website). The next five major producers, in decreasing amounts of production, were India, China, Thailand, Pakistan, and Mexico (Figure 13).

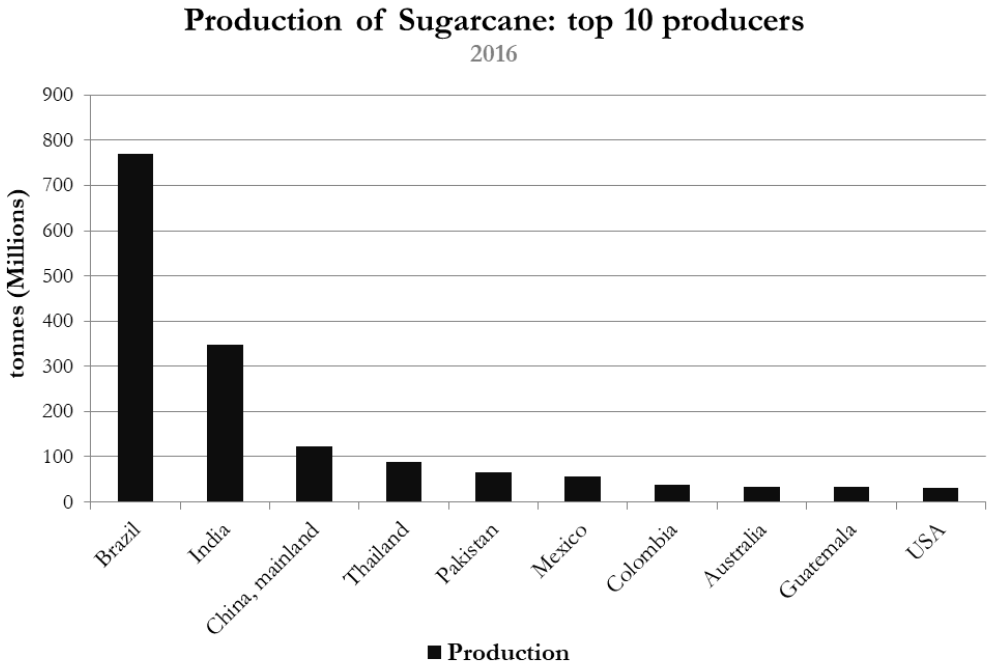

Figure 13. *Top 10 countries in sugarcane production in 2016* (FAOSTAT website).

2.1.2. Sugarcane Biology

Sugarcane belongs to the genus *Saccharum*, tribe *Andropogoneae*, native to the warm temperate to tropical regions of South Asia and Melanesia. Species of the *Saccharum* complex (sugarcane) are part of the Poaceae family and together with *Sorghum*, *Zea* and other genera comprise the Panicoideae super-family, one of the C4 photosynthetic grass lineages (Kellogg, 2000) (Figure 14).

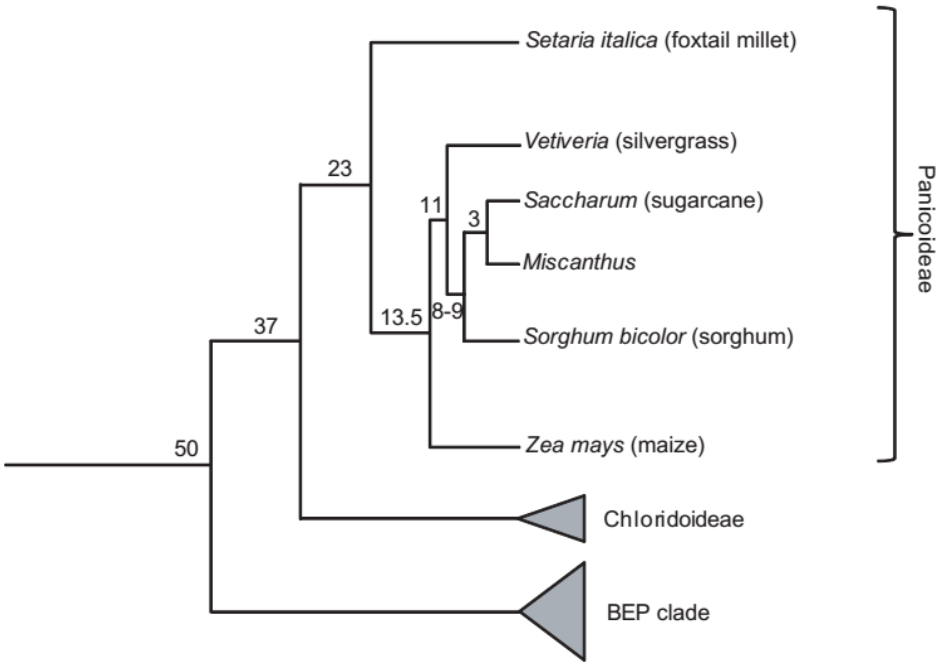

Figure 14. *Schematic diagram of evolutionary history of grasses and sugarcane*. BEP clade: Bambusoideae, Ehrhartoideae and Pooideae subfamilies. Numbers indicate divergence times in million-year units (Mya), relative to nearest common ancestors (reproduced from De Setta *et al.*, 2014).

The modern sugarcane cultivars are interspecific hybrids derived from crosses made at the end of nineteenth century by early sugarcane breeders in Java and India. They carried out crosses between *Saccharum officinarum*, the traditional cultivar grown by farmers (2n = 8x = 80) and *Saccharum spontaneum*, the wild cultivar (2n = 5x–16x = 40–128) (Grivet *et al.*, 2004). *S. spontaneum* was used in order to introduce vigor and resistance genes while *S. officinarum* was used because of its high sugar content (Grivet and Arruda, 2002). Both species were formed by two or more events of autopolyploidization (D'Hont *et al.*, 1996) and diverged between 2.5 and 3.5 Ma (Vilela *et al.*, 2017).

Modern hybrids have complex aneuploidy highly polyploidy genomes (2n=100 to 130), with unequal contribution from *S. officinarum* (80-90% of the chromosomes) and *S. spontaneum* (10-20%) and a small percentage of recombinant chromosomes (D'Hont, 2005). Reflecting the high degree of polyploidy, the size of sugarcane hybrids' genomes is very large: the monoploid genome is about 10 Gb (D'Hont and Glaszmann, 2001) (Figure 15). For each gene, up to 14 distinct sequence haplotypes originating from the two species may be distinguished among transcripts.

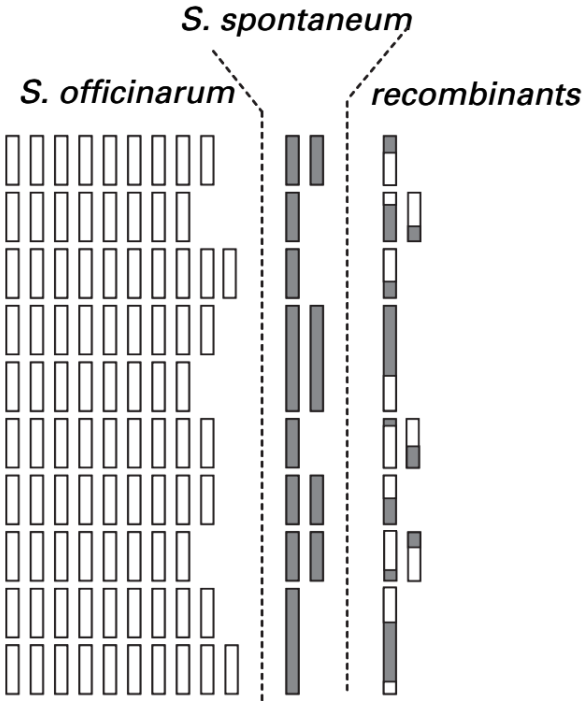

Figure 15. *Schematic representation of the genome of modern cultivars.* White and gray bars correspond to *S. officinarum* and *S. spontaneum* chromosomes or chromosome segments, respectively. Chromosomes from the same row are homologous (or homeologous) (reproduced from D'Hont, 2005).

Despite its economic importance, genomic aspects of sugarcane are little studied compared to other plants of economic interest. Due to the complexity and size of its genome, sugarcane was not among early candidates for whole genome sequencing. However, the transcriptome of sugarcane provided an alternative approach to study coding regions. The program SUCEST (Sugarcane EST Project - Vettore *et al.* (2001)) generated 26 cDNA libraries from different sugarcane organs and tissues sampled at various developmental stages from several Brazilian commercial cultivars. This program generated 237,954 partial sequences of cDNA called ESTs (Expressed Sequence Tags), which were grouped into nearly 43,000 SAS (Sugarcane Assembled Sequences).

2.1.3. Study of *thi1* homologues in sugarcane

Two SAS present in the SUCEST database were identified as putative sugarcane *At-thi1* homologues (SAS1 and SAS2) (De Almeida, 2004). These SAS sequences were used as probes to identify genomic sequences in bacterial artificial chromosome (BAC) library prepared from the sugarcane cultivar R570 (Tomkin *et al.*, 1999). The results revealed that sugarcane has two

genomic copies of *thi1* (*sc-thi1.1* and *sc-thi1.2*, corresponding, respectively, to SAS1 and SAS2), with nine BACs identified using SAS1 as a probe and ten using SAS2 as a probe.

We hypothesized that this duplication may be associated with differential expression profiles of THI1 in sugarcane, differential function of each copy, or differential organelle targeting. The presence of these two gene copies suggests at least the potential for differential gene regulation, compared to *A. thaliana*, which has only one copy of the gene.

In this work, we report the gene structure of both *sc-thi1* copies, their genomic context and synteny analyses, the genetic diversity of *thi1* gene copies in the genus *Saccharum*. Differential gene expression pattern of the two gene copies was also studied. Thiamine biosynthesis was examined using yeast complementation assays.

The results supported that each SAS represents *sc-thi1* copies located at two different genomic loci. A deep phylogeny coupled with comparative sequence analyses revealed that the ten *sc-thi1.2* sequences actually represent two different *sc-thi1* versions and were subdivided in *sc-thi1.2a* and *sc-thi1.2b*. The orthology analysis showed that this duplication is also present in the C4 grasses *Zea mays*, *Sorgum bicolor*, *Setaria italica* and *Panicum virgatum*.

Sc-thi1.1 and *sc-thi1.2*b have the same cellular distribution pattern (in the mesophyll cells), distinct from the observed for *sc-thi1.2a* (in the edges of the epidermal cells, in a punctate pattern). Moreover, *sc-thi1.1* and *sc-thi1.2b* are able to complement thiamine auxotrophy in a *thi4* yeast mutant, if the N-terminal chloroplast transit peptide of the encoded sc-THI1 protein is removed.

2.2. Material and Methods

2.2.1. Screening sugarcane BAC libraries for *sc-thi1* homologues

A sugarcane BAC library (de Setta *et al.*, 2014) is available. A pair of primers (thi1_F: CAC CAT GGC CGA GAA CAG; thi1_R: CGT ACG AGC TCT CCA AGG AC) was synthesized, corresponding to a conserved region of *thi1*, based SAS1 and SAS2 from the SUCEST Database (Vettore *et al.* (2003)) that were identified as putative *thi1* homologues. The amplification of this region generated a 108 bp fragment that was used to screen the library. The screening, sequencing and assembly of the BACs were done as described in de Setta *et al.* (2014).

2.2.2. Synteny and phylogeny analyses

Synteny analysis was carried out to compare BAC sequences containing both *thi1* copies and the related genomic region of six plants: *Arabidopsis thaliana*, *Oryza sativa*, *Brachypodium distachyon*, *Sorghum bicolor* and *Zea mays*, retrieved from *in silico* DNA alignment comparison of the predicted protein sequences of the BACs against the selected genomes present in the Phytozome v9.1 database (Goodstein *et al.*, 2012).

The Coding DNA Sequences (CDSs) of the *thi1* gene from the selected BACs and plant genomes were aligned using ClustalW2 (Thompson, Higgins and Gibson, 1994) with default parameters. A Neighbor-joining phylogenetic tree was inferred using MEGA 5.1 program (Tamura *et al.*, 2011) with the following parameters: Analysis - Phylogeny Reconstruction; Statistical Method - Maximum Parsimony; number of sites: 1008; No. of bootstrap reps = 1000; CI = 0.743520; RI = 0.747651.

2.2.3. Plant *thi1* phylogeny

In order to generate a plant *thi1* phylogeny, proteins homologues to *At-thi1* were selected from the Phytozome v9.1 database (Goodstein *et al.*, 2012). Ninety-three sequences, present in thirty-eight plant species, were found. However, only sixty of them were selected for analysis because some sequences were not complete or they displayed alternative splicing of transcripts derived from the same gene. Table 1 shows the name of the plants that contain *thi1* genes, the number of transcripts that were found in the database and how many transcripts were selected for this study. A multiple alignment of CDSs from all the transcripts selected plus the sc-*thi1* CDS was done using ClustalOmega program (Sievers *et al.*, 2011) with default parameters. A

neighbor-joining tree was inferred using MEGA6.1 (Tamura *et al.*, 2013) with the highest ranked substitution model (Tamura 3-parameter), 500 bootstrap replications, Gamma Distributed (G) = 0.36 and number of sites = 951.

Table 1. Organisms from Phytozome v9.1 database which present *At-thi1* homologues sequences in their genomes. The raw number of transcripts found in the database for each organism and number of transcripts selected to be used in this study are shown.

# transcripts in database	Organism Name	# selected transcripts
3	*Aquilegia coerulea*	1
1	*Arabidopsis lyrata*	1
1	*Arabidopsis thaliana TAIR10*	1
1	*Brachypodium distachyon*	1
3	*Brassica rapa Chiifu-401 v1.2*	3
1	*Capsella rubella*	1
1	*Carica papaya*	1
1	*Chlamydomonas reinhardtii*	1
3	*Citrus clementina*	1
4	*Citrus sinensis*	3
1	*Coccomyxa subellipsoidea C-169*	1
1	*Cucumis sativus*	1
2	*Eucalyptus grandis*	1
2	*Fragaria vesca*	2
6	*Glycine max*	4
6	*Gossypium raimondii*	3
2	*Linum usitatissimum*	1
3	*Malus domestica*	1
3	*Manihot esculenta*	1
1	*Medicago truncatula*	1
4	*Mimulus guttatus v1.1*	3
2	*Oryza sativa v7_JGI*	1
2	*Panicum virgatum v0.0*	2
2	*Phaseolus vulgaris*	2
7	*Physcomitrella patens v1.6*	4
6	*Populus trichocarpa*	3
1	*Prunus persica*	1
1	*Ricinus communis*	1
1	*Selaginella moellendorffii*	1
2	*Setaria italica*	1
1	*Solanum lycopersicum*	1
1	*Solanum tuberosum*	1
2	*Sorghum bicolor v1.4*	2
1	*Thellungiella halophila*	1
2	*Theobroma cacao*	2
2	*Vitis vinifera*	1
7	*Volvox carteri*	2
3	*Zea mays v1.1*	1

2.2.4. *Saccharum thi1* diversity network analysis

In order to study the *thi1* homologues diversity in the *Saccharum* complex, a network analysis was done. Sequences for network were obtained through PCR amplification using a pair of primers (thi1Conserved_F: CTC CTC AAG TCC TCC TTC GC and thi1Conseved_R: TCA TGC CGA TGT CCT GGA G) which correspond to a conserved region of *sc-thi1* from the nineteen BACs. Genomic DNA from *Miscanthus* sp., *S. spontaneum* (Mandalay)*, S. spontaneum* (IN-84-58), *S. officinarum,* the hybrids from Brazil breeding programs SP8032-80, R570 (BACs), SP7011-43, SP8132-50, RB835486, RB72454, RB867515, and the hybrids POJ-2878 (from Java), NA56-79 (from Argentina), NCo-310 (from South Africa) and Co-290 (from India) were used as template in the PCR. As positive control, the same region also was amplified from *S. bicolor*. These amplicons were then sequenced at the ABI PRIS 3730 DNA ANALYZER platform (Applied Biosystems™). Phred-Phrap-Consed package (Ewing and Green, 1998; Ewing *et al.*, 1998; Gordon, Abajian and Green, 1998; Gordon, 2003) was used to sequencing quality control, assembly and sequence edition. Each gene was analyzed individually using default parameters. Only bases with phred quality ≥ 20 were used, resulting in core sequences of about 330 bp (Figure 22). The sequence of this region was also obtained from *sb*A, *sb*B, *zm*A e *zm*B by blasting in Phytozome v9.1 database (Goodstein *et al.*, 2012).

All the resulting sequences were aligned using ClustalOmega (Sievers *et al.*, 2011) with default parameters. A network analysis were done using DnaSP5 (Librado and Rozas, 2009) and NETWORK 4.6.1.2 (Bandelt, Forster and Rohl, 1999) software considering the gaps, with other default parameters.

Based on the molecular characterization, three *sc-thi1* sequences from the BACs were selected to represent different versions of *sc-thi1* in subsequent analyses. The *sc-thi1* sequence found in the 108_C04 BAC represents *sc-thi1.1*, the one found in the 017_B18 BAC represents *sc-thi1.2a* and the 094_O04 BAC represents *sc-thi1.2b*.

2.2.5. RNA-seq analyses

Reads of three RNA-seq databases were mapped against the variable region of the N-terminal (corresponding to amino acids 1-89 of the encoded polypeptide, Figure 22) of the three *sc-thi1* versions. This region was used as it is the only part of the protein which differentiates between the *sc-thi1* copies. The three RNA-seq available databases used were:

- GaTE Lab database of HiSeq 1500 system (Illumina™) contains paired-end reads from samples of whole root and kernel top of a 3 months old R570 sugarcane plants (unpublished data).
- LAFIECO database of 454 sequence (Roche™) contains single-end reads from roots tips (first 4 cm from tip, divided into 1 cm segments) of a 3 months old SP80-3280 sugarcane plants (unpublished data).
- Schaker *et al.* (2016) generated a database of HiScanSQ (Illumina™) containing pair-end reads from RB92-5345 sugarcane plants either infected, or not, with *Sporisorium scitamineum* (smut disease) at two developmental stages. The first stage comprises buds collected at 5 DAI (days after inoculation), and the second stage comprises the base of the whips, up to 2 cm below the culm (the site of intensive sugarcane cell division and fungal sporogenesis) at 200 DAI, when the disease symptoms were evident.

Mapping of the reads was done using default parameters of Bowtie2 software (Langmead *et al.*, 2009) in Galaxy version 2.2.6.2 platform (Afgan *et al.*, 2016), available in CEFAP website (the Facility for Research Support of University of São Paulo). The mapping was visualized Unipro-UGENE software (Okonechnikov, Golosova and Fursov, 2012), which gives the total of reads mapped.

In order to measure the abundance of transcripts mapped to each *sc-thi1* copy, FPKM (Fragments Per Kilobase Million, used for paired-end databases) and RPKM (Reads Per Kilobase Million, used for single-end databases) normalized expression unit were calculated (Mortazavi *et al.*, 2008).

2.2.6. Functional complementation assay

The complementation assay was carried out using commercially synthesized copies of the *sc-thi1* gene. The three *sc-thi1* sequences from the selected BACs were used as templates for coding DNA sequences (CDS) synthesis by Blue Heron® Biotech, LLC.

Versions of these CDSs lacking the N-terminal chloroplast transit peptide (DelN) were obtained through PCR amplification on the commercial CDS, using a pair of primers (thi1DelN_R: GTC GAC TCA GGC GTC CAC for all; and thi1.1DelN_F: GGA TCC ATG ACC CGC CGC TA for *sc-thi1*.1; and thi1.2DelN_F: GGA TCC ATG ACC CGG CGG TA for

both *sc-thi1.2a* and *sc-thi1.2b*) corresponding to polypeptide sequence starting from the second initiation codon (amino acid 78 in Figure 22) to the stop codon (amino acid 360).

The CDS, containing the restriction enzyme sites of BamHI at 5' and Sal1 at 3', were inserted in the yeast expression vector pG-1 (Schena, Picard and Yamamoto, 1991) using T4 ligase (Promega®) according to the manufacturer's protocol. Two more constructs were used as control: A184V as positive control, which is the CDS of the *A. thaliana tz-201* mutant and is able to complement the thiamine auxotrophy in the *thi4* yeast mutant (Papini-Terzi *et al.*, 2003); and DelN as negative control, which is the *A. thaliana* wild-type (WT) *thi1* lacking the N-terminal chloroplast transit peptide (Galhardo, 2003). All constructs were transformed in KBY5 strain by the heat shock method (Schiestl and Gietz, 1989).

S. cerevisiae strains used in this work were W303 (*mata, ade2-1, trp1-1, leu2-3-112, can1-100, ura3-1, his3-11-115*) and KBY5 (as W303, THI4::*URA3* (Praekelt, Byrne and Meacock, 1994). Selection of transformants with pG-1 constructs was performed on yeast nitrogen base (YNB) (BD Biosciences®) medium lacking tryptophan.

For the thiamine auxotrophy experiment, cells were grown overnight in liquid YNB without tryptophan, centrifuged, and re-suspended in 10 mM $MgSO_4$. From an OD = 1, three serial and, tenfold dilutions were prepared. These were then plated on minimal medium (same as yeast nitrogen base, but lacking both thiamine HCL (YNB -thia) and tryptophan, from USBiological®) plates, either with or without thiamine and tryptophan, and incubated for 28 days at 30°C and, analyzed every 4 days thereafter.

2.2.7. Subcellular localization

In order to evaluate the subcellular localization of each *sc-thi1* copy, the N-terminal region (N-term) containing both the chloroplast transit peptide (CTP) and the mitochondria pre-sequence (MPS) was fused to the green fluorescence protein (GFP) reporter. Based on the work of Chabregas *et al.* (2003), which examined the subcellular localization of *At-thi1*, the N-terminal (CTP+MPS) was defined as the first 127 amino acids of Figure 22. This region was amplified from each of the *sc-thi1* CDSs (commercially synthesized as described above) using a pair of primers (thi1-NT-Gat_F: TAC AAA AAA GCA GGC TAT GGC CAC CAC CGC GG; thi1-NT-Gat_R: CAA GAA AGC TGG GTT CGG GGA CAC CGA CTG CTC) containing part of the *att*B adaptors (underlined) to the Gateway cloning system (Invitrogen™). A second PCR reaction was done to complete the *att*B adaptor, using another pair of primers (ATTB1.1: GGG

GAC AAG TTT GTA CAA AAA AGC AGG CT; ATTB2.2: GGG GAC CAC TTT GTA CAA GAA AGC TGG GT).

The final *att*B-PCR products were cloned into the destination vector pK7FWG2.0 (Karimi, Inzé and Depicker, 2002) (Figure 16) through the Gateway™ cloning Technology (Invitrogen™) following the manufacturer's specifications, resulting in three expression vectors, each one containing one of the *sc-thi1* N-termini fused to the N-terminus of the GFP fluorescence reporter gene, under the control of the constitutive Cauliflower Mosaic Virus 35S promoter (CaMV 35S).

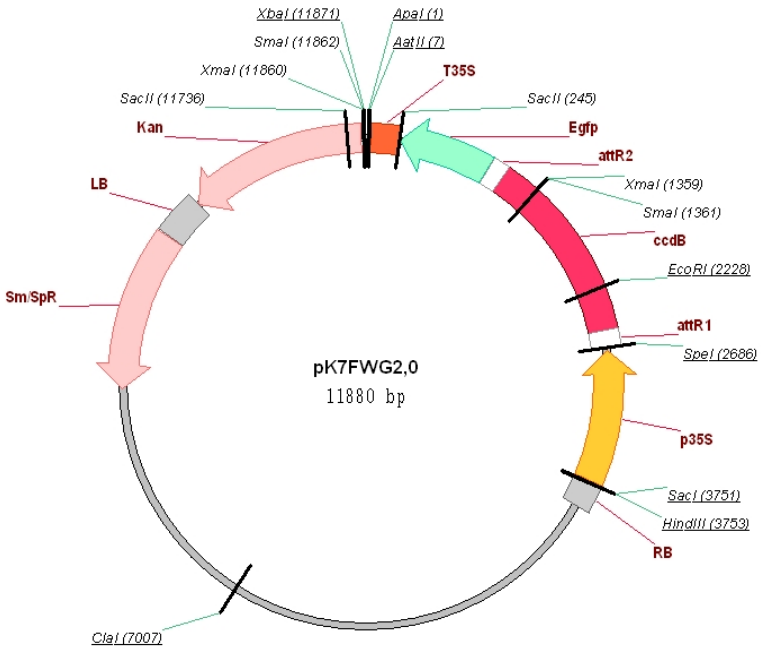

Figure 16. *Destination vector pK7FWG2.0*. Three *sc-thi1* N-terminal (CTP+MPS) versions were independently inserted between the *att*R1 and *att*R2 sites, replacing the lethal *ccd*B gene, using the Gateway™ Technology, resulting in a C-terminal fusion of the GFP fluorescence tag (reproduced from: Karimi, Inzé and Depicker, 2002).

The 35S-GFP-At5g47540 construct provided by Fernanda Marisca Bizotto (*Laboratório de Evolução e Diversidade II* from Federal University of ABC) was used as a positive control. For this construct the GFP expression is expected to be found in the cytoplasm of epidermal cells of tobacco leaves.

The three final vectors (one for each *sc-thi1* copy; 35S-scTHI1.1(N-term)-GFP, 35S-scTHI1.2a(N-term)-GFP and 35S-scTHI1.2b(N-term)-GFP) and the positive control vector were

transformed independently into *Agrobacterium tumefaciens* C58C1-pMP90 and then transfected into *Nicotiana tabacum* leaves, both following the protocol described by (Sparkes *et al.*, 2006). Two different sections of two different leaves of two different plants in different ages (5 and 8 weeks old) were infiltrated with each construction. An absorbance of 0.1 for the *Agrobacterium* culture was used.

The infiltrated leaves were covered with aluminum foil and kept under summer conditions at the greenhouse for two days. The samples were collected at 9 a.m. in the third day after infiltration. The expression of the transgene within the abaxial side of the leaf was examined by confocal microscope LSM880 (Zeiss™) using a 10x objective. GFP was excited with the argon laser (488 nm), and emitted light was collected between 498 and 510 nm. Chloroplasts were excited with the argon laser (488 nm), and emitted light was collected from 610 to 669 nm. The GFP and chloroplast signals were collected separately and later superimposed computationally with ZEN black edition (Zeiss) software.

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2.3. Results

2.3.1. Genomic identification of *sc-thi1* homologues

In order to identify genes homologues to *At-thi1* in sugarcane, an R570 cultivar 3D genomic library (de Setta *et al.*, 2014) was screened using PCR primer pairs designed from SAS1 and SAS2 transcripts. Nineteen BACs were selected, fully sequenced and annotated. The minimum, maximum and average sizes of BAC assemblies were 85.05 Kb, 164.89 Kb and 121.61 Kb, respectively (Table 2).

Table 2. Selected BACs for *sc-thi1* presence

Probe	SHCRBa BAC name	BAC size (bp)	*thi1* CDS size (bp)
SAS1_*sc-thi1*	021_C22	135265	1068
	045_A10	145524	1068
	086_A19	101298	1068
	093_A03	139698	1068
	107_N16	87694	1068
	108_C04	101728	1068
	134_H07	97816	1068
	145_O03	157314	1068
	149_E16	164894	1068
SAS2_*sc-thi1*	017_B18	131240	1059
	030_H05	141404	1059
	092_F09	85055	1059
	251_N23	130963	1059
	094_O04	102271	1056
	109_G13	100204	1056
	183_N05	129178	1056
	184_H17	115565	1056
	190_F02	105074	1056
	222_C03	138508	1056

An alignment analysis of all *thi1* homologue sequences found in these BACs with the *At-thi1* showed that, while *At-thi1* has two introns, the nineteen *sc-thi1* contain only one intron (whose size varies from 63 to 149 bp), as well as the other *thi1* homologues of the Poaceae family (Figure 17).

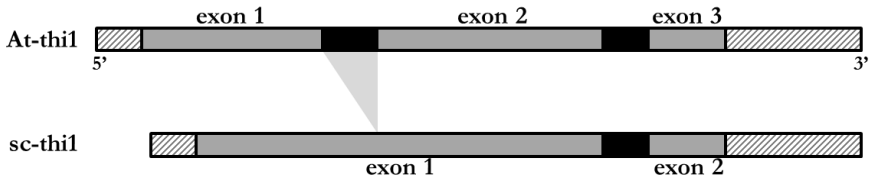

Figure 17. *Structure of the thi1 gene in A. thaliana and in the Poaceae family*. Black boxes represent introns, gray boxes represent exons and dashed boxes represent untranslated regions (5'UTR to the left and 3'UTR to the right).

2.3.2. Synteny and Phylogeny of *thi1* in grasses

Synteny analysis was performed using the nineteen sugarcane BACs sequences, the *thi1* containing genomic region of *A. thaliana* (Eudicot) and other five grass genomes aiming to understand the evolutionary history from THI1 coding genes. As presented in Figure 18, this analysis supports that there are two copies of *thi1* in sugarcane genome, found in two distinct genomic loci. Nine clones correspond to *sc-thi1.1*, and ten correspond to *sc-thi1.2*. Differences in intergenic regions length along the BACs are due mainly to differential transposable element insertions (data not shown).

Thi1 genes are found on the chromosomes 2 (sbA) and 3 (sbB) of *S. bicolor*, the chromosomes 3 (zmA) and 8 (zmB) of *Z. mays* and in the chromosomes 2 (siA) and 4 (siB) of *S. italica*. Only one copy of *thi1* has been identified in *O. sativa* and *B. distachyon*. Using a comparative genomics approach, we reveal that among the plant genomes analyzed, there are five different genomic environments (A-E) surrounding *thi1* gene (Figure 18). The plant *thi1* gene is found in at least 5 independent genomic locations. First, in the common ancestor of the *Andropogoneae* tribe (sugarcane, sorghum, and maize) two loci carry *thi1* copies (*thi1.1* – present in region A and *thi1.2* – present in region B), which is in accordance with the observed high collinearity between sugarcane and sorghum chromosomes (Ming *et al.*, 1998). Interestingly, *Z. mays* duplicated the entire region A and despite having region B, which carries a copy of *thi1* in the other genomes, *Z. mays* has lost *thi1* from that location, as well as, a neighboring gene (gene 17 in the figure 18). Second, *S. italica* has two *thi1* copies, one in a region syntenic to those of *O. sativa* and *B. distachyon*, and another copy located in a different region of all species analyzed in this study. Finally, the *A. thaliana thi1* region is non-syntenic to any region of grasses studied.

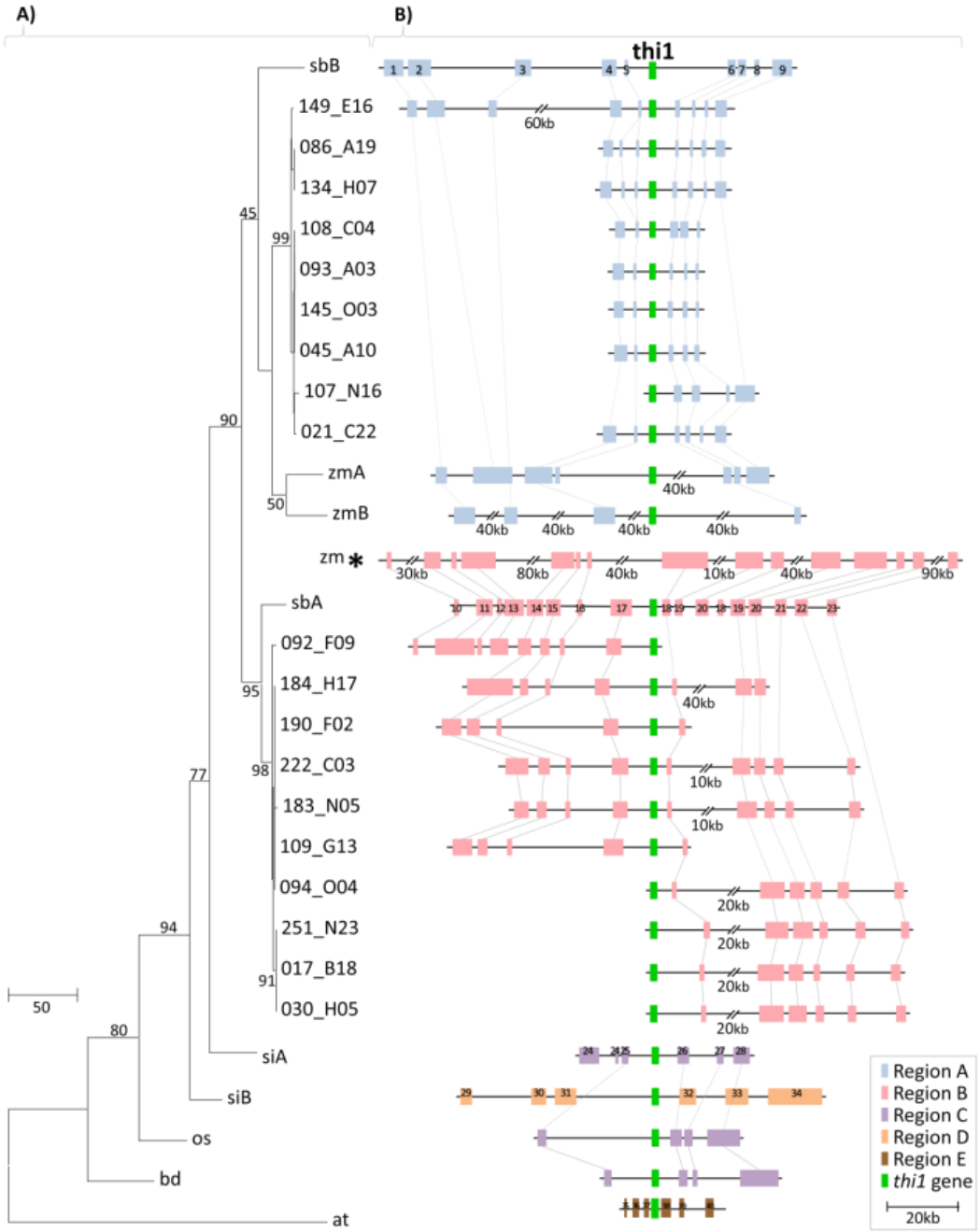

Figure 18. *Comparison of thi1 gene and its genomic flanking regions in sugarcane and other plants through phylogenetic and synteny analyses.* A) Maximum Parsimony tree inferred by MEGA5.1 using Phylogeny reconstruction parameter and 1000 bootstrap replicates. The tree is based on the multiple alignment of the two exons of the single *thi1* copy from *A. thaliana* (at), *O. sativa* (os) and *B. distachyon* (bd) and two copies of *thi1* on chromosome 2 (siA) and 4 (siB) in *S. italica*, 2 (sbA) and 3 (sbB) in *S. bicolor*, and 3 (zmA) and 8 (zmB) in *Z. mays*. B) Synteny analysis was performed using blastx between sugarcane BAC sequences and sequences from the other genomes obtained from the Phytozome 9.1 database. Rectangles indicate genes. Accession number of the genes: 1-Sb03g025470; 2-Sb03g025480; 3-Sb03g025490; 4-Sb03g025500; 5-Sb03g025510; 6-Sb03g025530; 7-Sb03g025540; 8-Sb03g025550; 9-Sb03g025560; 10-Sb02g039980; 11-Sb02g039990; 12-Sb02g039995; 13-Sb02g040000; 14-Sb02g040010; 15-Sb02g040020; 16-Sb02g040040; 17-Sb02g040050; 18-Sb02g040070; 19-Sb02g040110; 20-Sb02g040120; 21-Sb02g040140; 22-Sb02g040150; 23-Sb02g040160; 24-Si033048; 25-Si029341; 26-Si030570; 27-Si033272; 28-Si030843; 29-Si006377; 30-Si006932; 31-Si005733; 32-Si006773; 33-Si008008; 34-Si005676; 35-AT5G54745; 36-AT5G54750; 37-AT5G54760; 38-AT5G54780; 39-AT5G54790; 40-AT5G54800; *Part of the *Z. mays* chromosome 7, which is syntenic to region B, but does not have either *thi1* copy II or gene 17.

2.3.3. *Thi1* nucleotide diversity in plants

In order to study the *thi1* nucleotide sequence diversity in plants, sixty CDS similar to *thi1* were selected from thirty-eight plants from Phytozome v9.1 database (Goodstein *et al.*, 2012). These sequences were aligned with the nineteen sugarcane versions and used to perform a phylogenetic reconstruction.

The alignment shows that the cysteine residue that is known to be the sulfur donor in yeast (Chatterjee *et al.*, 2011) and in archaea (Hwang *et al.*, 2014), is conserved for all the plant THI1 selected sequences (Figure 19). This suggests that the sulfur donation function of THI1 is present in all plants and is indicative of its role in thiazole ring biosynthesis. In addition, both start codons are present in all *thi1*-like sequences selected, suggesting that the dual targeting to mitochondria and chloroplast (Chabregas *et al.*, 2001, 2003) could occur for all encoded THI1 polypeptides. In spite of these features, the encoded amino acid sequence between both start codons is highly variable, both in length and amino acid content for each gene.

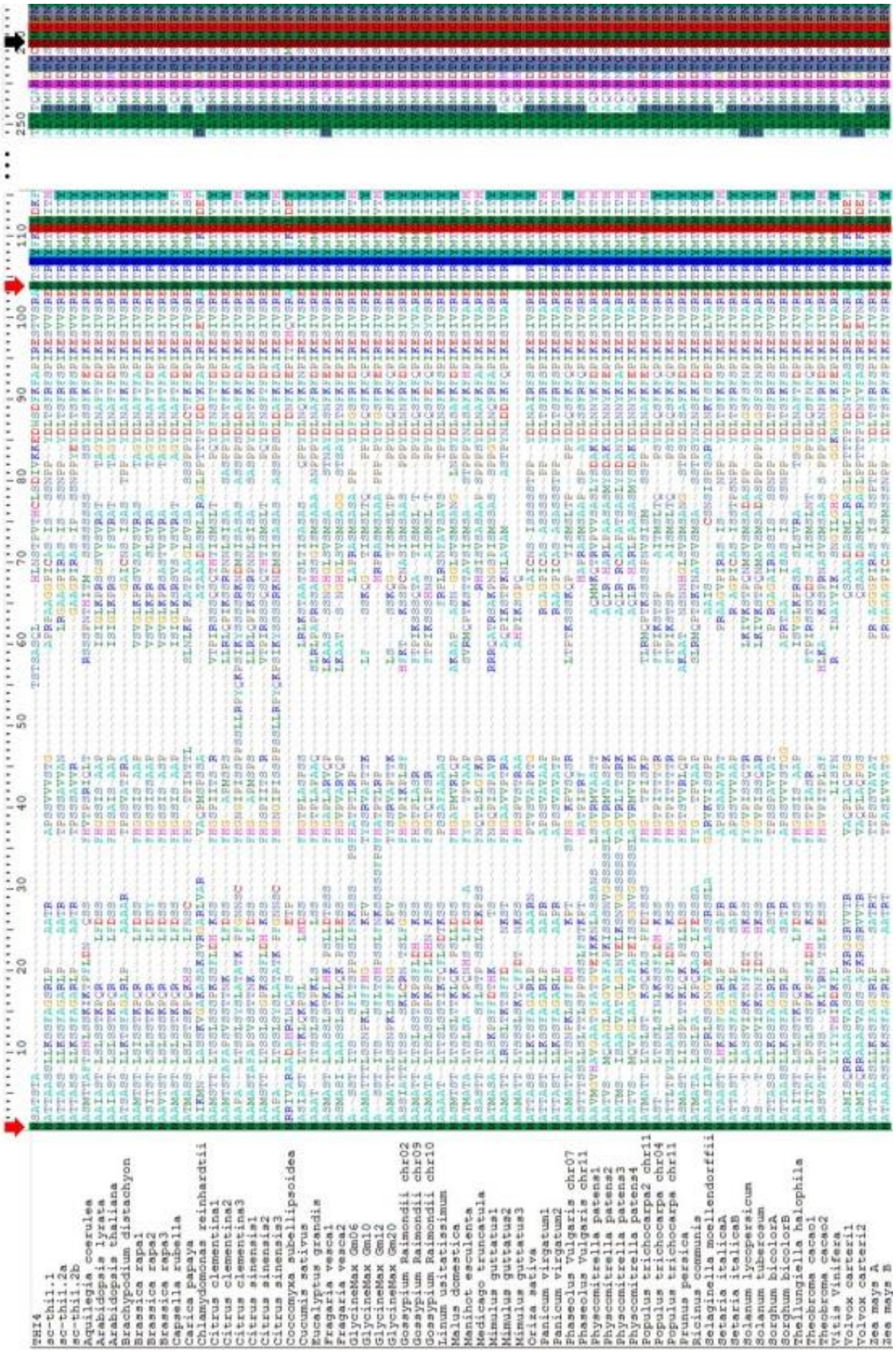

Figure 19. *Plants THI1 alignment.* The red arrows indicate the presence of both initiation codons. The black arrow indicates the conserve cysteine residue. For reference, the THI14 (*S. cerevisiae*) is also shown in the alignment.

The phylogenetic tree (Figure 20) illustrates that the *thi1* selected sequences groups in four well-supported clades, according to the species taxonomy (Eudicots, Monocots, Bryophytes and Chlorophytes), but the basal evolutionary relationships were not resolved.

While C3 metabolism grasses (*Brachypodium distachyon* and *Oryza sativa*) have just one *thi1* copy, all C4 metabolism grasses (*Panicum virgatum*, *Setaria italica*, *Zea mays* and *Sorghum bicolor*) also present two copies homologous to *thi1* gene. In accordance with the synteny above, each *thi1* copy of *S. bicolor* is grouped with different *sc-thi1* clades.

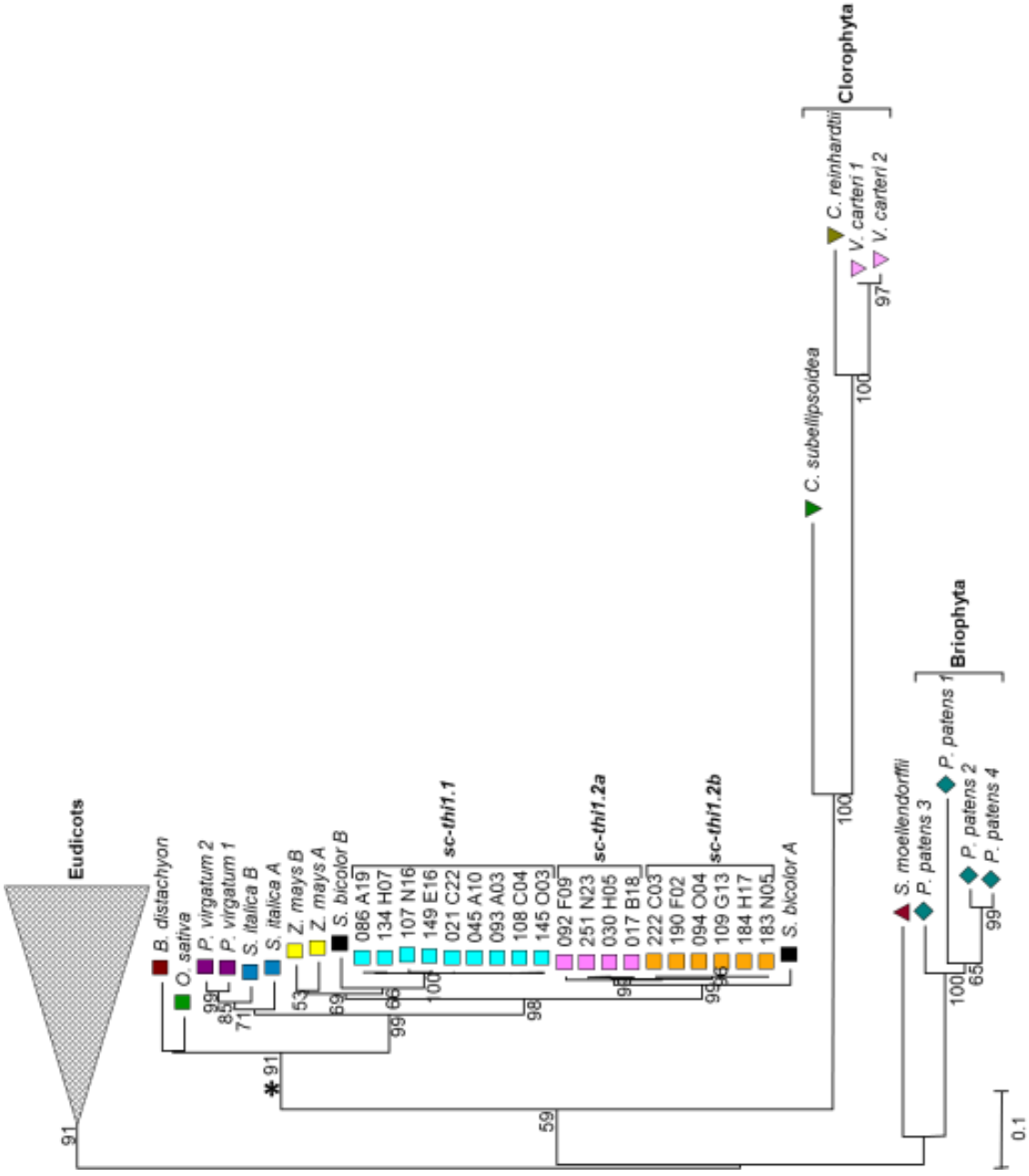

Figure 20. *Comparison of THI1 protein among sugarcane and other plants.* Neighbor-joining *thi1* phylogenetic tree inferred by MEGA6.1 using *thi1* CDS present in 38 plants. Highest ranked substitution model (Tamura 3-parameter) and 500 bootstrap replicates were used. The * indicates the Monocot branch.

The two well-supported sugarcane clades reinforce the presence of two copies (bootstrap 100 to *sc-thi1.1* and 99 to *sc-thi1.2*). However, the clade of *sc-thi1.2* is divided in two subclades, *sc-thi1.2a* (BACs 017_B18, 030_H05, 251_N23 and 092_F09) and *sc-thi1.2b* (BACs 094_O04, 109_G13, 183_N05, 184_N05, 190_F02 and 222_C03), also well-supported by bootstrap 99 and 96 respectively. Thereby, one sequence of each *sc-thi1* version was chosen to represent each copy: *sc-thi1* from BAC 108_C04 represents *sc-thi1.1*, BAC 017_B18 represents *sc-thi1.2a* and BAC 094_O04 represents *sc-thi1.2b*. When the CDS sequence is compared, the three *sc-thi1* copies present high nucleotide identity and similarity (above 89%) (Table 3).

Table 3. Percentage of identity among the three *sc-thi1* CDS.

sc-thi1.1	100%		
sc-thi1.2a	89.5%	100%	
sc-thi1.2b	90.26%	95.71%	100%
CDS	***sc-thi1.1***	***sc-thi1.2a***	***sc-thi1.2b***

The main difference among the sequences of the two subgroups lies within the 5' region, where the CTP is predicted (Machado *et al.,* 1996). The Figure 21 shows an overview of nucleotide genomic sequence of the three selected sequences.

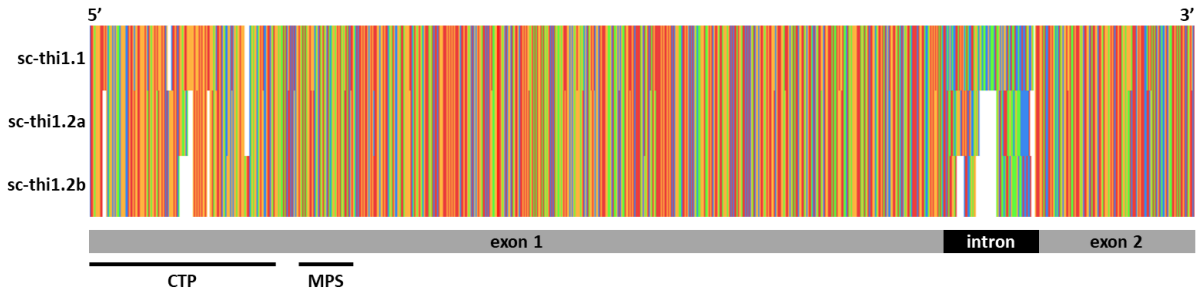

Figure 21. *Overview of the genomic sequence alignment of the three sc-thi1 selected to represent each group. Sc-thi1* is represented by 108_C04 BAC; *sc-thi1.2a* by 017_B18, and *sc-thi1.2b* by 094_O04. The grey/black bar represents the *sc-thi1* gene structure. CTP = chloroplast transit peptide; MPS = mitochondrial pre-sequence; Green =A; Blue = T; orange = C; red = G.

Figure 21 also shows nucleotide sequence differences outside of the N-terminal region. Most of these result in synonymous amino acid substitutions. The sc-THI1.1 protein differs from both sc-THI1.2 subgroups in one residue of MPS (Chabregas *et al.*, 2003), and other seven residues along the protein (Figure 22). This figure also highlights the different segments used in subsequent analyses discussed in the following sections.

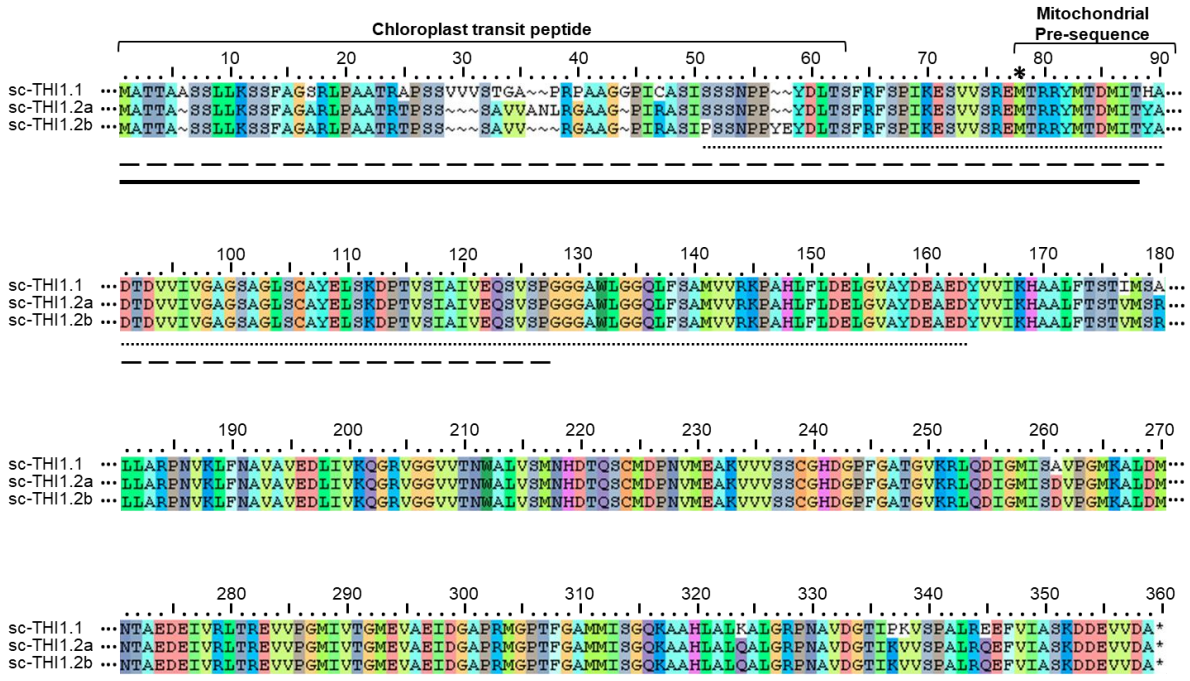

Figure 22. *Amino acid sequence alignment of the three sc-thi1 selected to represent each group.* The lines below the sequence represent parts of the protein select for different analyses: dotted line region was used in the Network analysis (section 2.3.4); dashed line region was used in the subcellular localization analysis (section 2.3.3); and solid line region was used expression analysis (section 2.3.5). The black arrow indicates the second initiation codon, which is the first amino acid of the Del-N constructs for complementation assay (section 2.3.6). Color shades represent the identities and similarities among amino acids, with a threshold of 50%.

2.3.4. Genomic diversity in sugarcane cultivars

A conserved region (amino acid 59 to 233 in Figure 22) of *thi1* was chosen in order to analyze the haplotype diversity of *thi1* in *Saccharum* complex. A pair of primers was designed based on the *thi1* CDS sequence from sugarcane BACs. This conserved region was amplified from 10 modern sugarcane hybrids cultivars, the parental species *S. officinarum* and *S. spontaneum* and closely related plant species, *Miscanthus* sp. Table 4 shows the cultivars used, how many sequences were amplified for each, and how many of them are related to each *sc-thi1* copy of this study.

Table 4. Number of assembled clones from each genome (total number and number related to each *sc-thi1* copy).

Cultivar name	# Related to each *sc-thi1* copy		# Total
	sc-thi1.1	***sc-thi1.2***	
R570 (BACs)	9	10	19
***Miscanthus* sp.**	8	3	11
S. spontaneum	9	9	18
***S. spontaneum* (IN-84-58)**	7	5	12
S. officinarum	6	2	8
Co-290	10	6	16
NA56-79	7	3	10
NCo-310	7	2	9
POJ-2878	15	2	17
RB72454	10	7	17
RB835486	8	9	17
RB867515	8	2	10
SP7011-43	3	8	11
SP8032-80	9	3	12
SP8132-50	2	8	10
TOTAL	118	79	197

One hundred and ninety seven amplicons (varying between 521-530 bp each) corresponding to positions 53-233 of the protein were sequenced, assembled and aligned against the nineteen BAC sequences. The overall nucleotide identity is high, at 95.2%. Neighbor-joining and maximum likelihood phylogenetic analyses were not able to resolve *thi1* evolutionary relationships among sugarcane species and cultivars (data not shown). Thus, a Network analysis was generated (Figure 23).

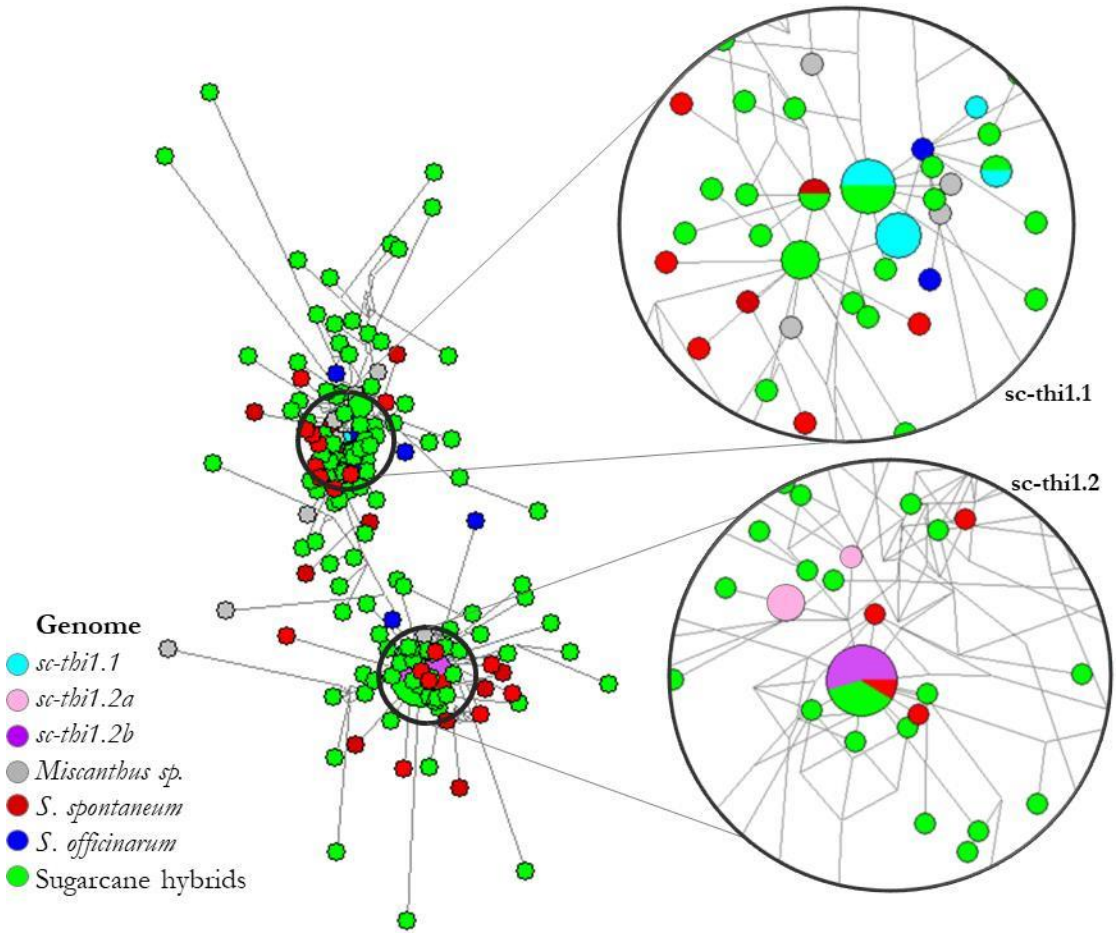

Figure 23. *Network analysis of thi1 gene in Saccharum complex*. The network was constructed using the NETWORK 4.6.1.3 software (Bandelt *et al.*, 1999) with default parameters. A 539 bp alignment of 210 sequences was used to construct the network. The right part of the figure is a close-up of the entire network shown in the left. The size of the circles is proportional to the number of sequences in the haplotype; the distance between clusters is proportional to the number of substitutions observed between sequences.

The network analysis showed two sequence groups within the species analyzed in the *Saccharum* complex. These groups were not species- or cultivar-specific, but were, instead, *sc-thi1* copy-specific (Table 4), supporting the notion that all sugarcane varieties studied have the two different loci for *sc-thi1*, including the parental cultivars *S. spontaneum* and *S. officinarum,* and *Miscanthus* sp. This is in accordance with the reconstructed phylogenetic tree and the synteny analyses, suggesting early *thi1* duplication in C4 grasses.

Network analysis also supports the diversification of two subgroups *sc-thi1.2*. The six sequences from *sc-thi1.2b* fall into one haplotype (along with one *S. spontaneum* and four modern cultivars sequences), whereas the four sequences from *sc-thi1.2a* fall into two different haplotypes, one composed by several BACs (017_B18, 030_H05 and 251_N23) and a second composed by 092_F09.

2.3.5. Differential expression analysis of *sc-thi1*

In order to analyze if there is differential expression among *sc-thi1* versions, three RNA-seq databases available were analyzed. N-terminal sequences corresponding to the first 89 amino acids (Figure 22) were used to map the RNA-seq reads. This option was chosen because when whole CDS sequences were used, individual reads mapped to different *sc-thi1* sequences (data not shown). This occurs because, as discussed previously, the three *sc-thi1* isoforms are highly identical and differ mainly at the N-terminal region. Therefore, the N-terminus is the only region diverse enough to allow unambiguous assignment to individual transcript sequences.

The mapping was done using Bowtie2 software (Langmead *et al.*, 2009) in Galaxy version 2.2.6.2 platform (Afgan *et al.*, 2016). The total number of reads mapped was obtained by using the Unipro-UGENE software (Okonechnikov, Golosova and Fursov, 2012). The abundance of transcripts mapped to each *sc-thi1* copy was calculation of FPKM (used for paired-end databases) and RPKM (used for single-end databases) normalized expression units (Mortazavi *et al.*, 2008).

2.3.5.1. Whole root and kernel top

Our group generated an RNA-seq database of two tissues (whole root and kernel top) derived from 3 months-old R570 sugarcane plants (unpublished data from GaTE Lab – Dr. Marie-Anne Van Sluys, University of São Paulo). RNA-seq was performed using the HiSeq 1500 system (Illumina™), resulting in paired-end reads. The mapping of these reads against each of the three N-terminal *sc-thi1* copies is shown in Figure 24 to each tissue of the database.

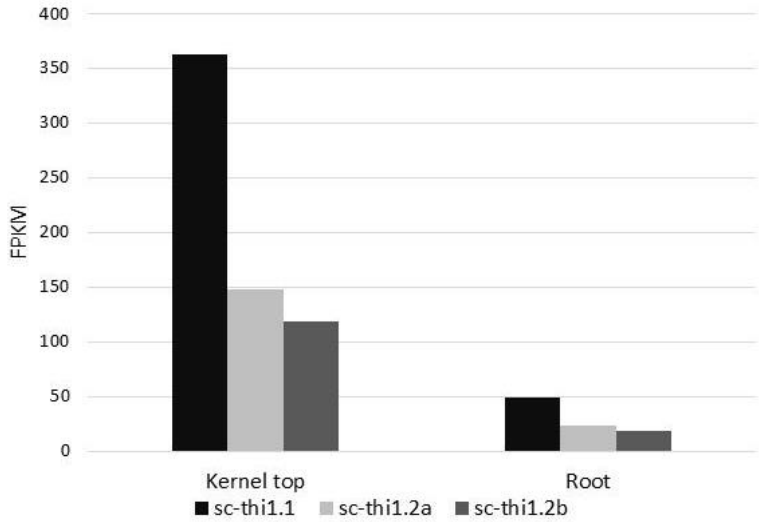

Figure 24. *Comparative transcript expression of sc-thi1 versions in two tissues of a 3 months old R570 sugarcane plants.* FPKM per tissue and *sc-thi1* copy.

The results show that all *sc-thi1* copies are differentially expressed in kernel top and root, with significantly higher expression in the kernel top tissues. Moreover, in both tissues, *sc-thi1.1* is the most highly expressed isoform (about 2-fold).

The expression of *sc-thi1.2a* and *sc-thi12.b* is also distinct, with *sc-thi1.2b* being the least expressed gene. This reinforces the subdivision of *sc-thi1.2* in two subgroups.

2.3.5.2. *Sporisorium scitamineum* (smut disease) infection

Schaker *et al.* (2016) generated and published an RNA-seq database to evaluate the effects of *Sporisorium scitamineum* (smut disease) infection in RB92-5345 sugarcane plants, using HiScanSQ (Illumina™) (pair-end reads). Two developmental stages were collect from infected (test) and uninfected (control) plants. The first developmental stage collected were buds 5 DAI (days after inoculation) and the second was the base of the whips, up to 2 cm below the culm, which is a region of intensive sugarcane cell division and fungal sporogenesis at 200 DAI, when the disease symptoms are evident. Experiments were performed in triplicates for each treatment (5 DAI control, 5 DAI infected, 200 DAI control, and 200 DAI infected), and each was mapped against the N-terminal *sc-thi1* copies independently. Figure 25 shows the results of this analysis.

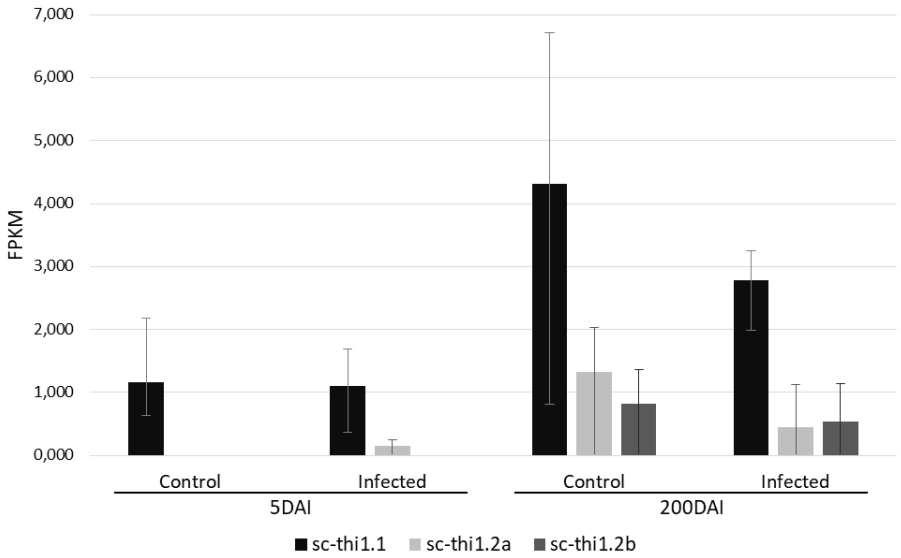

Figure 25. *Comparative transcript expression of sc-thi1 versions in two development stages of RB92-5345 sugarcane plants infected or not (control) with Sporisorium scitamineum (smut disease).* FPKM per sample and *sc-thi1* copy. DAI = days after inoculation. Minimum and maximum values are represented by the error bars.

Sc-thi1.1 is the only gene family member expressed in all conditions, being highly expressed at 200 DAI. Significant expression of both *sc-thi1.2a* and *sc-thi12.b* occurs only at 200 DAI, and at levels that are about 2-fold lower than *sc-thi1.1*, in accordance with the results

observed previously in kernel top and roots. No relevant differences were perceived between infected and control plants, indicating that *sc-thi1* is not involved in the defense response to smut disease at these time points.

2.3.5.3. Roots tips

LAFIECO group (Dr. Marcos Buckeridge, University of São Paulo) generated an RNA-seq database of four segments of 1 cm of root tips (first 4 cm from tip), of a 3 months old SP80-3280 sugarcane plants, using the 454 sequence (Roche™) platform (unpublished data). The database (single-end reads) was kindly provided to us to map against the N-terminal *sc-thi1* copies. However, no reads from any of the root tips samples mapped to the *sc-thi1* variants, indicating that this region most likely receives thiamine (or thiazole) synthesized from other tissues.

Although the experimental design of the three RNA-seq experiments used was not originally intended for this work, it still can contribute to our understanding of the biological roles of *sc-thi1* as it shows that individual *sc-thi1* copies are differently expressed at some level in different tissues, preferentially in the aerial parts, and there is no expression of any of them in root tips.

2.3.6. Subcellular localization

An *in silico* search for subcellular location signals in the *thi1* mRNA sequences was done starting from the first start codon (1ºATG) or the second (2ºATG), using five different software predictors available on-line. A chloroplast transit peptide from the 1ºATG is predicted, but no significant match for mitochondrial transit peptide was identified. There is no consistency in prediction starting from the 2º ATG (Table 5).

Table 5. Prediction in subcellular localization of silico using different predictors.

	targetP[1]		**Psort[2]**		**BaCelLo[2]**		**PlantMPloc[4]**		**Protein prowler[5]**	
	1ºATG	**2ºATG**	**1ºATG**	**2ºATG**	**1ºATG**	**2ºATG**	**1ºATG**	**2ºATG**	**1ºATG**	**2ºATG**
sc-thi1.1	C	none	C	ER	C	S	C/M/N	C/M/N	C	other
sc-thi1.2a	C	none	C	Cy	C	S	C	C/M/N	C	other
sc-thi1.2b	C	none	C	Cy	C	N	C/M/N	C/M/N	C	other

C = chloroplast; M = mitochondria; N = nuclear; ER = endoplasmic reticulum; Cy = cytoplasm; S = secretory. [1]Emanuelsson *et al.*, 2007. [2]Nakai and Kanehisa, 1991 [3]Pierleoni *et al.*, 2006. [4]Chou and Shen, 2010. [5]Bodén and Hawkins, 2005.

In order to test the subcellular localization *in vivo*, the N-terminal region containing both the CTP and the MPS of each *sc-thi1* version was fused to the green fluorescence protein (GFP) reporter in an expression vector.

Almeida (2004) has already used the three sugarcane N-terminal versions fused to GFP under the control of the 35S promoter to evaluate their subcellular targeting following transient expression in *Allium cepa* epidermis. Fluorescence, derived from GFP, was observed only in the chloroplasts. These authors suggested that perhaps this plant does not have the cellular machinery required to transport *sc-thi1* into mitochondria. Since dual targeting to chloroplast and mitochondria was demonstrated by Chabregas *et al.* (2003), using 35S-AtTHI1(N-term)-GFP transformed into WT tobacco leaf protoplasts, we decided to use *N. tabacum* as a model to evaluate the *sc-thi1* version subcellular targeting. Thus, the three constructs were transfected separately into *N. tabacum* leaves by *Agrobacterium* infiltration. The distribution of GFP expression was analyzed by confocal microscopy. Figure 26 shows the results.

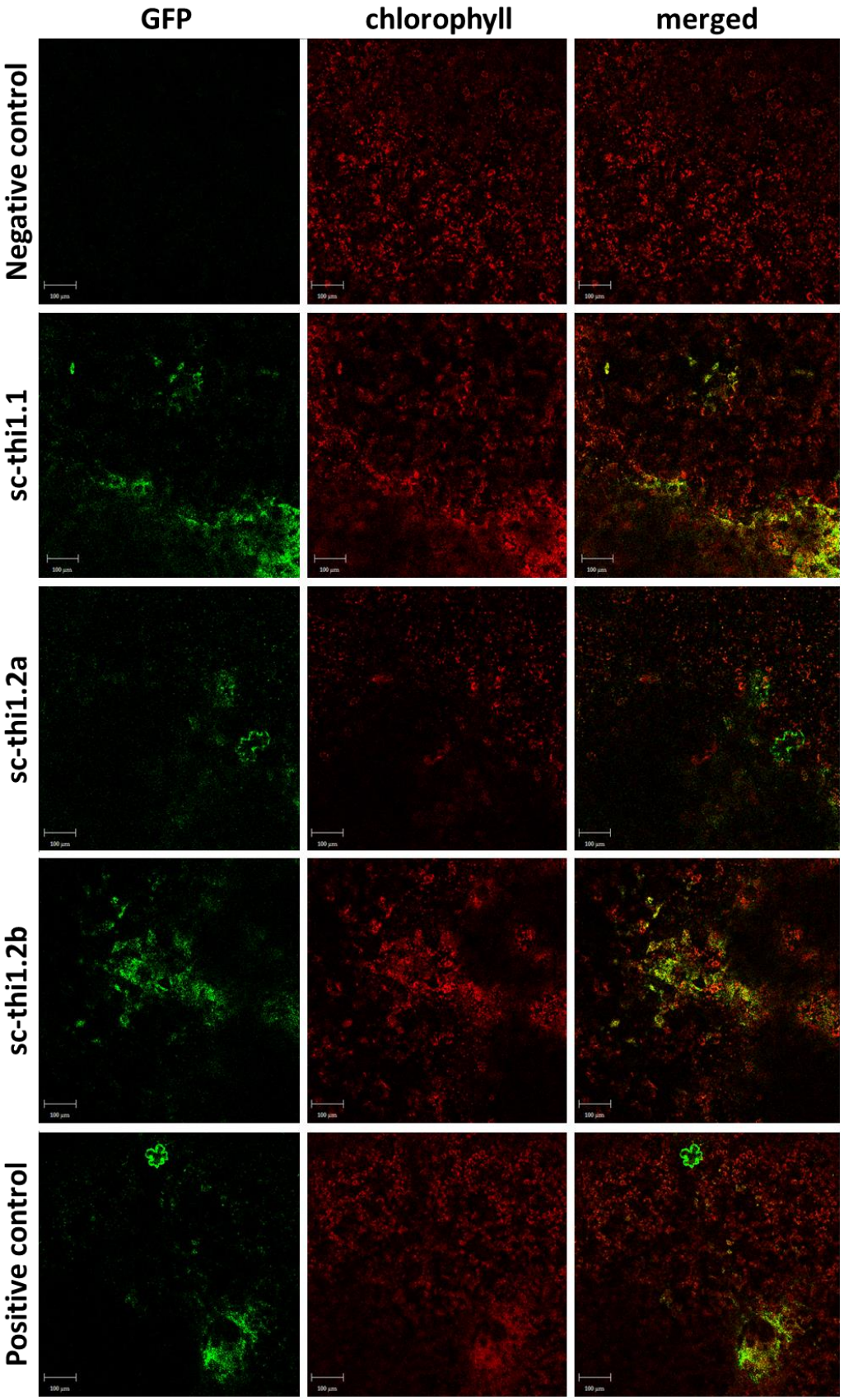

Figure 26. *Transient expression of 35S-sc-thi1Nterminal-GFP fusions in WT*. The images were taken by confocal microscope LSM880 (Zeiss™). The GFP and chloroplast signals were collected separately and later overlapped. Scale bar =100µm.

It is not possible to drive a final conclusion from the experiments above. Nonetheless, the three N-terminus-*sc-thi1* constructs display a distinct GFP distribution pattern from the positive control, which is targeted to cytoplasm. In this image, it appears that the *sc-thi1.1* N-terminal and *sc-thi1.2b* N-terminal sequences target GFP to mesophyll cells, while the *sc-thi1.2a* N-terminal sequence targets GFP to the edges of epidermal cells, in a punctate pattern. No clear chloroplast targeting is observed, and the punctate spots may correspond to mitochondria, or to inclusion bodies. Further analyses with more resolution should be done to clarify the subcellular localization.

Woodward *et al.* (2010) showed that one of the *thi1* homologue copies of *Zea mays* (THI2) accumulates in punctate spots within the chloroplast-containing mesophyll cells, using 35S-THI2-GFP construct transiently expressed in *Nicotiana benthamiana* leaves. They speculated that the unusual punctate pattern of accumulation reflects the formation of inclusion bodies, which may be a result of overexpression from the cauliflower mosaic virus 35S promoter of THI2.

Almeida, (2004) tried to express both the whole AtTHI1 protein and just the AtTHI1 N-terminal fused to GFP under the control 35S promoter in *A. thaliana*, but without success. However, under the control of the At-THI1 promoter, the AtTHI1-GFP fusion expression was found mostly in chloroplasts, in different stages of development and tissues.

A further complication is that overexpression of THI1 paralogs under the constitutive 35S promoter may trigger posttranscriptional gene silencing or bias protein targeting, resulting in inconsistent observations, depending on the system and THI1 paralog used for the analysis.

2.3.7. Functional complementation assay

It was previously shown that the *S. cerevisiae* strain KBY5 (THI4::URA3), which has a disruption in the *thi4* gene, cannot grow in minimal medium without thiamine supplementation. Significantly, the *A. thaliana* homologue (*At-thi1*) is able to restore thiamine auxotrophy of this strain (Machado *et al.*, 1996). Therefore, a functional analysis of *sc-thi1* was performed using the *S. cerevisiae* thiamine auxotrophic strain KBY5 to test the thiazole synthetic properties of *sc-thi1.1*, *sc-thi1.2a* and *sc-thi1.2b*.

The three entire CDSs representing each *sc-thi1* group, as well their corresponding DelN versions (that is the CDS lacking the N-terminal chloroplast transit peptide sequences) were synthesized (Figure 22) and transferred to the yeast expression vector pG-1 (Schena, Picard and

Yamamoto, 1991). Two constructs, A184V and DelN present in the same expression vector were used as controls (Papini-Terzi *et al.*, 2003; Galhardo, 2003). A184V is the cDNA of the *thi1*gene from the *A. thaliana tz-201* mutant and was used as positive control (in fact, it serves as a control for partial complementation, since Papini-Terzi *et al.* (2003) showed that the thiamine auxotrophy complementation was incomplete and the complemented strain grew more slowly than did the wild-type yeast). DelN contains a cDNA for the *thi1* gene of WT *A. thaliana* without the N-terminal chloroplast transit peptide (first 69 amino acids). This was used as negative control, as Galhardo (2003) showed previously that the *A. thaliana* DelN was unable to complement yeast thiamine auxotrophy.

The constructs were transformed in KBY5 strain and plated on minimal medium lacking thiamine (YNB –thia), along with the WT strain, W303a. Growth was evaluated during the following 28 days of incubation at 30ºC. The results are shown in Figure 27. Panel (a) of Figure 27, shows all transformants plated in YNB –thia medium that was supplemented with both thiamine and tryptophan, showing that all strains are able to grow efficiently in the absence of selection. Figure 27 (b) shows the results of growth of these strains on medium supplemented only with thiamine; thus, only transformants with tryptophan vector-based complementation are able to grow. Since neither W303a nor KBY5 were able to grow but all transformants did grow, this shows that transformation was efficient and that the presence of the plasmid complemented tryptophan auxotrophy in the recipient strain. Figures 27 (c) and 27 (e) show the results of growth on plates supplemented only with tryptophan, after 4 days and 28 days of incubation, respectively. In this plate, only strains W303a and auxotrophic strains that were complemented by the recipient plasmid were able to grow. Figures 27 (d) and 27 (f) show the results of growth on plates lacking both thiamine and tryptophan, after 4 days and 28days of incubation, respectively.

In Figures 27c-e, none of the *sc-thi1* entire CDSs were able to complement thiamine auxotrophy, as was also the case for the negative control and for strains expressing s*c-thi1.2a*DelN. However, both DelN versions of *sc-thi1.1* and *sc-thi1.2b* were able to complement thiamine auxotrophy. However, complementation is less efficient than with the A184V allele, with the yeast forming smaller colonies. Papini-Terzi *et al.* (2003) showed that the KBY5 strain bearing the A184V construction grows poorly after 4 days. This result was reproduced, although *sc-thi1.1*DelN and *sc-thi1.2b*DelN transformants took longer (28 days) to grow.

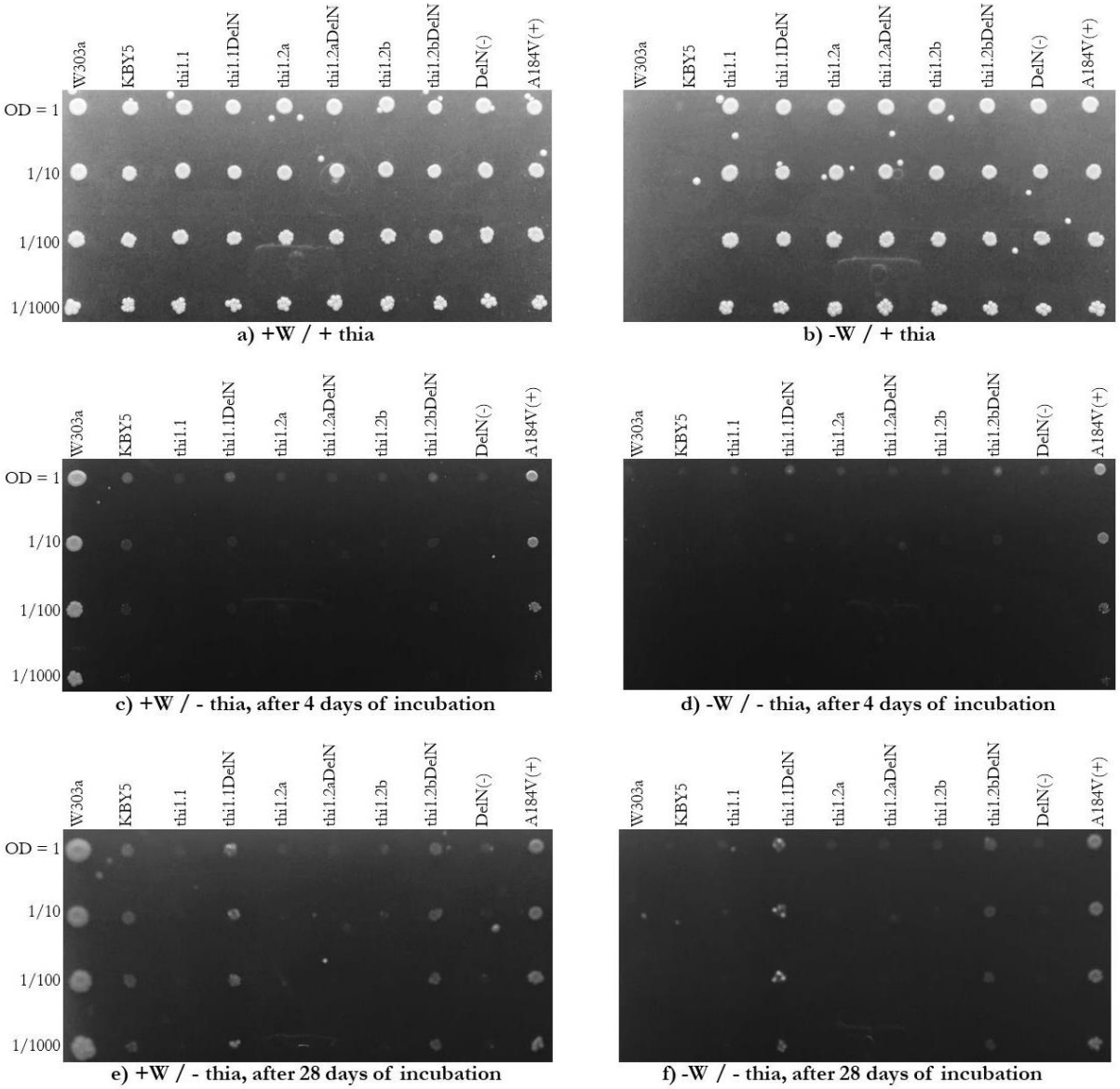

Figure 27. *Functional yeast complementation assay. S. cerevisiae* strains W303 and KBY5 were transformed with the three versions of *thi1* gene found in sugarcane genome, and with the positive control A184V and the negative control and DelN, and plated in YNB media with or without tryptophan and/or thiamine. W = tryptophan and "thia" = thiamine. Each column represents one transformant. Lines represent dilution series. a) and b) are the experimental controls to check if all strains are able to grow (a) and if all transformed ones can grow without tryptophan (b). c) and d) show the growth after 4 days of incubation, while e) and f) is after 28 days of incubation, both cases at 30ºC.

This result indicates that the N-terminal chloroplast transit peptide from sugarcane somehow interferes in the complementation efficiency in KBY5 yeast strain, while the DelN version of *A. thaliana* blocks the complementation effect. This is not surprising as this region is highly variable, as discussed above. Nonetheless, without the N-terminal chloroplast transit peptide, only two of the three representative of *sc-thi1* are able to complement the lost function within the thiamine pathway.

2.4. Discussion

All plants sequenced to date present at least one *thi1* homologue gene. The phylogenetic tree inferred using plants *thi1*-like genes is in accordance with the species taxonomy (Eudicots, Monocots, Bryophytes and Chlorophytes), indicating that this gene evolved along with the rest of the plants' genomes. An alignment of these *thi1*-like sequences showed that most of the nucleotide changes have resulted in synonymous substitution, except within the N-terminal region, which is highly variable. The maintenance of this gene throughout the evolution of the plants and the amino acid sequence of the catalytic sites can be explained by the proposed *thi1* dual function (Machado *et al.*, 1996). *Thi1* has a key function in thiamine biosynthesis as an important cofactor for many metabolic pathways, such as the carbohydrate pathway (Belanger *et al.*, 1995). It also has a less well understood function in organellar DNA damage tolerance and in other stress related pathways (see section 1.2.1 for a review of this subject).

The variability found in the N-terminal region could be explained by its targeting function, which depends on the kinds of amino acids present (hydrophobic and positively charged) (van Heijne and Gavel, 1988; Käll, Krogh and Sonnhammer, 2004) rather than on specific amino acid sequences. This would make it a region able to accommodate non-synonymous substitutions without loss of function. Although *thi1* homologues are present in all plants, the synteny analysis showed that *thi1* genes from different plants are found in different genomic environments.

Our molecular characterization of the sugarcane *thi1* gene revealed that gene duplication has not only occurred in C4 photosynthetic plants but that the genes are located on different genomic regions. Five non syntenic genomic environments were identified. There have been duplications and translocations of the gene, as well as duplications of entire regions containing the gene. Based on genomic diversity analysis, we conclude that the two copies of *thi1* present in sugarcane arose in the ancestral Panicoideae subfamily and remains in many modern cultivars of sugarcane.

Genomic identification of sugarcane *thi1* homologues revealed nine alleles of *sc-thi1.1* and ten alleles of *sc-thi1.2*. Phylogenetic and network analyses of its sequences showed that, despite the similarity of all *sc-thi1.2* genes, a diversification of *sc-thi1.2* is nonetheless occurring. Two subgroups were identified (comprising four alleles of the *sc-thi1.2a* group and six alleles of the *sc-thi1.2b* group).

Analysis of tree sugarcane RNA-seq databases revealed that all *sc-thi1* versions are more highly expressed in older plants (six months) when compared to young buds. Also, they are up to 7-fold more highly expressed in kernel top than in roots. This correlates with the *At-thi1*

expression pattern, which showed higher expression in leaves and very low expression in roots (Papini-Terzi *et al.*, 2003). None of the *sc-thi1* versions are expressed at root tips, in accordance with the fact that *At-thi1* expression is restricted to the vascular system in root (Ribeiro *et al.*, 2005).

Low levels of *thi1* in roots could be due the state of plastid maturity and, consequently, their readiness for thiamine synthesis, which would suggest a dependence on inter-cellular transport of thiamine and/or the thiamine precursor thiazole, and explaining why isolated roots from most plant species cannot grow *in vitro* without thiamine supplementation (Bonner, 1940).

Sc-thi1 is the most expressed gene, being 2-fold more highly expressed than the two *sc-thi1.2* gene, in all studied cases. *Sc-thi1.2a* is slightly more expressed than *sc-thi1.2b*. The differential expression in times and/or tissues of *sc-thi1* isoforms is in accordance with observations in *Zea mays*, which also has two *thi1* copies (*zm-thi1* and *zm-thi2*). These genes also have a distinct pattern of accumulation within the plant, which reflects their sub-functionalization, with zm-THI1 being predominantly in mature green leaves, and zm-THI2 expressed preferentially in young, rapidly dividing tissues (Woodward *et al.*, 2010).

Although rice *thi1* homologues have been related to biotic stress (specifically, resistance to *X. oryzae* pv. o*ryzae* (Wang *et al.*, 2006)), the comparison between the expression pattern of sc-*thi1* version in control plants and plants infected with smut disease (*Sporisorium scitamineum*) indicates that *sc-thi1* is not involved in the response to this biotic stress.

Differences in expression pattern are also observed in the subcellular localization of sc-*thi1* copies. *Sc-thi1.1* and *sc-thi1.2b* have a similar pattern, being targeted to the mesophyll cells, while, *sc-thi1.2a* is found in the edges of epidermal cells, in a punctuated pattern.

Although both *zm-thi1* and *zm-thi12* can restore thiamine prototrophy in yeast (Belanger *et al.*, 1995), only two of the three *sc-thi1* isoforms can complement the KBY5 strain *thi4* mutation, albeit less efficiently. However, *sc-thi1.1* and *sc-thi1.2b* are able to do so when the chloroplast transit peptide is removed (DelN), indicating that this region may interfere somehow with mitochondrial translocation in yeast.

The differences observed in cellular localization, expression, and capacity for functional complementation, supports the presence of three *thi1* homologues in sugarcane and opens avenues for further functional studies of these genes.

2.5. Conclusions

- The *thi1* gene is present in all sequenced plants, with different copy numbers and in different genomic contexts. This thesis expanded this observation.

- While the N-terminus of THI1 protein is highly variable, the rest of the protein is well conserved.

- Sugarcane has three copies of *thi1*: *sc-thi1.1*, which is the most different among the three, and *sc-thi1.2a* and *sc-thi1.2b*, which are similar and yet seem to be diverging from each other.

- *Sc-thi1.1* is the most highly expressed isoform in different tissues and developing stages.

- *Sc-thi1* is not involved in smut disease response.

- *Sc-thi1.1* and *sc-thi1.2b* are capable to partially complement thiamine auxotrophy in a mutant yeast strain.

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3. CONSTRUCTION OF PLANT MODEL FOR FUNCTIONAL STUDY OF *THI1* HOMOLOGUES

ABSTRACT

The use of complementation assays to identify and study *thi1* gene homologues from other species is not possible in *A. thaliana* as the only *At-thi1* mutant able to survive (*tz-201* mutant) is not transformable. Therefore, plant models with modified *thi1* genes are needed. *Physcomitrella patens* was chosen as it shares fundamental genetic and physiological processes with vascular plants, and can be used in studies of plant evolution, development and physiology. *P. patens* is a small organism with a short life cycle. Moreover, *P. patens'* dominant phase is the haploid gametophyte, which facilitates genetic analyses. Interestingly, even though it is an ancient plant, it has six *thi1* homologues, in contrast to C4 grasses, which contain only two copies. The copies were named based on the chromosome number on which they are located and on their chromosomal orientation (*PpThi1.20F*, *PpThi1.20*R, *PpThi1.23F*, *PpThi1.23*R*1*, *PpThi1.23*R*2* and *PpThi1.24*). Two of these (*PpThi1.23*R*1*, *PpThi1.23*R*2*) are identical and are treated here as one class (*PpThi1.23*R). In order to produce *thi1* gene modified plant lines, Homologous Recombination technology was used. Five plasmids were made in order to knockout each of the five *PpThi1* gens individually, resulting in the replacement of the native gene with a resistance gene cassette that also allows for the selection of the transformants. Gene knockout plasmids were transformed into protoplasts, which were analyzed for survival and regeneration capabilities. Single *PpThi1* mutant lines were obtained for all *PpThi1* copies. Many regenerants were obtained for all *PpThi1* copies knockouts, except for *PpThi1.20F*, which appeared to affect the ability of protoplasts to survive and regenerate. Morphological analysis, growth area measurement, fresh and dry weight analyses and soluble carbohydrate content quantification showed that both protonemal and gametophyte growth of the knockouts plants were normal, although subtle effects morphological and physiological were observed. Because the experimental design allows for recycling of the resistance gene cassette, it is possible to sequentially construct strains in which multiple, and indeed all, of the *thi1* genes are mutated and/or deleted. Such multiple mutants will be useful for *thi1* functional complementation experiments and for discerning the specific function of individual *thi1* gene family members.

Keywords: *Physcomitrella patens*; Plant model; *Thi1* gene; Mutant lines; Homologous Recombination; Soluble sugar; Protonemata regeneration

3.1. Introduction

3.1.1. *Physcomitrella patens* as a plant model

Our group has used a number of different techniques to transform the only available *At-thi1* mutant (*tz-201*). To date, these efforts have not been successful and no plants have been regenerated from transformation experiments (Papini-Terzi *et al.*, 2003). Thus, a complementation assay in *A. thaliana tz-201* mutant is not possible. To bypass this problem, other plant models should be tested. Thus, *Physcomitrella patens* was chosen to generate *thi1* mutants.

Physcomitrella patens, a moss (Bryophyta) from the family Funariaceae, is used as a model organism for studies on plant evolution, development and physiology. Mosses share fundamental genetic and physiological processes with vascular plants, although the two lines diverged early in land plant evolution (Kamisugi *et al.*, 2006). A comparative study between modern representatives of the two lines should give insight into the evolution of the mechanisms behind the complexity of modern plants (Kamisugi *et al.*, 2006). Moreover, *P. patens* can be studied in two distinct phases of its life cycle, 1) a haploid gametophyte (the dominant phase) that produces gametes and 2) a diploid sporophyte wherein haploid spores are produced (Figure 28). Also, during the haploid phase we can perform analyses with photosynthetic (chloronema) and non-photosynthetic (caulonema) tissues. The whole life cycle can be achieved under optimal conditions in less than 12 weeks.

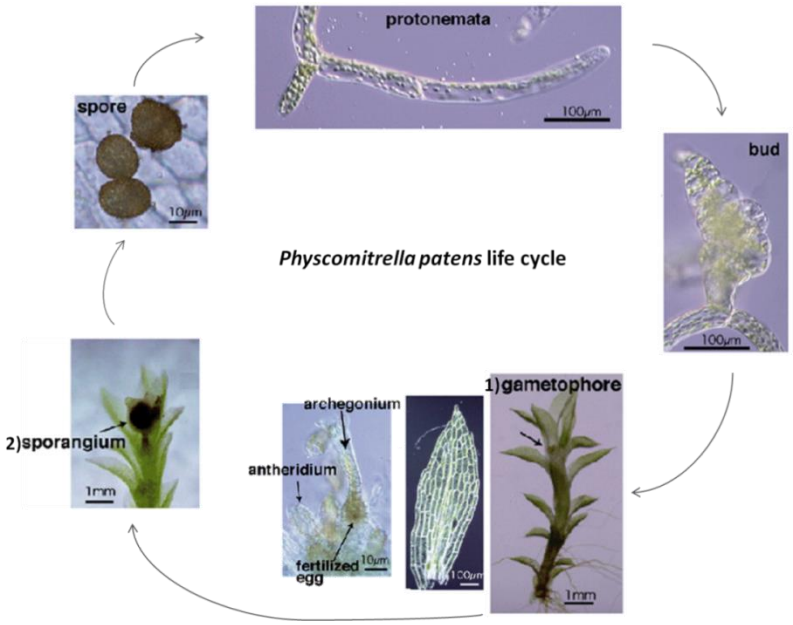

Figure 28. *Physcomitrella patens* life cycle: haploid spores produce protonemal filaments that extend by apical division and tip growth. The filaments produce buds that develop into leafy gametophores, which enlarge by diffuse growth. Haploid spores are produced by diploid sporophytes, which develop from eggs fertilized by swimming sperm at the gametophore apex. Modified from PHYSCObase database.

The haploid genome of *Physcomitrella patens* ssp. *patens* ecotype Gransden 2004 is estimated to be ~480 Mbp contained in 27 pairs of chromosomes, and was sequenced to approximately 8.1x depth (Rensing *et al.*, 2008).

A preliminary BLASTn analysis at Phytozome 10.2 database showed that *P. patens* has at least 4 *thi1* genes while *A. thaliana* has only one copy, sugarcane has two copies as other C4 metabolism plants. It is intriguing that a supposedly simple plant, *P. patens*, contains more gene copies of *thi1* than higher plants and it will be interesting to find out if there is a greater degree of biochemical specialization of THI1 functions in the moss than in the other plant species.

P. patens is one of a few known multicellular organisms that displays highly efficient Homologous Recombination (HR) (Schaefer and Zryd, 1997; Schaefer, 2002), meaning that its can be used to create knock-out or knock-in moss lines relevant for plant biology functional studies. Because the haploid phase is dominant in *P. patens*, this also means that the phenotypes of gene knockouts reveal themselves immediately. Therefore, to create mutants for each *thi1* gene copy, we used an HR strategy.

3.1.2. Homologous Recombination

HR (also called general recombination) results in the transfer of genetic information between two DNA duplex segments of similar nucleotide sequence. It is an essential cellular process required for generating genetic diversity, ensuring proper chromosome segregation, and repairing certain types of DNA damage, like DNA double-strand breaks (DSBs) (Krebs, Goldstein and Kilpatrick, 2014).

During meiosis, HR results in DNA crossover events between homologous chromosomes (Figure 29) that produce the genetic diversity inherent in germ cells. HR is also involved in maintenance of somatic cell genomic stability by restoring replication after a stalled replication fork has encountered a DNA lesion or strand break, as well as following exogenous stresses such as ionizing radiation that induce DNA double-strand breaks. The importance of HR can be gauged by the conservation of HR genes and functions from bacteria to man (Amunugama and Fishel, 2012).

Although it can occur anywhere on a DNA molecule, HR requires extensive base-pairing interactions between complementary strands of the two interacting DNA duplexes (Alberts *et al.*, 2008). Mismatch repair (MMR), a conserved process that corrects DNA mismatches formed during replication and recombination, ensures that HR occurs between perfectly homologous sequences and suppresses recombination between sequences that contain

partial homology (Amunugama and Fishel, 2012). Genetic studies in yeast indicate that even a single mismatch reduces the recombination rates by at least fourfold compared to recombination between substrates of perfect homology (Datta *et al.*, 1997).

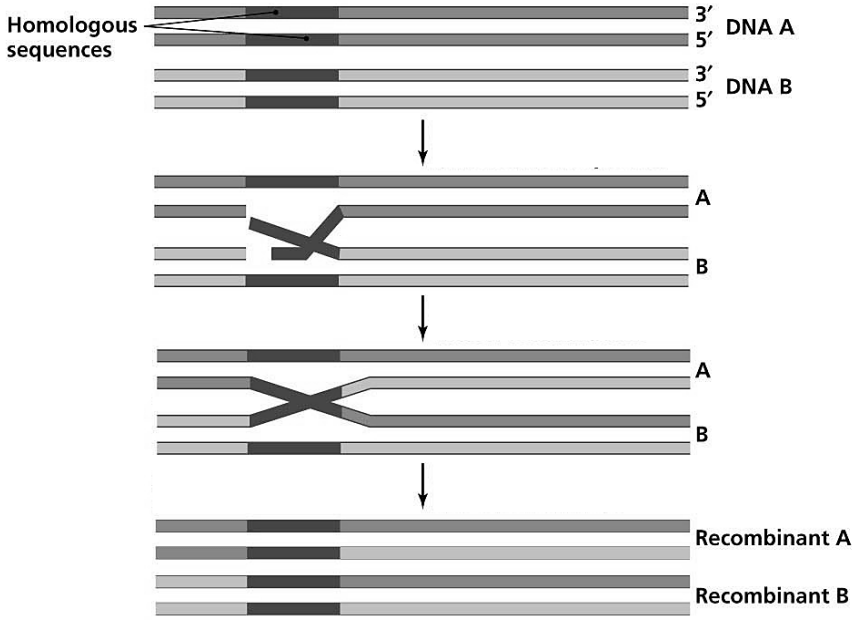

Figure 29. *Homologous recombination during meiosis.* Modified from http://academic.pgcc.edu.

HR can be used to create recombinant DNA and genetically modified organisms, by targeting an exogenous DNA sequence to a specific genomic position to create knock-out or knock-in organism lines. This approach is called reverse genetics and it is a powerful and sensitive tool to study the function of genes. HR occurs between each end of a targeting construct and the native locus occurs when either single or multiple targeting vectors are delivered, resulting in a 'Targeted Gene Replacement' (TGR) (Kamisugi *et al.*, 2006). The method is especially common in yeast and is also used in mouse genetics to create 'knockout' mice lines.

Among the plants, *P. patens* displays highly efficient rates of HR. If the transforming DNA contains homology to the *Physcomitrella* genome, integration can occur at the cognate genetic locus occurs at frequencies of up to 100% (Kamisugi, Cuming and Cove, 2005), whereas in flowering plants, HR occurs at far low frequencies (typically ~ 10^{-4} – 10^{-5}) (Britt, 2003).

In this work we created gene knockouts for each of the *PpThi1* genes using the HR strategy described above. Mutants were selected for the presence of the antibiotic selection cassette and validated by PCR analysis of recombination borders. Finally, knockout plants were characterized for anatomical, physiological and metabolic phenotypic differences.

3.2. Material and Methods

Construction of the plasmids used for HR of *PpThi1* genes, along with a knowledge of the methods used for cultivation, protoplasts production, transformation and regeneration of *P. patens*, was carried out at the School of Environmental and Biological Science of Rutgers University, The State University of New Jersey (United States of America), under the supervision of Dr. Michael Lawton and collaboration of Dr. Rong Di, during the internship abroad established in the Double Degree PhD program, with FAPESP BEPE fellowship from September 2015 to September 2016. HR lines and subsequent analyses were performed at GaTE Lab (IB-USP).

3.2.1. Plant material and growth conditions

All experiments were performed using the *Physcomitrella patens* ssp. *patens* ecotype Gransden 2004. Gametophores were cultured aseptically on BCDAT medium (0.1 mM $MgSO_4$, 1.84 mM KH_2PO_4, 1 M KNO_3, 4.5 mM $FeSO_4$, 5 mM Ammonium tartrate, 50 mM $CaCl_2$, 8% agar) (PHYSCO manual), in growth chamber at 25º ± 1ºC, with 12/12h dark/light periods. Protonemata-rich cultures were obtained and kept in BCDATG medium (BCDAT medium supplemented with 0.5% [w/v] glucose) (PHYSCO manual).

3.2.2. Gene copies identification

The genome of *Physcomitrella patens* is already sequenced and available (COSMOSS; PHYSCObase database). A BLASTn analysis against the Phytozome v.12 database shows the presence of six gene copies homologous to *At-thi1* at the *P. patens* v3.3 genome. They were named *PpThi1.20F*, *PpThi1.20*R, *PpThi1.23*F, *PpThi1.23R1*, *PpThi1.23*R*2* and *PpThi1.24* based on the chromosome number on which they are located (chromosomes 20, 23 or 24) and their respective chromosomal orientation (F or R) on it. As *PpThi1.23R1* and *PpThi1.23R2* are 100% identical, they were treated as one unit, since both gene copies can be deleted simultaneously by a single HR event.

3.2.3. Constructs for Homologous recombination

Five vectors were constructed to individually knock out each copy of *PpThi1* by HR. To construct these vectors, flanking regions of each gene copy were selected and amplified from genomic DNA by PCR. Three different expression vectors containing three different selection markers were used as the plasmid backbones for the construction of the final gene targeting plasmids: pTN82 (G418 geneticin resistance; PHYSCObase database) used to knock out *PpThi1.20F* and *PpThi1.23F*; pBSMDIIIa (hygromycin resistance; which is identical to pBSMDII (Finka *et al* ., 2008), except that the Neo/Kan^{R} cassette is replaced with AphIV/Hygromycin^{R}) used to knock out *PpThi1.20R* and *PpThi1.23R*; and p35S-zeo (zeocin resistance; PHYSCObase database) used to knock out *PpThi1.24*. pNT82 and pBSMDIIIa contain two loxP sites in the same orientation and which flank the resistance genes. This feature allows the selection marker cassette to be excised by the later expression of *Cre* site-specific recombinase. The utility of this feature is that selection cassettes can be used to create multiple knockouts through repeated rounds of HR and recycling of the selection marker cassette. Figure 30 shows the scheme for replacing the gene of interest (*thi1*) by the selection marker using HR.

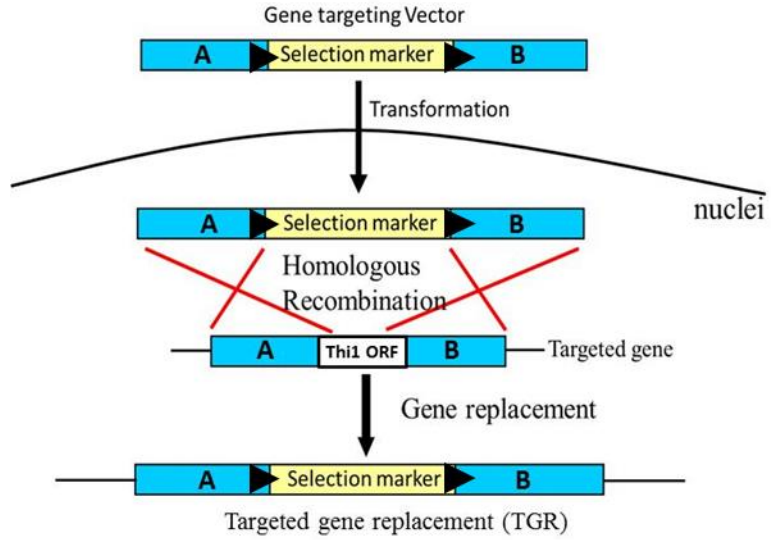

Figure 30. *Homologous recombination scheme:* The target gene represents any *thi1* copy. The regions A and B were chosen as HR targeting sequences. The gene targeting plasmid contains a selection marker in between regions A and B. After cell transformation, HR occurs between the gene targeting plasmid and the targeted native gene locus, resulting in Targeted Gene Replacement (TGR) or gene knockout (KO). The black arrowheads represent loxP sites (present only in the constructions using pNT82 and pBSMDIIIa vectors) which can be used to remove the selectable marker from the target gene locus. Modified from Kuwayama (2012).

Specific pairs of primers were designed to PCR amplify specific A and B regions for each of the five *PpThi1* genes. These primers also add restriction sites to the ends of each amplicon in order to facilitate sub-cloning into the backbone vectors by restriction digest (Table 6). The resulting amplicons were cloned into their respective gene targeting plasmids.

Table 6. Primer design to construction of HR plasmids.

Plasmid #	Final plasmid name[1]	*PpThi1* region	Amplicon size	Primer name[2]	Primer sequence
1	*PpThi1.20F*-G418	A	938 bp	A Foward+XhoI	**ggCTCGAG**ACATCAGTAGGAGTGCTAGATGC
				A Reverse+SalI	**ggGTCGAC**GTCAACACAATGCAGAAGCAAG
		B	987 bp	B Foward+SpeI	**ggACTAGT**GATTGCTTGTGCAGGGATTT
				B Reverse+SacI	**ggGAGCTC**ACTTGTTCACAGTGAGGTGGTG
2	*PpThi1.20R*-hyg	A	850 bp	A Foward+SalI	**ggGTCGAC**GAGAAAGCGGAGCTCAGTCA
				A Reverse+HindIII	**ggAAGCTT**CCGGAATCAGCACTTCAGAAT
		B	982 bp	B Foward+SphI	**ggGCATGC**GATCAAGAGAATGCCTGGGAGTG
				B Reverse+BamHI	**ggGGATCC**TGCTGTCGATGCCTTCCTATCAT
3	*PpThi1.23F*-G418	A	518 bp	A Foward+SalI	**ggGTCGAC**GAAATCAAACTTGATGTATCTACG
				A Reverse+ClaI	**ggATCGAT**GTGGAGATAGCGAAAGATGG
		B	869 bp	B Foward+SpeI	**ggACTAGT**GAGGAGCACAATGGATACTG
				B Reverse+SacI	**ggGAGCTC**TTCTTCCAGCAGCTTGTC
4	*PpThi1.23R*-hyg	A	927 bp	A Foward+SalI	**ggGTCGAC**GAGCCATTGCATAGAAAGC
				A Reverse+HindIII	**ggAAGCTT**ACAGTAGTTAGCGATCAGAGC
		B	1014 bp	B Foward+SphI	**ggGCATGC**CTTTCGTCATCGAACTGG
				B Reverse+BamHI	**ggGGATCC**CTTTATCAGTGGGGCTGTAG
5	*PpThi1.24*-zeo	A	800 bp	A Foward+SalI	**ggGTCGAC**GCGCACAGCAATGTCAGAGT
				A Reverse+EcoRI	**ggGAATTC**AGACTACGATCGCTGCTCACAC
		B	1024 bp	B Foward+SacI	**ggGAGCTC**GCTGAGAGAGTCCCTTAAAACCT
				B Reverse+XbaI	**ggTCTAGA**CTGCACATTCATCTGCCATT

[1]Plasmid name: Pp = *Physcomitrella patens*; *thi1* gene; # = chromosome; zeo = zeocin resistance plasmid; hyg = hygromycin resistance plasmid; G418 = G418 resistance plasmid
[2]Primer name indicates the amplified region, the orientation and the restriction site added
Bold letters indicate the restriction site in the primer sequence.
.

The final plasmids were verified by restriction enzyme assay and PCR, and transformed independently into *P. patens* protoplasts.

3.2.4. Transformation of Moss protoplasts

Protoplast preparation and polyethylene glycol (PEG)-mediated transformation of *P. patens* was performed according to PHYSCO manual (Figure 31).

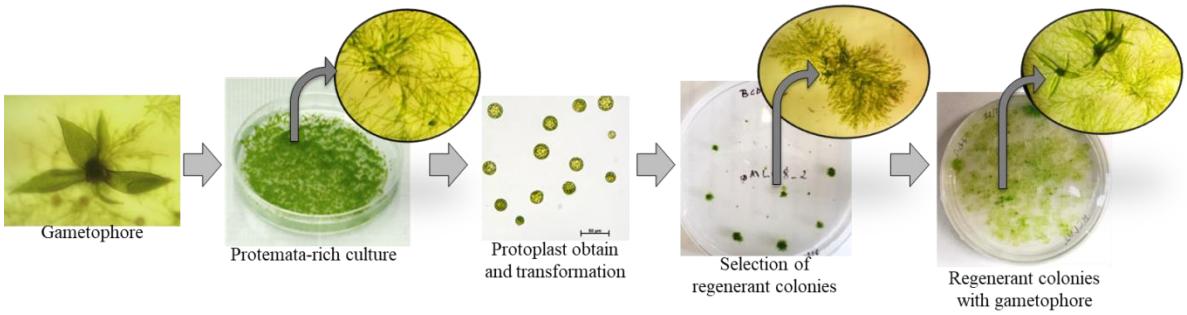

Figure 31. *Experimental scheme comprising protoplast isolation, PEG-mediated transformation, antibiotic selection and regeneration of P. patens transformants.*

The protonemata-rich culture is obtained after seven cycles of wild-type (WT) tissue fragmentation with a homogenizer followed by incubation on cellophane sheets layered onto BCDATG medium plates, every seven days. This culture is then used to prepare protoplasts by cell wall digestion with 1% Driselase™ (Sigma-Aldrich®) enzyme solution.

About 6 µg of each plasmid was transformed independently into $1.2x10^6$ cell/ml, using a 0.4 mg/mL PEG4000 solution. As negative control, WT *P. patens* protoplasts were submitted to the same transformation process without the addition of plasmid DNA. At least three independent transformations events were carried out for each plasmid.

Transformed protoplasts were incubated for 5 days on BCDAT medium without antibiotics and then transferred to BCDAT medium containing either 20 mg/L hygromycin or 20 mg/L G418 or 50 mg/L zeocin for 3 weeks. False positives that arise due to episomal expression of the selective marker were removed by a sequential regimen of selection/non-selection/selection. This effectively kills any 'escapes' due to episomal maintenance of the gene targeting plasmids. Since *P. patens* is haploid, 0.5 mg/L of thiamine was included in the growth medium to complement possible loss of thiamine biosynthetic capability.

The regeneration rate was evaluated for each transformation experiment. Genomic DNA of plants which still retained resistance to antibiotics was extracted by Invisorb® Spin Plant Mini Kit (Stratec Molecular GMBH). Integration of the resistance gene was confirmed by PCR analysis with primers specific for each resistance gene (zeocin: ZeoR 1F: CTT ATC CTC AGT CCT GCT CCT CT and ZeoR 377R: CAA GTT GAC CAG TGC CGT TC; hygromycin:

HygF: CTA TTT CTT TGC CCT CGG ACG and HygR: GCG ACG TCT GTC GAG AAG TT; geneticin: nptIIF: GAG GCT ATT CGG CTA TGA CTG and nptIIR: TCG ACA AGA CCG GCT TCC ATC).

To prepare protonemata that were at similar developmental stages, this tissue was fragmented with a homogenizer and transferred onto new medium every five days (Nishiyama *et al.*, 2000). On this culture schedule, the protonemata usually did not develop any buds. Protonemata-rich mutant cultures were stored aseptically in water in 50 mL conical tubes at 4ºC in the dark. When necessary, they were spread on cellophane sheets layered onto BCDATG medium plates, and kept in normal growth conditions for one week to recover.

3.2.5. Morphological analysis

The regeneration of the protonemata-rich mutants in BCDAT and in BCDATG (0.5% [w/v] glucose) was observed by stereomicroscopy and finer-scale observations were made by light microscopy.

3.2.6. Effect of thiamine on mutant growth

The effect of thiamine in the medium on the growth rate of mutant colonies was evaluated. As a starting material for this experiment, ten protonemal colonies (~1 mm in diameter) of each mutant line were inoculated the same distance apart on BCDAT agar plates supplemented or not with 0.5 mg/L thiamine and kept in normal growth conditions. The protonemal colonies and the gametophores were observed and photographed under the stereomicroscope after 1 and 2 weeks.

The growth of the culture was measured using the area obtained from the analysis of the microscopy images. The original image was filtered to obtain a mask only in the desired color spectrum. For the analysis the HSV (Hue, Saturation, Value) representation was used with the limits presented in Table 7. Each parameter can vary its value between 0 and 1. These limits maximize the visualization of the shades of green.

Table 7. HSV limits used to create the image mask

	Lower limit	Upper limit
Hue	0.077	0.430
Saturation	0.258	0.952
Value	0.369	0.784

A Matlab code was implemented to create the masks for each image iteratively. An example of the created masks can be seen in Figure 32.

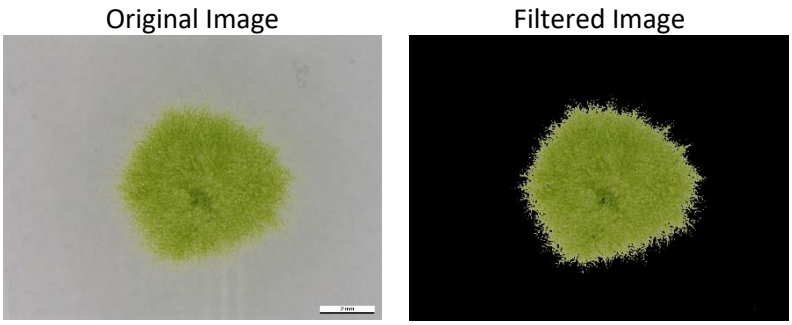

Figure 32. *Image filtering process.*

The original image is composed by 1440 x 1920 pixels. The 2 mm scale bar in the original image corresponds to 275 pixels. From that, the area of one pixel ($Pixel_{area}$) corresponds to:

$$Pixel_{area} = \frac{(2\ mm)^2}{(275\ px)^2} = 5.289 \cdot 10^{-5}\ mm^2$$

The mask indicates the number of pixels that are green, which corresponds to the colony. Thus, it is just necessary to multiply the number of green pixels by the $Pixel_{area}$ to obtain the total cultured area. By measuring the cultured area after one and two weeks it is possible to evaluate the culture growth using the following formula:

$$Growth\ (\%) = \frac{Area\ after\ 2\ weeks}{Area\ after\ 1\ week} \cdot 100$$

3.2.7. Fresh and dry weights

Fresh and dry weights were measured for four colonies with gametophores of each *PpThi1* mutant, except for the *PpThi1.20F* mutant, for which only two colonies were obtained. Dry material was obtained by drying the colonies for 2 hours at 110º C in an incubator. The ratio between dry weight and fresh weight was determined.

3.2.8. Carbohydrate analysis

Plant material prepared to determine dry weight was used to quantify soluble carbohydrates. First the material was pulverized using a mortar and pestle. Total soluble carbohydrates were extracted by six washes with 1 mL of 80% ethanol and concentrated by SpeedVac™. The material was resuspended in 1 mL of Milli-Q™ water and pigments were removed by chloroform extraction. Carbohydrate composition was determined by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD) in a Dionex DX-500 system using a Carbo-Pac SA10 column.

3.3. RESULTS AND DISCUSSION

3.3.1. Genomic identification of *PpThi1* homologues

A BLASTn analysis against the Phytozome v12.0 database reveals the presence of six gene copies homologous to *At-thi1* at the *P. patens* v3.3 genome. These copies are distributed on chromosomes 20, 23, 24 as represented the Figure 33. Each copy was named in relation to its chromosomal location and orientation (F – forward; R – reverse).

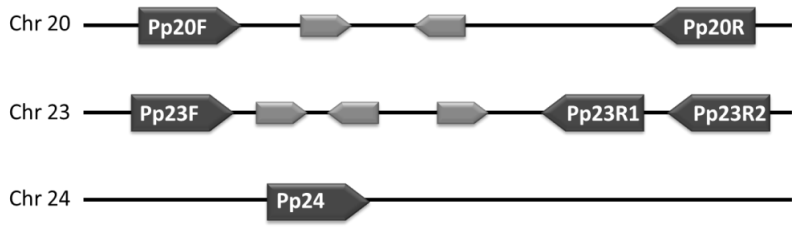

Figure 33. *P. patens thi1 copies:* The chromosomes 20, 23 and 24 are represented, showing the regions containing *thi1* copies (dark grey). Other genes at the same region are showing in light grey. The arrow points to the gene orientation.

The two copies downstream on chromosome 23 were named as R1 and R2 as they display 100% nucleotide identity (Table 8) and are, thus, analyzed as single species.

Table 8. Percentage of nucleotide identity among *PpThi1* copies. Highlighted in black is the identity between *PpTthi1.3* R copies of chromosome 23.

PpThi1-20F	100%					
PpThi1-20R	96.23%	100%				
PpThi1-23F	33.34%	33.88%	100%			
PpThi1-23R1	33.97%	33.88%	97.79%	100%		
PpThi1-23R2	33.97%	33.88%	97.79%	**100%**	100%	
PpThi1-24	79.79%	79.79%	33.79%	33.51%	33.51%	100%
Genomic sequence	*PpThi1-20F*	*PpThi1-20R*	*PpThi1-23F*	*PpThi1-23R1*	*PpThi1-23R2*	*PpThi1-24*

Interestingly, *PpThi1* copies present on the same chromosome (F and R) present high nucleotide identity (96.23% for *PpThi1.20* and 97.79% for *PpThi1.23*). While comparing copies from different chromosomes, the identity is low (about 33% between copies of chromosomes 20 and 23, and 24 and 23; 79.79% between copies of chromosomes 24 and 20). This is consistent with the more recent duplication of tandemly arrayed gene copies. These nucleotide differences can be seen in the nucleotide alignment (Figure 34).

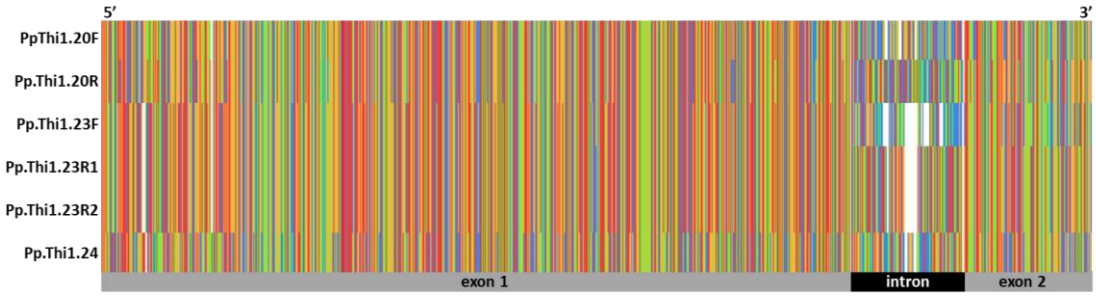

Figure 34. *Overview of the alignment of the six PpThi1 genomic.* The grey/black bar represents the gene structure. Green =A; Blue = T; orange = C; red = G.

All six *PpThi1* copies have just one intron, whose size varies from 101 to 405 bp (below in Figure 34). This is different from *At-thi1* (which has 2 introns), but the same as the *thi1* homologues found in the of the Poaceae family.

Despite the nucleotide sequence differences, the amino acid sequence identity of the encoded proteins is high (above 88%, Table 9), indicating the high degree of conservation of the protein.

Table 9. Percentage of amino acid identity among PpTHI1 copies.

PpTHI1-20F	100%				
PpTHI1-20R	98.34%	100%			
PpTHI1-23F	91.98%	91.16%	100%		
PpTHI1-23R	91.16%	90.33%	99.17%	100%	
PpTHI1-24	88.98%	88.15%	88.67%	88.95%	100%
Protein sequence	PpTHI1-20F	PpTHI1-20R	PpTHI1-23F	PpTHI1-23R1	PpTHI1-24

The main differences are in the N-terminal region (Figure 35), where a CTP is predicted for *At-thi1* (Chabregas *et al.*, 2003) and for *sc-thi1* (Chapter 2). This is in accordance with what is seen for other *thi1* homologues in plants (Figure 19 – Chapter 2).

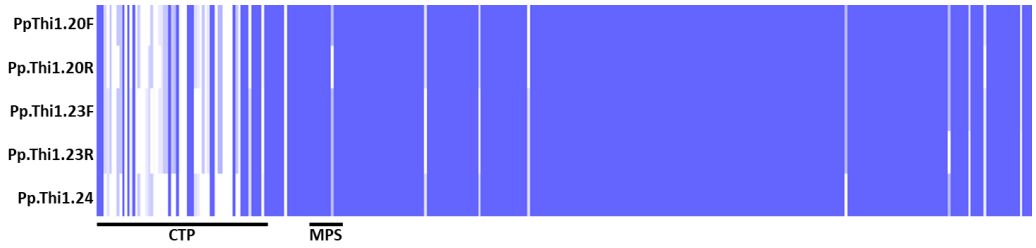

Figure 35. *Overview of protein alignment among the five PpTHI1.* The blues shading indicates regions of protein identity (dark blue = 100% identity and white = 0%). CTP = chloroplast transit peptide; MPS = mitochondrial pre-sequence. CTP and MPS were marked based on predictions made for these sequences in *At-thi1* (Chabregas *et al.*, 2003).

3.3.2. Mutants lines generation by Homologous recombination

Figure 36 summarizes the experimental design to create *P. patens* lines mutants for single *thi1* copies by HR. Five HR plasmids where constructed in order to create single *thi1* copy mutants, replacing a substantial segment of the coding region of each native gene with a resistance gene cassette. Plasmids with hygromycin and geneticin (G418) resistance genes present loxP sites in the same orientation and flanking the resistance gene, so that the selection marker cassette can be excised by the *Cre* site specific recombinase. This allows the resistance gene cassette to be recycled and to be used again in a new HR event. Thus, a single mutant line can be used to create double, triple, quadruple, quintuple and sextuple *thi1* mutant lines.

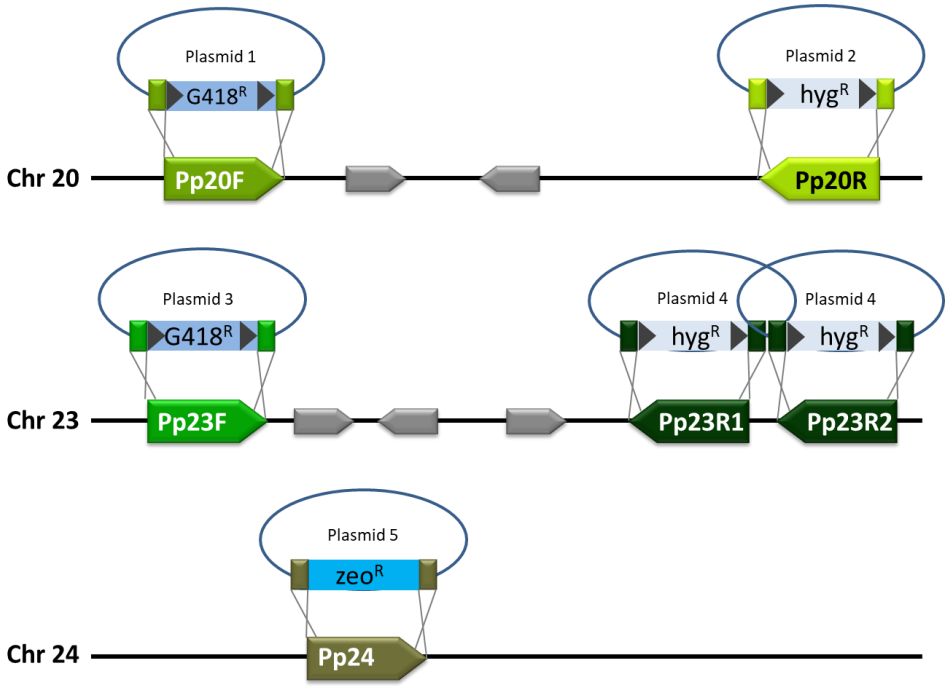

Figure 36. *Experimental design for constructing P. patens line mutants for single thi1 copy, by HR.* The chromosomes 20, 23 and 24 are represented, showing the regions containing *thi1* copies (different shades of green). Other genes at the same region are showing in light grey. The block arrows indicate gene orientation. The solid black arrowheads represent loxP sites. Grey lines indicate the HR process, which replaces each *PpThi1* by a different resistance gene. (zeo = zeocin; hyg = hygromycin; G418 = geneticin).

The plasmids were transformed independently into *P. patens* protoplasts. After transformation, the regeneration rate in specific antibiotics was evaluated (Table 10). *PpThi1.24-zeo* regeneration rate was about the same as WT. *PpThi1.20R-hyg*, *PpThi1.23R-hyg* and *PpThi1.23F-G418* regeneration resulted in many colonies, but about fivefold less than wild type. After many attempts for *PpThi1.20F-G418*, only two regenerants were obtained, however, after regeneration, they were able to develop normally. It is possible that *PpThi1.20* is important for *P. patens* growth and regeneration from protoplasts and that its loss compromises this ability far more than the similar deletion of other *PpThi1* gene family members.

Table 10. *P. patens* regenerants colonies after three or more attempts to transform the HR plasmids.

Plasmids	Attempts	Regenerants colonies
WT (control)	3	Over one thousand
PpThi1.20F-G418	3	Over one hundred
*PpThi1.20*R-hyg	8	Two
PpThi1.23F-G418	3	Over one hundred
*PpThi1.23*R-hyg	3	Over one hundred
PpThi1.24-zeo	3	Over one thousand

Different rates of regeneration may indicate different functions for each copy, as it is not an antibiotic specific bias. *PpThi1.20*R-hyg and *PpThi1.23*R-hyg are both resistant to hygromycin, while *PpThi1.23F*-G418 is resistant to geneticin and shows same regeneration rates.

PCR amplification of the correspondent resistance gene cassette using genomic DNA of individual confirmed that mutant line for each single *PpThi1* copies were obtained by HR (Figure 37).

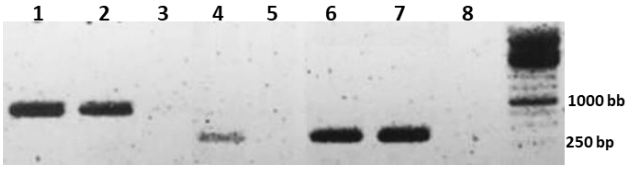

Figure 37. *PCR amplification of internal sequence from inside each correspondent resistance gene from mutants genomic DNA.* 1 – *PpThi1*.20R-hyg; 2 – *PpThi1*.23R-hyg; 3 – WT (hyg); 4 – *PpThi1*.24-zeo; 5 – WT (zeo); 6 – *PpThi1*.20F-G418; 7 – *PpThi1*.23F-G418; 8 – WT (G418).

3.3.3. Morphological analysis of the protonemata

In order to analyze the overall morphology and development of *PpThi1* mutant line, protonemata-rich mutant cultures were fragmented (blended) transferred onto medium in the presence or absence of glucose (0.5% [w/v]). Medium containing glucose is used to keep the moss in the protonematal phase, while the absence of glucose promotes gametophore development.

No substantial visual differences were observed in WT or mutant plants growing in the presence or absence of glucose. No gametophore grew in presence of glucose. However, we did observe some subtle morphological differences in growth of mutants compared to WT.

A qualitative morphological description of each *PpThi1* mutant line was constructed based on preliminary observations of four plates of protonemata-rich KOs of each line, made under the stereomicroscope and microscope. Figure 38 represents one example of what was observed for each *PpThi1* mutant line at two and nine days after blending (DAB) and plating onto new medium.

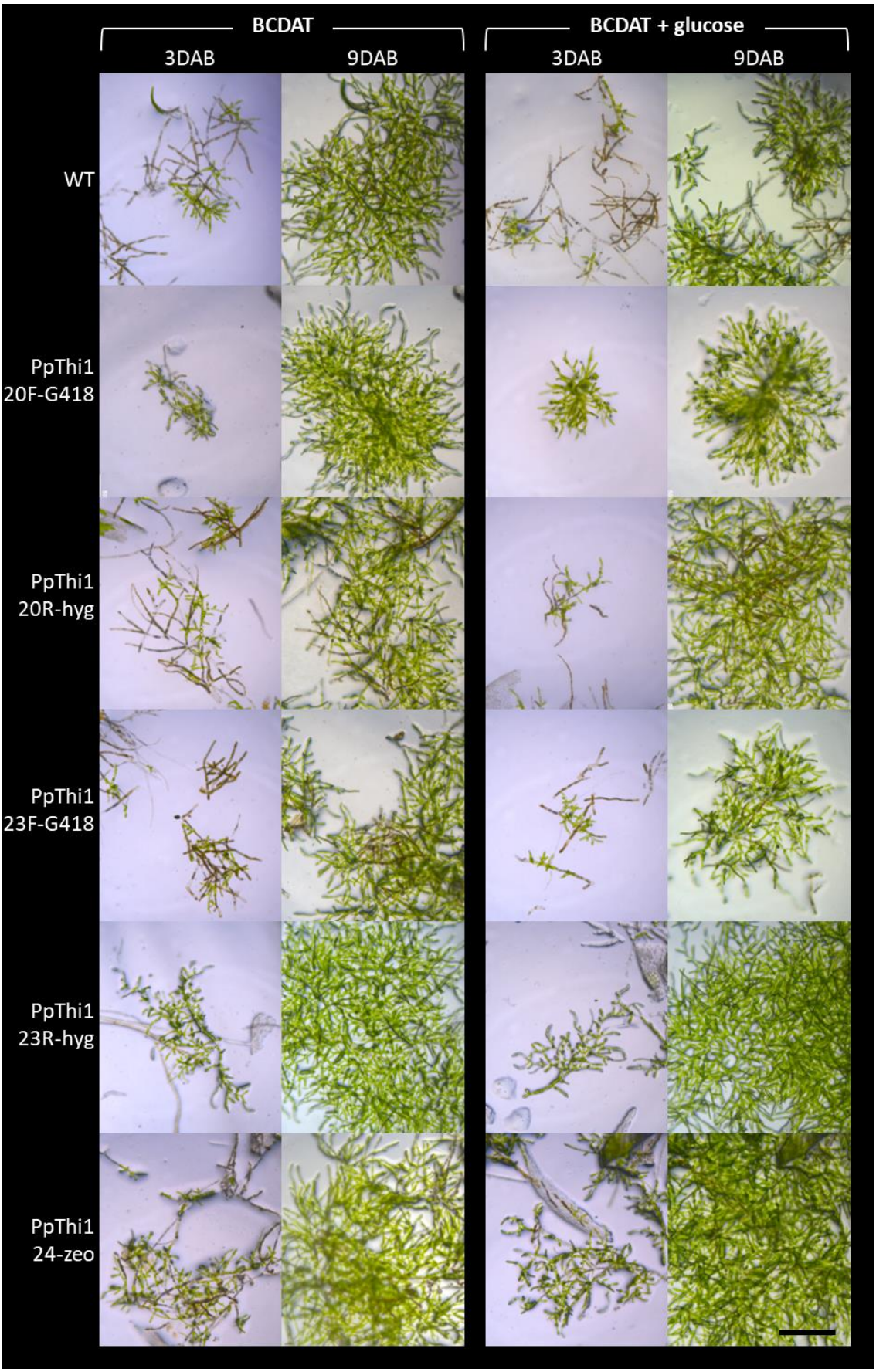

Figure 38. *Protonemata-rich mutant cultures were fragmented (blended) and transferred onto BCDAT and BCDATG (which is the same as BCDAT supplemented with 0.5% glucose) medium*. DAB = days after blend. Scale bar = 500μm.

After blending and replating, some protonemata do not survive and turn brow. These preliminary observations indicated an increased number of brown cells in *PpThi1.23F* line and a decrease in *PpThi1.20F* and *PpThi1.23*R lines, when comparing with WT. Also indicates that *PpThi1.24* develops more biomass, with longer protonemata, than the WT, and that *PpThi1.23*R and *PpThi1.20F* present a different pattern of cellular growth and division. However, further, more quantitative studies are needed to confirm these observations.

Unlike the WT, the *PpThi1.20F, PpThi1.20*R *and PpThi1.23*R mutant line present a globular structure at the protonemal tips (Figure 39), except for the *PpThi1.23F*. However, further measurements should be done to confirm these observations.

Figure 39. *Examples of PpThi1 mutant colonies presenting a globular structure at the tip of the protonemata.* From left to right: 5x, 20x and 40x magnification in microscopy.

Despite the morphological differences observed, none of the *PpThi1* copies are vital at the protonemata stage. While these observations were consistent, they clearly need to be followed up using more quantitative methods.

3.3.4. Effect of thiamine on mutant growth

In order to analyze if any of the *PpThi1* copies knockout mutants are thiamine auxotrophic, protonemal colonies (~1 mm in diameter) were grown with or without 0.5 mg/L of thiamine. Colonies growth was evaluated based on the increase of total cultured surface from one week to two weeks of cultivation. Figure 40 shows the results for all lines.

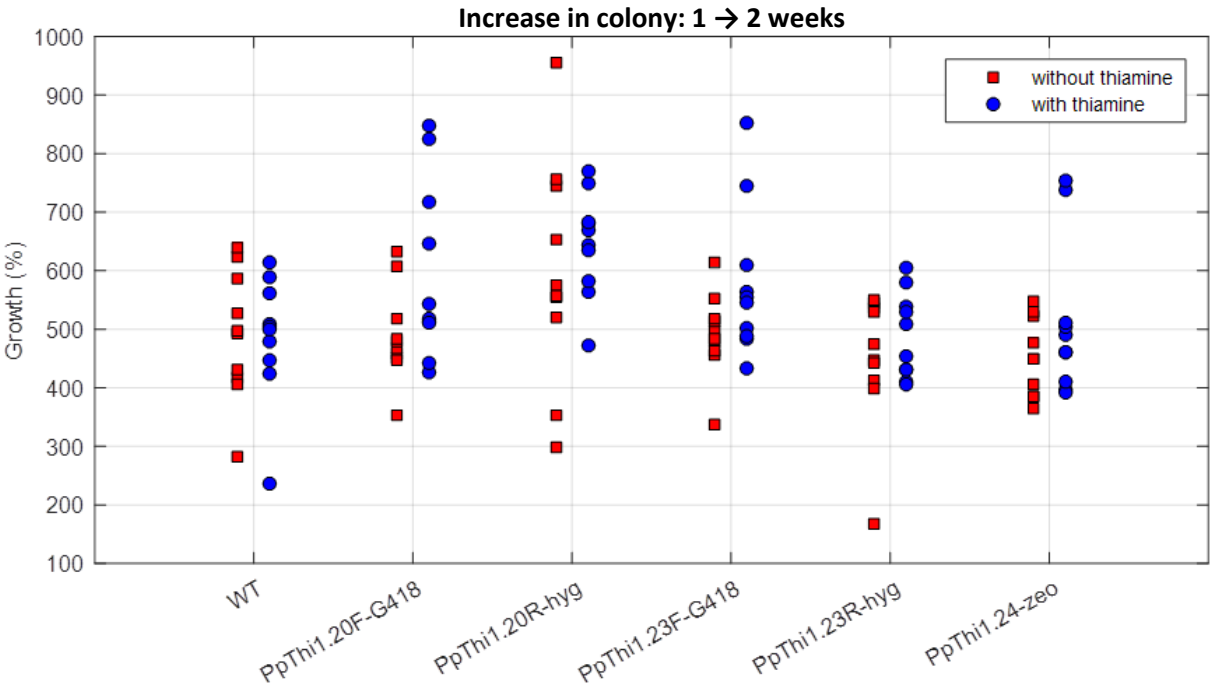

Figure 40. *Increase in culture area from 1 to 2 weeks in the presence or absence of thiamine (0.5 mg/L).*

The same analysis was done after three weeks of cultivation when the gametophyte starts to develop. Figure 41 shows the results for the analysis of increase in area from one week to three weeks.

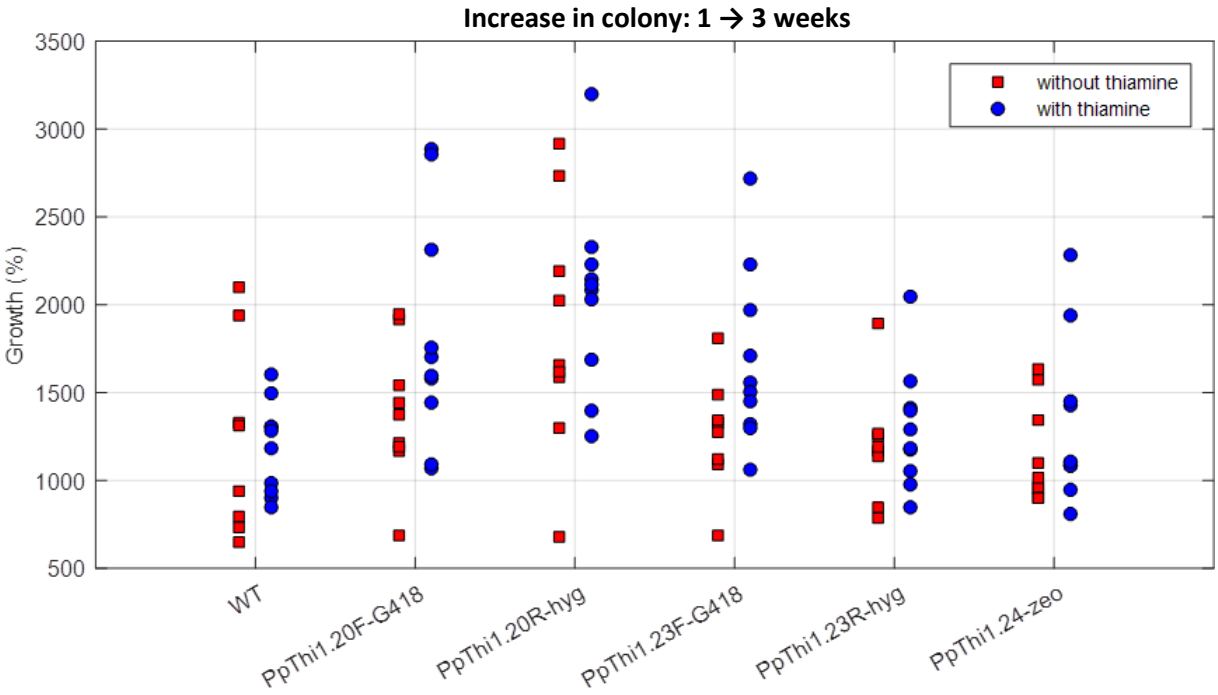

Figure 41. *Increase in culture area from 1 to 3 weeks in the presence or absence of thiamine (0.5 mg/L).*

Wild type colonies' area of growth is slightly affected by the absence of thiamine in the medium when compared to thiamine containing media. Of the mutant lines, only the *PpThi1*.23R line displays similar pattern to wild type. All the remaining lines seem to respond with increased colony growth to the presence of thiamine in the medium, compared to WT plants. Interestingly, no evident limitation in area growth was observed for any particular line which supports functional redundancy.

In the presence of thiamine, the correlation is weaker for the mutant lines than for WT, as a higher dispersion of the data points is observed. The table 11 shows the average increase of the colonies area between 1 and 2 weeks, and between 1 and 3 weeks, and the correspondent standard deviation.

Table 11. Average increase of the colonies areas between 1 and 2 weeks, and 1 and 3 weeks.

	Without thiamine				**With thiamine (0.5 mg/L)**			
	1 → 2 weeks		1 → 3 weeks		1 → 2 weeks		1 → 3 weeks	
PpThi1-WT	4,9x	± 1,10	12,24x	± 5,51	4,86x	± 1,07	11,84x	± 2,59
PpThi1-20F	4,89x	± 0,81	13,86x	± 3,92	5,99x	± 1,52	18,28x	± 6,52
PpThi1-20R	5,96x	± 1,94	18,55x	± 6,98	6,45x	± 0,88	20,46x	± 5,41
PpThi1-23F	4,91x	± 0,71	12,68x	± 3,25	5,78x	± 1,29	16,82x	± 4,96
PpThi1-23R	4,47x	± 1,13	11,93x	± 3,35	4,89x	± 0,73	12,93x	± 3,41
PpThi1-24	4,51x	± 0,66	11,53x	± 2,89	5,12x	± 1,31	13,24x	± 4,64

On average, in the absence of thiamine all the mutant lines grow in the same rate as the WT, except for the *PpThi1.20R* which is positively affected by the gene knockout. In the presence of thiamine, the growth rate of the WT, as well as *PpThi1.23R*, is not affected. However, the other four mutant lines are positively affected by the presence of thiamine.

Figure 42 shows colonies of each *PpThi1* mutant line after 1, 2 and 3 weeks of cultivation, with the area closest to the average area of each group, in order to illustrate the phenotype.

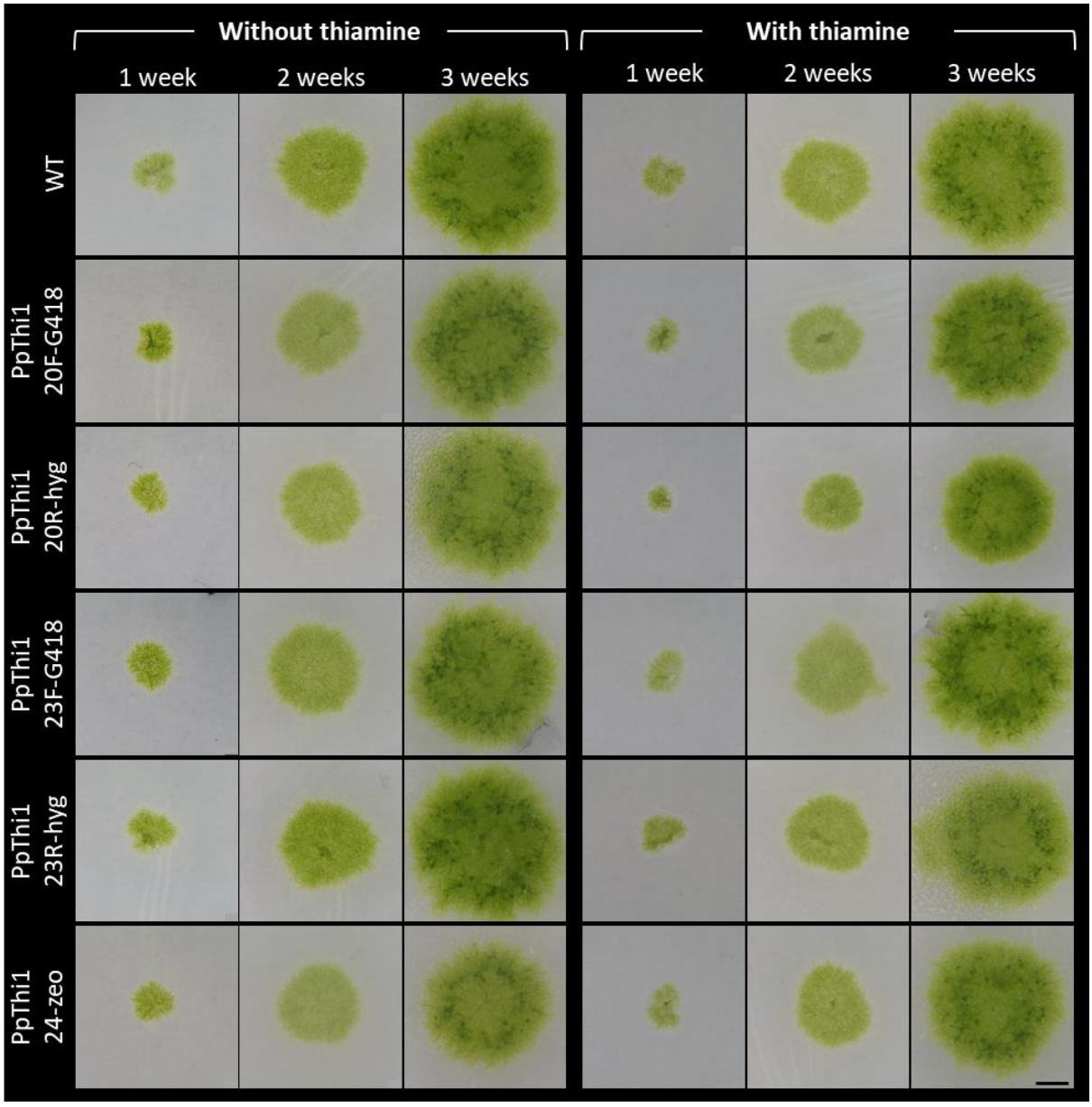

Figure 42. *Examples of PpThi1 mutant colonies grown with or without 0.5 mg/L thiamine addition*. Scale bar = 2 mm.

3.3.5. Fresh and dry weight

Momoli (2008) reported that the *At-thi1* mutant (*tz-201*) has a higher root fresh weight, while the WT has a higher shoot fresh weight after 6 weeks. In order to evaluate if *PpThi1* mutations also causes general physiological changes in moss, the ratio between dry and fresh weight was evaluated (Figure 43).

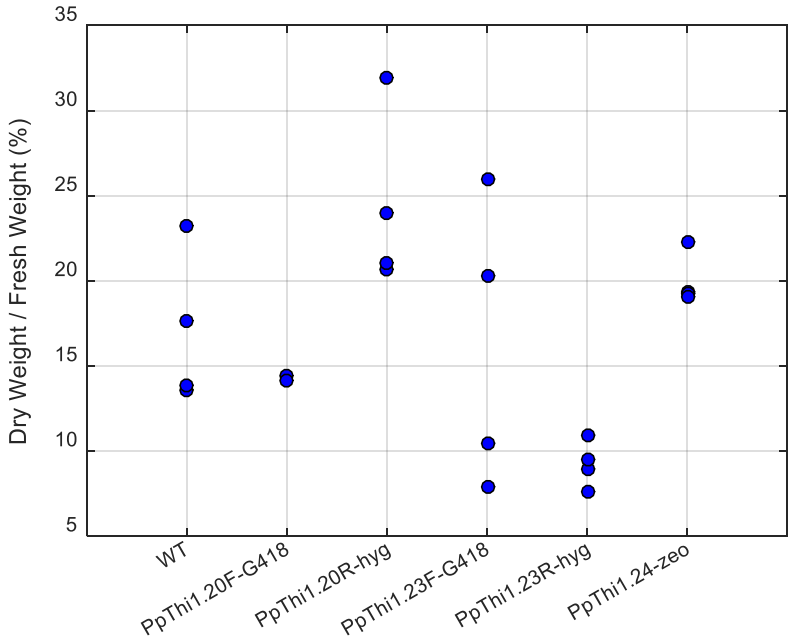

Figure 43. *Dry and fresh weight ratio for different PpThi1 mutants samples.*

The percentage of dry weight is high variable among the samples and does not differ from what is observed for the WT, except for *PpThi1.23R* mutant which has significantly lower dry weight content than does the WT and the other mutant lines.

Unlike *A. thaliana*, the bulk of mass of *P. patens* grows very close to the culture medium. This may interfere with measuring the water content of this plant, at the harvesting for the experiment. Another possibility for these weight measure differences is the differences in the dispersal of the blended protonemata on the petri dish.

3.3.6. Carbohydrate analysis

Sucrose is a disaccharide (glucose + fructose) essential to the translocation of sugars from photosynthetic source leaves to other plant organs while glucose and fructose represent the hexoses that partition carbon into the plant cell metabolism. Vitamin B1 is necessary to some of the key pathways in which those soluble sugars enter metabolism. Raffinose, different from sucrose, is a trisaccharide (galactose, glucose and fructose) that is mostly related to plant cell wall metabolism.

Momoli (2008) demonstrated that the *At-thi1* mutant (*tz-201*) has an increased level of sucrose and corresponding decrease in glucose and fructose, relative to the WT plant. Thus, the soluble carbohydrate content was evaluated for all *PpThi1* mutant lines. Figure 44 (A) shows the sucrose content while (B) shows glucose, fructose and raffinose content.

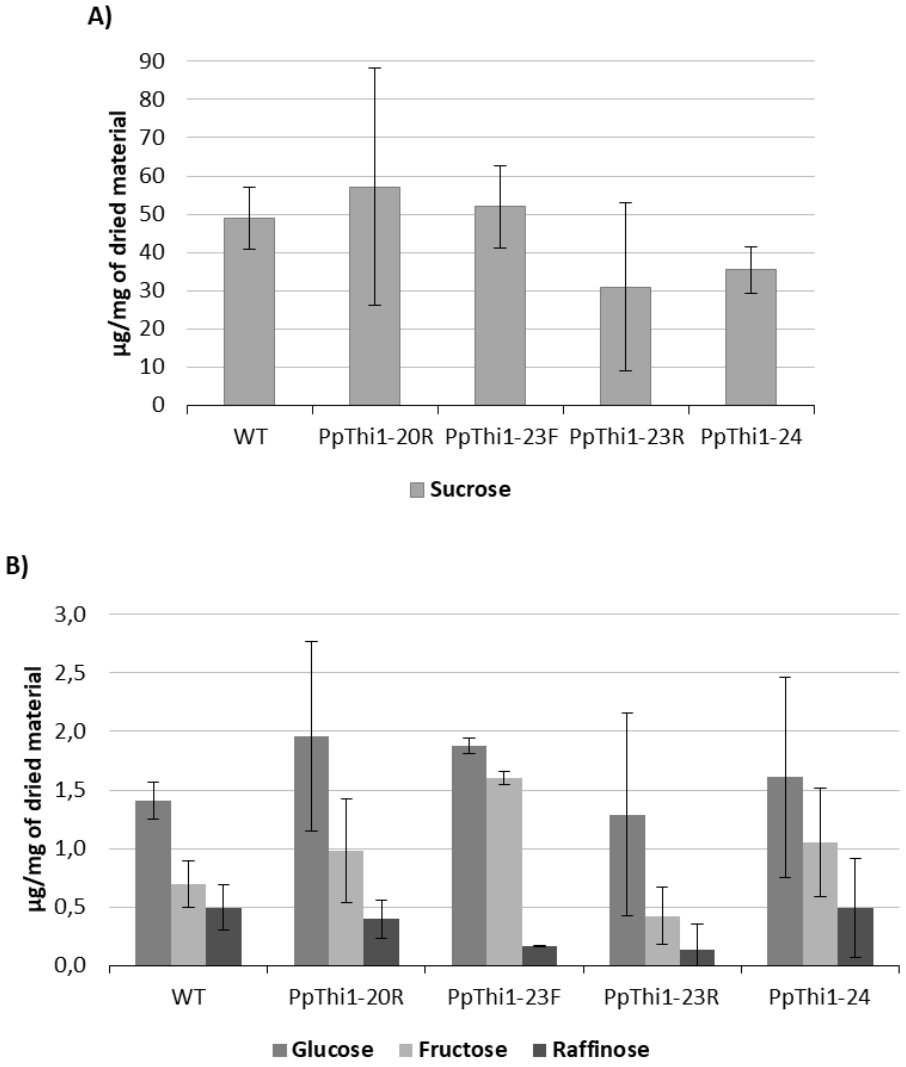

Figure 44. *Carbohydrate content of PpThi1 mutant lines*. The vertical bar indicates the standard deviation.

Preliminary results support that each KO line present differences in soluble sugar content when compared to WT strain. *PpThi1.23*R and *PpThi1.24* have decreased levels of sucrose (Figure 44 A) while *PpThi1.20*R has slightly higher content (0.8-fold higher) and *PpThi1.23F* is similar to WT. Hexose monomer content is variable among the KO lines in comparison to WT. *PpThi1.20*R, *PpThi1.23F* and *PpThi1.24* have increased levels of glucose while *PpThi1.23*R has less when compared to WT. Fructose content also varies in a similar trend. *PpThi1.23*R has the least fructose content probably resulting in the total decrease in sucrose content observed in Figure 44 (A). *PpThi1.20*R, *PpThi1.23F* and *PpThi1.24* have all higher levels when compared to WT but *PpThi1.23F* has similar levels of both hexoses. Interestingly, *PpThi1.23F* and *PpThi1.23*R have the least content of raffinose. Further experiments are needed towards understanding and validating the changes in the soluble sugars content.

3.4. Conclusions

- Single *PpThi1* knockout lines were obtained for all *PpThi1* copies.
- The absence of *PpThi1.20F* affects drastically the protoplast survival and regeneration.
- None of the *PpThi1* copies are vital for the protonemata or the gametophyte survival, supporting functional redundancy.
- Discrete changes in morphology and growth rate were observed in the presence of thiamine.
- Changes in soluble sugars are detected among KO lines when compared to WT.

3.5. Perspectives

The generation of plant mutants for *thi1* homologues represents an important advance and a potentially useful genetic reagent for future studies. To date, the only extant *At-thi1* mutant (*tz-201*) able to survive is not transformable (Papini-Terzi *et al.*, 2003) and therefore of limited utility..

Single gene *PpThi1* KOs mutants can survive with minor effects on morphology and physiology. However, further studies are needed to provide a more detailed description of the knockout lines and to better understand how the absence of these genes contributes to altered phenotype.

The constructs used to generate these mutant lines were designed in such a way that the generation of multiple *PpThi1* copy gene knockouts is feasible, in different combinations, including multiple knockouts comprising the entire *PpThi1* gene family. Whether such a sextuple mutant could survive in the presence of exogenous thiamine would have to be established experimentally. It is possible that individual *PpThi1* KOs may be refractant to subsequent transformations, as occurs with the *A. thaliana tz-201* mutant. If so, the same plasmids constructed in this work can be modified to allow the creation of *P. patens* versions of the *tz-201* mutation, or other point mutations, that allow the plant to survive. Only by attempting such an iterative deletion of *PhThi1* genes, will the individual roles that they play become apparent.

Nonetheless, each single line is already available for complementation experiments using *Arabidopsis* and sugarcane *thi1* homologues. They can also be used to address the involvement of each copy in DNA protection and other stress-related pathways (induced, for example by exposure to bleomycin or MMS or UV light).

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4. FINAL CONSIDERATIONS

Results from the work described in this thesis reveal that sugarcane and *Physcomitrella patens* harbor more than one copy of *thi1* in their genomes.

Sugarcane has two genomic loci containing *thi1* (*sc-thi1.1* and *sc-thi1.2*) each displaying synteny to other Poaceae. *Sc-thi1.2* locus discloses that the alleles of sc-thi1.2 are diverging from each other. Moreover, these three *sc-thi1* gene versions have differential expression patterns and complements differentially the KBY5 thiamine auxotrophic yeast strain.

P. patens has six different *thi1* copies, two on chromosome 20, three on chromosome 23 and one on chromosome 24. The knockout of each of them is not lethal, although generates gene mutations leads to some differences in morphology and growth. Of the knockout mutants generated, deletion of *PpThi1.20F* results in a marked decrease in regeneration efficiency.

The redundancy described in chapter 2 and 3 in both sugarcane and *P. patens* may indicate a specialization of functions other than thiamine biosynthesis, such as the organellar DNA protection/repair described for *Arabidopsis thaliana thi1* single copy (Machado *et al.*, 1997). As describe for yeast (Chatterjee *et* al., 2011), THI1 acts as a reagent donating the sulfur for the thiazole ring formation. Taken together, the results presented here advance our knowledge and understanding of the thiazole biosynthetic protein and its cellular and biochemical functions.

Finally, the generation of *P. patens* single *thi1* copy knockouts lines provides a useful plant system for further functional studies of this gene family.

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APPENDIX - *Brachypodium distachyon thi1* mutant lines

ABSTRACT

In order to obtain mutants for the study of the gene *thi1*, in addition to *Physcoitrella patens* discussed in Chapter 3, we also tried to obtain *thi1* mutants for *Brachypodium distachyon*. *B. distachyon* is a grass related to the major cereal grains and is used as a model due to its relatively small genome, short life cycle and small size. *B. distachyon* has only one *thi1* copy (*Bd-thi1*). To produce *thi1* mutant plant line, CRISPR/Cas9-gene editing technology was applied. Two vectors containing different resistance genes were constructed to edit *Bd-thi1*. After transformation and selection, 22 plants regenerated and from them, 475 T1 seeds were obtained. The germination rate of these seed was very low. The 11 plants able to germinate and grow were tested for CRISPR/Cas9 *thi1* gene edition and did not present the expected edition.

Keywords: 1. *Brachypodium distachyon*; 2. Plant model; 3. *Thi1* gene; 4. Mutant lines; 5. CRISPR/Cas9

Introduction

Brachypodium distachyon (Figure 45) is a grass related to the major cereal grain species including wheat, barley, oats, maize, rice, rye, sorghum, millet and sugarcane. It has several advantages as an experimental model organism for understanding the genetic, functional, and cellular and molecular biology of grasses. Its genome is composed of five chromosomes containing 272 million nucleotides, which is a small genome size for a grass species. (The International *Brachypodium* Initiative, 2010). *B. distachyon* is a self-fertile, inbreeding annual plant with a life cycle of less than 4 months. These features, coupled with its small size (approximately 20 cm at maturity), lack of seed-head shatter, and undemanding growth requirements should make it amenable to high-throughput genetics and mutant screens (Draper *et al.*, 2001). Moreover, a BLASTn analysis with Phytozome 10.2 database shows that *B. distachyon* has only one *thi1*-like copy, which makes the genetics analyses easier.

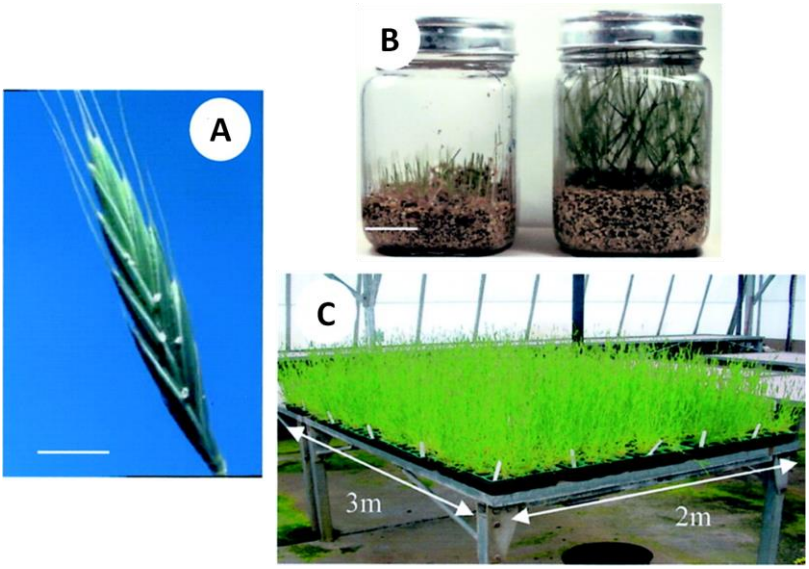


Figure 45. *Brachypodium distachyon*: A) Spikelet containing typically around 10 to 12 seeds. B) *B. distachyon* exhibit undemanding maintenance requirements, growing successfully under sterile conditions in glass jars on vermiculite supplemented with 0.5x Hoagland solutions. Bar = 5 cm C) The smaller stature of *B. distachyon* allows typical planting densities of at least 300 plants square meter in ordered arrays for seed production or mutant screening. Modified from (Draper *et al.*, 2001).

From these characteristics, generate *thi1* gene disruption lines of *Brachypodium distachyon* is relevant for the study of *thi1* homologues from grasses, like sugarcane, as it facilitates the model-to-crop transfer of knowledge. The strategy selected to generate this mutant line was the CRISPR/Cas9 Technology.

CRISPR/Cas

CRISPR/Cas9 technology is based on the acquired immunity system of bacteria and archaea against viruses and plasmids. The CRISPR/Cas (clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas)) protein systems use sequences derived from plasmids and phages to activate Cas endonucleases to neutralize those plasmids and phages via RNA-guided sequence-specific DNA cleavage, thus blocking their transmission and creating acquired immunity. By replacing the guide-RNA with a synthetic version (gRNA) directed against a gene of interest, CRISPR/Cas technology permits gene cleavage and gene editing by targeting nucleic acids in a sequence-specific manner (Horvath and Barrangou, 2010).

To achieve that, a specific gRNA target the Cas9 protein to the gene to be disrupted. Then, the Cas9 will make a double-strand brake. It has been shown that in plants, the major repair in this CRISPR/Cas-caused double-strand brakes is made by non-homologous end joining (NHEJ) resulting in INDELs (Xie *et al.*, 2014), which disrupt the targeted gene.

Material and Methods

Plant material and growth conditions

Wild type *B. distachyon* Bd21, which has been fully sequenced, was grown in soil. Immature seeds were used to produce calli for transformation by gene gun bombardment (mature protocol). *In vitro* plants were grew in Murashige and Skoog (1692) medium, supplemented with 0.5 mg/L thiamine for transformants. Plants were kept in normal growth conditions (25ºC, 12h-12h light-dark).

CRISPR/Cas9 technology

A CRISPR gene editing vector (pRD214) designed and constructed by Dr. Lawton and Dr. Di's laboratory (Department of Plant Biology Rutgers, The State University of New Jersey) was used as backbone. It contains the wheat U6 sRNA promoter-driven gRNA cassette, and the maize ubiquitin promoter-driven, monocot codon-optimized Cas9 gene, which can be used to edit genes in monocotyledonous plants.

The target sequence of *B. distachyon thi1* gene (*Bd-thi1* gRNA) was incorporated into pRD214 by the Q5 Site-Directed Mutagenesis Kit (New England BioLabs®), using a specific pair of primers (BdThi1_gRNA F: TGT CCC CGG GGT TTT AGA GCT AGA AAT AGC AAG TT and BdThi1_gRNA R: CGG ACT GCT CAA GTC TGA TGC AGC AAG CGA G).

The cassette containing $P_{TaU6}/Bd_{thi1}/crRNA::P_{ZmUbi}/MoCas9/T_{nos}$ was sub-cloned into pCAMBIA1300, which contains the hygromycin resistance gene (Hyg^R), resulting in the pML27 plasmid and pCAMBIA2300, which contains gentamicin resistance gene ($G418^R$), resulting in the pML28 plasmid (Figure 46 and Table 11).

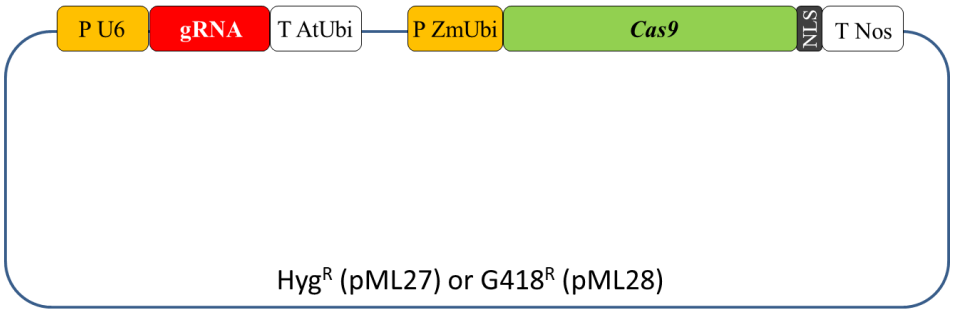


Figure 46. *Final expression plasmid to gene edition of monocotyledonous plants by CRISPR/Cas9.* The gRNA is the genome specific part and, here, is the *bd-thi1* gRNA. Two version were obtained: one with the hygromycin resistance gene (Hyg^R), and the other with the geneticin resistance gene ($G418^R$).

Table 12. Description of the plasmids used to CRISPR/Cas9 gene edition of the *B. distachyon thi1* gene.

Plasmid Name	Plasmid description
pML27-Hyg	Bd-thi1sgRNA+pCAMBIA1300 to CRISPR/Cas9
pML28-G418	Bd-thi1sgRNA+pCAMBIA2300 to CRISPR/Cas9

Both *Bd-thi1* CRISPR vector containing different selective marker were used to transform *B. distachyon* Bd21 calli by gene gun bombardment (mature protocol). After a recovery period on non-selective media, the calli were kept on selective media for 6 weeks then transferred to regeneration media plus silver nitrate (5 mg/L). Calli resulting from the transformation of pML27 were called *Bd-thi1.27*, and resulting from the transformation of with pML28 were called *Bd-thi1.28*.

Resulting shoots were transferred to rooting media for 2 weeks and then transferred to soil. Supplemental thiamine (0.5 mg/L) was added to regeneration and rooting media. The plants in soil were irrigated with 100 µl of 1% thiamine solution once a week.

Seeds germination

The T1 seeds obtained were sterilized (30 seconds in 20 mL of 70% ethanol, 15 minuts in 10 mL of 30% bleach plus one drop of triton 100, and 3 washes with sterile water) and put to germinate into MS 30 medium supplemented with 0.5 mg/L thiamine. 89 *Bd-thi1.27* seeds and 53 *Bd-thi1.28* seeds were germinated in correspondent antibiotic (50 mg/L of hygromycin and 50 mg/L of geneticin). Then 20 *Bd-thi1.27* seeds and 20 *Bd-thi1.28* seeds were germinated without any antibiotics. After germination, the plants were transferred to soil and irrigated with 100 µl of 1% thiamine solution once a week.

CRISPR/Cas mutation analysis

The genomic DNA of the T1 plants germinated was extracted from leaves material by Invisorb® Spin Plant Mini Kit (Stratec Molecular GMBH). A pair of primers (BdThi1 29F: GCC TCC TCA AGA CCT CCT TC and BdThi1 618R: GAG CGC CCA GTT GGT GAC) was used to amplify a 500 bp region, which contains the gRNA target in the middle. The amplification was done using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Thermo Scientific®) and the amplicons were sequenced at the ABI PRIS 3730 DNA ANALYZER platform (Applied Biosystems™). The sequences quality was checked at the Sequence Scanner software (Applied Biosystems™) and the presence of CRISPR/Cas9-driven mutation in the sequence was manually analyzed.

Results

The choice of target sequence of *Bd-thi1*

To the *Bd-thi1* specific gRNA design, first was thought to use as target the same region mutated on the *A. thaliana tz-201* mutant line (Papini-Terzi *et al.,* 2003), the only *At-thi1* mutant able to survive (even if with thiamine supplementation). This mutation corresponds to a substitution of a C to a T that leads to a non-synonymous substitution, from Alanine to Valine, at position 140. This residue is part of the β5-sheet and its alteration in the mutant affects the conformational stability of the protein (Garcia *et al.,* 2014).

However, due to a limitation in the approach chosen for the CRISPR/Cas edition, the *Bd-thi1* gRNA needed to be designed in a region different from *tz-201* locus. This limitation is based on the Cas9 of the vector, which is codon-modified specifically to edit monocots. This Cas9 requires a gRNA composed of 20 nucleotides, designed from a G at 5', followed by 17 nucleotides, ending in a GG at 3'. The region correspondent to *tz*-locus in the *B. distachyon* genome do not contains a sequence with this requirement. A sequence like that was found just in one place of *Bd-thi1*. Figure 47 shows *At-thi1* and *Bd-thi1* cDNA sequences alignment, indicating the mutation in the *tz-201* mutant and the *Bd-thi1* gRNA target chose.

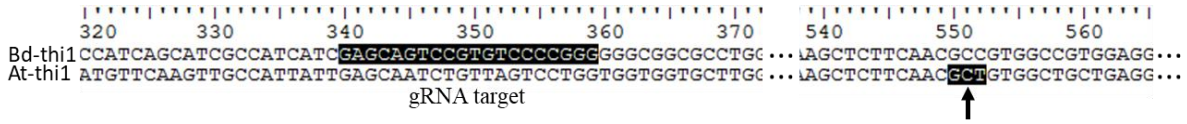


Figure 47. *At-thi1* and *Bd-thi1* cDNA alignment. The *Bd-thi1* gRNA target is highlighted to the left. The highlight to the right is the altered codon of *A. thaliana tz-201* mutant, and the C which is substituted by a T is pointed by the arrow. The differences in the same codon in *B. distachyon* are silent mutations.

The gRNA target is situated between the 340 and 359 nucleotides of the cDNA, which codifies to the EQSVSPG residues. This is a high conserved region of the THI1 protein in plants, as it is possible to see in the protein alignment of all *thi1*-like sequence from sequenced plants available in Phytozome v9.1 database (Goodstein *et al*., 2012)(Figure 48). This is part of the β2-sheet, which is responsible for the interaction of THI1 with AHZ ligand (Godoi *et al.* 2006).

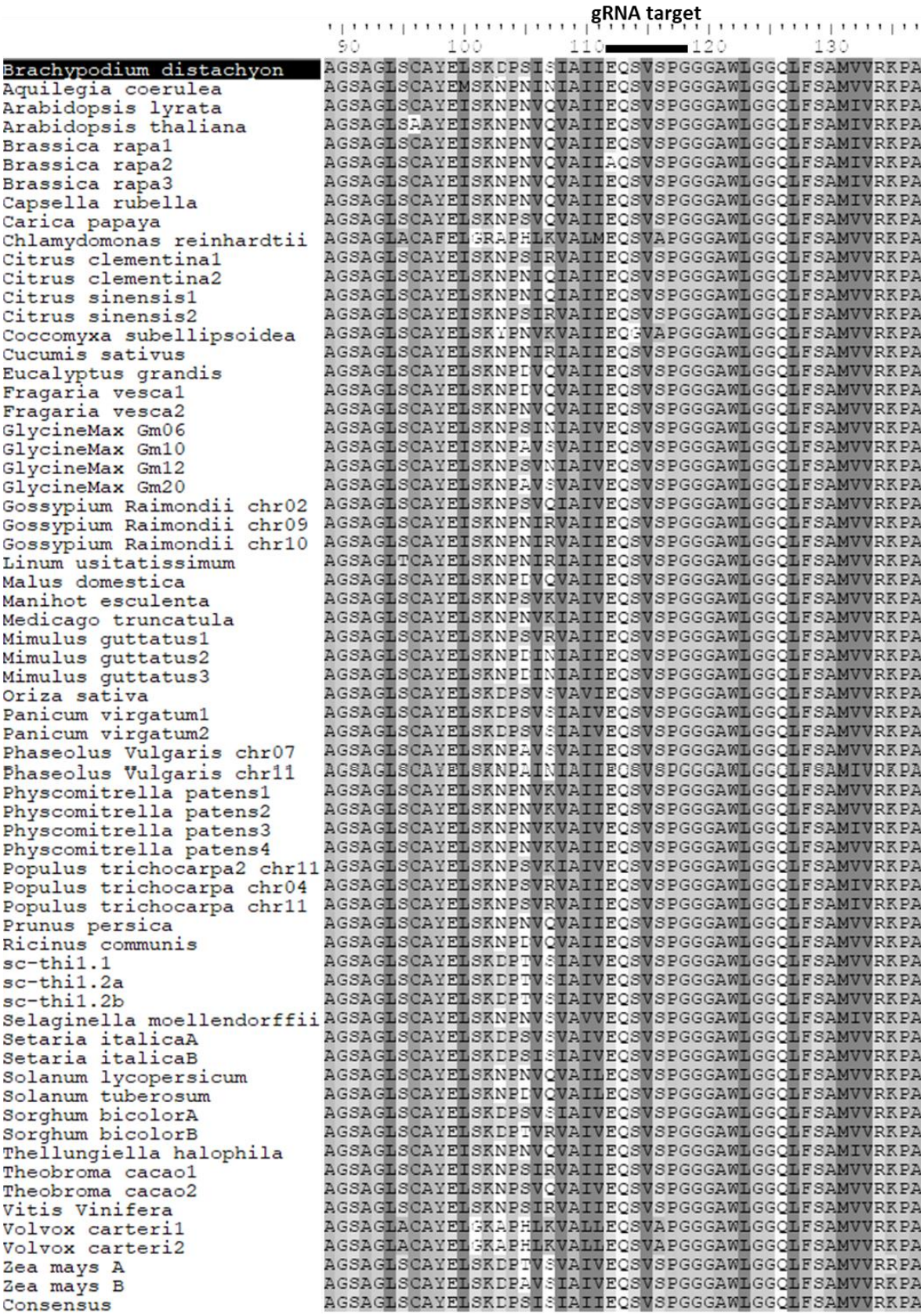


Figure 48. *Part of the protein alignment of all thi1-like sequence from sequenced plants available in Phytozome*, highlighting the gRNA tagert to CRISPR/Cas edition of the *thi1* gene in *B. distachyon*.

Brachypodium distachyon transformants obtaining

Three transformation batches were done with each plasmid, resulting in 12 selection/regeneration plates each. The survival rate is higher for calli transformed with the hygromycin resistance CRISPR/Cas9 plasmid (pML27) than for plasmids with a G418 (pML28) selectable marker gene (Figure 49).

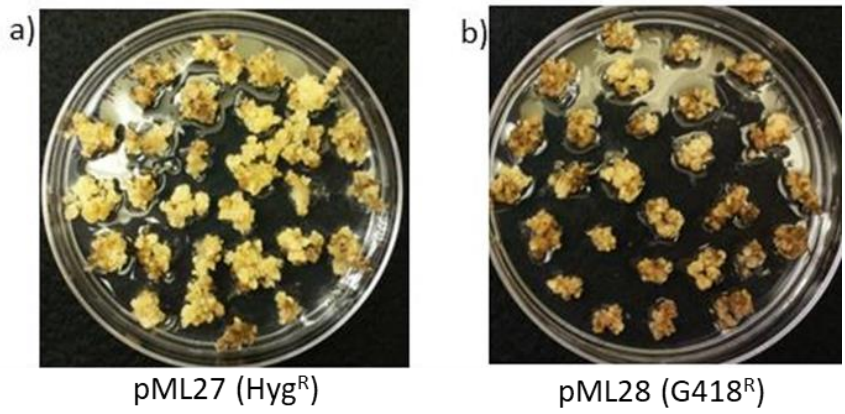


Figure 49. *B. distachyon calli* in selective media. Three weeks of selection after bombardment (35 mg/L of hygromycin; 25 mg/L of gentamicin). The brown *calli* will not survive. a) Transformants of hygromycin resistance CRISPR/Cas9 plasmid shows about 60% survivor rate. b) Transformants of G418 resistance CRISPR/Cas9 plasmid shows about 30% survivor rate.

The basic Murashige and Skoog medium used in the regeneration media contains a small amount (0.1 mg/L) of thiamine that was not enough for the regenerants to grow healthily as the plants were bleached. After addition of 0.5 mg/L of thiamine, the plants became green and were able to grow (Figure 50). This observation is important as the thiamine addition requirement for grow allows for selection of restored function.

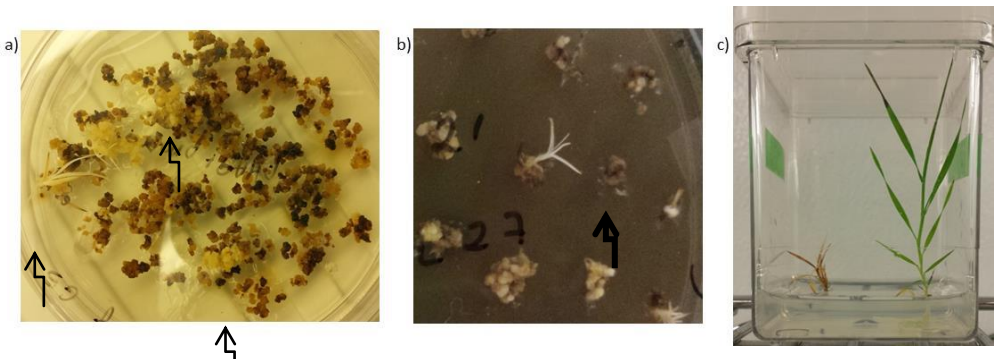


Figure 50. *B. distachyon calli in regeneration media.* a) After two weeks on regeneration selective media (5 mg/L of hygromycin; 10 mg/L of gentamicin), many *calli* died (brown) and some starts to regenerate (arrows). b) On media without thiamine addition the regenerants plants are bleached (arrow). c) Rooting media whit thiamine.

After all the process of selection, shooting and rooting inductions, 22 plants were obtained: 7 for calli transformed with pML27 (called *Bd-thi1.27*) and 15 for calli transformed with pML28 (called *Bd-thi1.28*). Figure 51 shows the regenerants plants one month prior to seed harvest.

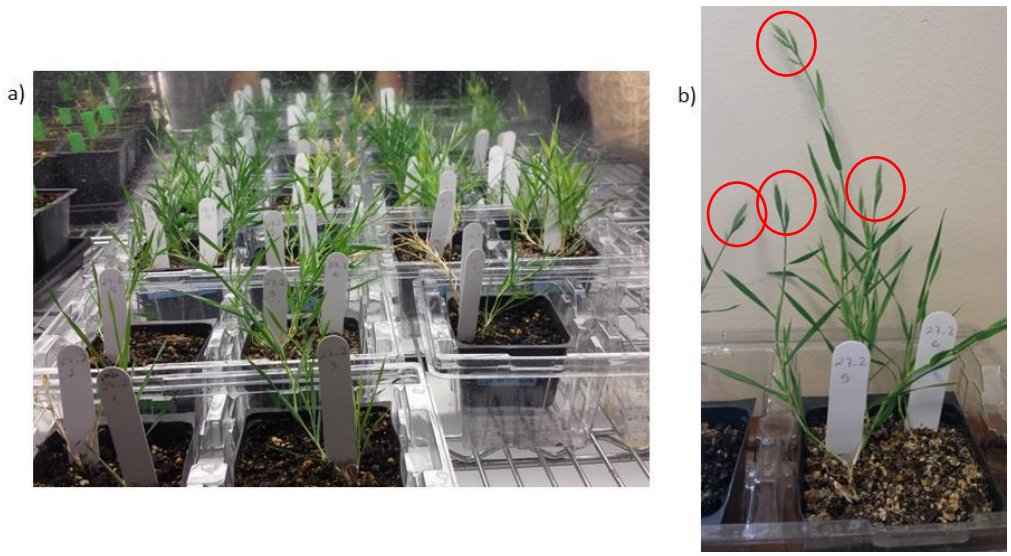


Figure 51. *B. distachyon regenerant plants.* a) Overview of the 22 regenerants. b) Highlighting of the immature seeds.

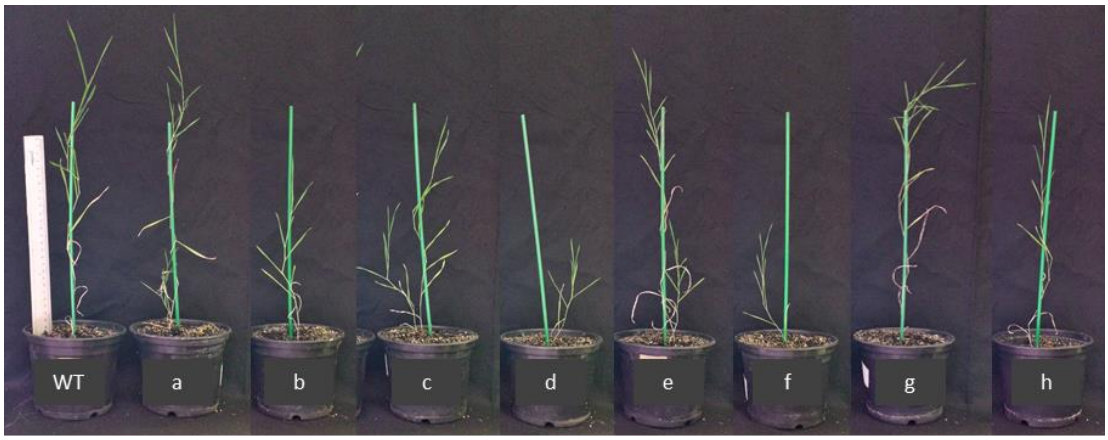
From the pML27-hyg plants regenerants, 287 seeds were obtained, while form the pML28-G418 just 188 plants regenerated (Table 13).

Table 13. *B. distachyon* regenerants and F1 seeds obtained after three attempts to transform the plasmids which direct the Cas9 protein to *Bd-thi1* in order to gene edition.

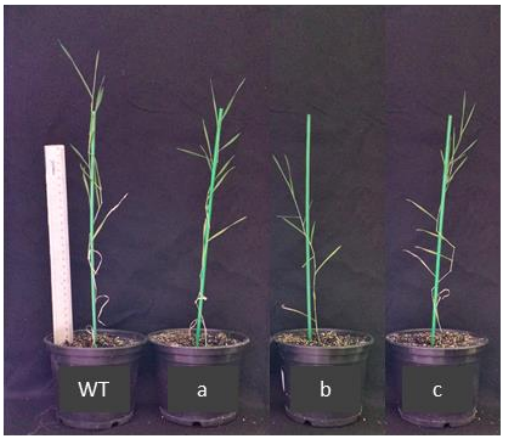
Plasmids	Attempts	Regenerants	F1 seeds
pML27-hyg	3	15 plants	287
pML28-G418	3	7 plants	188

T1 analysis

The germination of 50 seeds of each *Bd-thi1.27* and *Bd-thi1.28* were tried in MS30 supplemented with thiamine, in the presence of the specific antibiotics (50 mg/L of hygromycin and 50 mg/L of geneticin, respectively). However, the germination rate was below 10%, and from that, just two germinated plants from pML27-hyg survived and grew. Thus, 20 seeds of each were plated in the same medium, without antibiotics. While 100% of the wild-type (WT) seeds germinated, only 6 seeds from *Bd-thi1.27* and 3 from *Bd-thi1.28* were able to germinate and grew. Figure 52 shows all the germinated T1 plants.



a) T1 bd-thi1.27 plants



b) T1 bd-thi1.28 plants

Figure 52. *T1 germinated plants*. The scale bar is 30 cm ruler.

The genomic DNA of these resulting plants was obtained and the region containing the gRNA target sequence was amplified by PCR and sequenced. As CRISPR/Cas9 technology is supposed to be sequence-specific, the edition (INDEL) should occur upstream to 3' GG. The analysis of the sequences amplified form the T1 plants revealed that none of them had their DNA edited at the expected region or close by it (Figure 53).

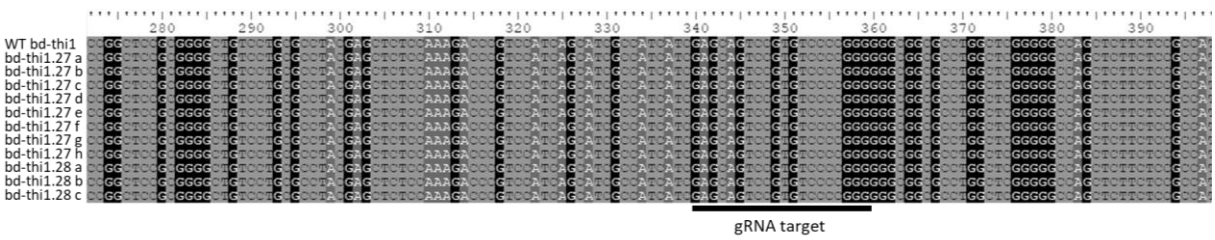


Figure 53. *Alignment of the region containing the gRNA target sequenced for the 11 plants obtained and the WT.*

Discussion

The selection of calli in medium containing antibiotics indicates that the plasmid was integrated in the cells genome. The absence of gene edition in the plants obtained can be explained by two situations:

1 – The plasmids do not work: the construction is not able to target properly the Cas9 to the gene target; the Cas9 codon modified is not compatible with *B. distachyon*; the whole system does not work in *B. distachyon*.

2 – The gene edition occurs but interferes in the plant regeneration/survival/seed germination: this hypothesis is based on the observation that the *tz-201* is the only mutant of *A. thaliana* for the *thi1* gene known to date. Probably because other mutations in the *At-thi1* are defective to the plant.

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