Investigation of the expression of *Glb-1* and *GluA2* promoters in *indica* and *japonica* rice in different tissues via GUS staining

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Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma to any academic institution, and to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

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List of abbreviations

Act	*actin*
*Act*p	*actin* promoter
CaMV 35S	Cauliflower Mosaic Virus 35S promoter
CRE	Cis-regulatory element
DC1	Double construct 1
E35S	Enhanced 35S promoter
GFP	Green fluorescent protein
Glb-1	*globulin-1*
*Glb-1*p	*globulin-1* promoter
GluA2	*glutelin A2*
*GluA2*p	*glutelin A2* promoter
GUS	β-glucuronidase
HvSUT1	*Hordeum vulgare sucrose transporter 1*
iGlb-1	*indica globulin-1*
*iGlb-1*p	*indica globulin-1* promoter
jAct	*japonica actin*
*jAct*p	*japonica actin* promoter
jGlb-1	*japonica globulin-1*
*jGlb-1*p	*japonica globulin-1* promoter
jGluA2	*japonica glutelin A2*
*jGluA2*p	*japonica glutelin A2* promoter
NC	Negative control
NTC	No-template control
OsNAS2	*Oryza sativa nicotianamine synthase 2*
PB-I/II	Protein body I/II
PC	Positive control
RT-qPCR	Real-time quantitative PCR
SC1/2	Single construct 1/2
SNP	Single nucleotide polymorphism
SSP	Seed storage protein
Ubi	*ubiquitin*
WT	Wild type

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Summary

This study investigated the gene expression patterns of the promoters governing two seed storage proteins in rice, the *globulin 1* (*Glb-1*) and *glutelin A2* (*GluA2*) promoters. Previous research in which these *japonica* rice-derived promoters controlled the expression of a barley sucrose transporter (*HvSUT1*) and a rice nicotianamine synthase (*OsNAS2*) in transgenic *indica* rice varieties has cast doubt on the long-held notion that their expression is confined to the endosperm. These transgenic plants showed increased or decreased vigour, depending on the transgene combination, compared to their wild-type counterparts, suggesting the leaky activity of these promoters. This research investigated this hypothesis by using the GUS reporter gene fused to the promoter elements. Previous research had generated transgenic lines with the *japonica*-derived actin and globulin-1 promoters (*jAct*p and *jGlb-1*p) driving GUS expression in *japonica* rice, and the *jGlu2A* promoter (*jGluA2*p) driving GUS expression in *indica* rice. To complete this study with appropriate controls, novel transgenic lines with the *indica Glb-1* promoter (*iGlb-1*p*)* fused to the GUS gene (*iGlb-1*p::GUS), and the *jGlu2A*p*::GUS* fusion construct were transformed into *japonica* rice via callus infection with *Agrobacterium*. The characterisation of the transgenic plants was performed by end-point PCR followed by gel electrophoresis to confirm the inheritance or integration of the GUS-containing constructs. GUS staining was used to visualise any promoter-driven activity in seed, leaf, crown, and root tissue to shed light on the role of these two promoters in the physiology of those transgenic lines expressing the sucrose transporter and nicotianamine synthase genes. However, preliminary results showed no evidence of ectopic expression of *Glb-1*p or *GluA2*p under the conditions of this experiment. Further research on these transgenic lines is warranted, but it is hoped that by exploring the expression patterns of these two promoters this study could encourage further investigation of their role and function to inform potential applications in plant biotechnology.

Chapter 1: Introduction

1.1 Introduction

1.1.1 Rice agriculture in times of climate change

Rice (*Oryza sativa* L.) stands at present as the most common crop used for human consumption, providing one fifth of the total caloric intake worldwide, with more than half of the population worldwide relying on it as a staple food in their diet (Fukagawa and Ziska, 2019) – in some developing nations accounting for as much as three quarters of the total caloric supply (Bin Rahman and Zhang, 2016). With a global population forecast to reach 9.7 billion people by the middle of the century (UN DESA, 2022), it is anticipated that global rice consumption will rise to 505 million tons in the same time frame, representing an 18% increase from 2010 (Kruseman *et al.*, 2020). The efforts to feed the global population are made all the more challenging by the heightened unpredictability of environmental stresses brought about by climate change, such as droughts, heatwaves, floods, soil degradation, diseases and pests (FAO, 2022). Unfortunately, rice is one of the crops most impacted by climate change (Saud *et al.*, 2022).

Climate change poses a huge threat to global agriculture due to the rise in atmospheric CO_2 levels and its repercussions, such as variations in temperature, changes in rainfall, and pests and pest-transmitted infections (Raza *et al.*, 2019). Higher CO_2 levels seem to have been beneficial to plant growth in some agricultural crops such as rice and wheat (*Triticum aestivum* L.) in recent years – a phenomenon sometimes referred to as “carbon fertilisation effect”, with these benefits expected to continue in the near future (van der Kooi *et al.*, 2016). However, a 2014 meta-analysis showed significant agreement among crop loss projections after this period: a large majority (70%) predict a decreasing trend in yields starting from the 2040s-2050s, with the latter half of the 21st century generally showing estimates of reductions of over 10% in

crop productivity compared to the first half (Challinor *et al.*, 2014). Moreover, elevated CO_2 levels can negatively impact the nutritional value of crops, including rice (Myers *et al.*, 2014).

Elevated temperatures are a significant risk to rice cultivation, especially true during the reproductive stage (Fahad *et al.*, 2019). High temperature can cause irreversible damage to plant growth and severely impact yield and quality, with a predicted crop loss estimated to be as high as 40% by the end of the century (Fahad *et al.*, 2018). Rising temperatures also affect insects, leading to larger populations and higher survival rates over the cold season, overall increasing the spread of pests and diseases (Skendžić *et al.*, 2021). Fluctuations in precipitations induced by climate change can lead to excessive rainfall, which has been shown to endanger crops just as much as high temperatures and drought (Li *et al.*, 2019) by flooding, soil erosion and nutrient loss (Slater *et al.*, 2021). This is especially true of agricultural soil, which is increasingly degraded (Austen *et al.*, 2022). To tackle environmental changes and ensuing food shortages, rice varieties that can withstand environmental stress and provide larger yields need to be developed (Clarke and Zhang, 2013).

Starting from the 1960s, novel crop breeding techniques and agricultural advancements ushered in the Green Revolution, ensuring that the burgeoning global population would be fed (Gogolev *et al.*, 2021). In Asia, the yields of rice and wheat doubled over the 1970s-1990s period (Hazell, 2009). Part of this success story relies on the introduction of semidwarf varieties of rice and wheat, which owe their reduced stature to a faulty *SD1* gene, which impairs gibberellic acid production (Ferrero-Serrano, Cantos and Assmann, 2019). As of 2021, semidwarf varieties make up over half of all rice grown in the United States alone (Peng *et al.*, 2021).

The extraordinary impacts the Green Revolution has had on agriculture, economy, and society at large are difficult to overstate, considering it took humanity nearly 10,000 years to

reach a global crop production of 1 billion tons of grains in 1960, and from there to 2 billion only the subsequent 40 (Khush, 2001). However, the conventional breeding techniques that have brought about these advancements are now too modest to address the world's needs (Ansari *et al.*, 2017).

On the other hand, genetic engineering has proven to be a successful strategy in rice (Zafar *et al.*, 2020) by widening the pool of genetic resources beyond those available in its close relatives utilised in conventional breeding programs, while also reducing the time and labour required to obtain results (Dong and Ronald, 2019). Novel technologies, such as genome engineering, can offer powerful new avenues to improve rice thanks to specific and efficient alterations to DNA sequences (Zafar *et al.*, 2020), instead of relying on the random integration of transgenes.

Genetic engineering can be utilised to develop varieties that are resistant to drought, high salt concentrations, and diseases and pests, while also improving the nutritional profile of rice (Karavolias *et al.*, 2021), as shown with the success of the Golden Rice project, a variety engineered to contain beta-carotene to counteract the vitamin A deficiency found in many regions where rice is a staple food (Tang *et al.*, 2009).

Whilst agricultural biotechnology offers tools to assist us in increasing productivity to feed the growing global population, the realisation of the full potential of genetically engineered crops hinges on the characterisation of the regulatory elements that govern transgene expression, such as promoters. Only with a more subtle, targeted, and effective approach to transgenic crops will biotechnology deliver on its promises to agriculture to improve yield and increase resilience in the face of an ever-changing climate.

1.1.2 Promoters in plant biotechnology

The investigation of promoters has been fundamental in our understanding of plant physiology (Freeman *et al.*, 2011; Jeong and Jung, 2015) and such knowledge has paved the way for biotechnological applications, such as crop improvement (Jeong and Jung, 2015; Ali and Kim, 2019). Promoters are found in the upstream region of a coding sequence, where the RNA polymerase and the transcription factors bind (Lis and Walther, 2016). Given their crucial role in dictating the temporal and spatial expression of a gene (Bentovim, Harden and DePace, 2017), promoters are also instrumental in genetic engineering, as they regulate the transcriptional levels of a transgene (Jeong and Jung, 2015)

When trying to express a transgene, gene silencing is a significant issue that renders biotechnological approaches less reliable and restricts any potential applications, such as crop improvement (Jeong *et al.*, 2002). Butaye *et al.* (2005) even observed that the use of several promoters might be required to reduce the effects of gene silencing as a result of promoter competition for the same transcriptional machinery.

Promoters are usually classified as constitutive, tissue-specific, or inducible. Constitutive promoters drive the expression of genes that are crucial to the plant metabolism throughout its life and as such are always active in every tissue. Conversely, tissue-specific promoters are either activated or repressed based on the tissue in which their gene is expressed and on the developmental stage in which the plant is found, so they are only active in certain tissues at certain stages. Lastly, inducible promoters trigger gene expression depending on a variety of stimuli, such as physical, chemical, and biotic signals (Liu, 2009; Grunennvaldt *et al.*, 2015).

To ensure that a newly inserted transgene will be transcribed, most of the promoters used in plant biotechnology are constitutive (Koetle *et al.*, 2017; Jiang *et al.*, 2018), such as the

cauliflower mosaic virus 35S promoter (CaMV 35S) (Amack and Antunes, 2020) and its enhanced version known as E35S (Heck *et al.*, 2005), the maize (*Zea mays* L.) *ubiquitin-1* (*Ubi-1*) promoter, and the rice *actin-1* (*Act-1*) promoter (Grunennvaldt *et al.*, 2015; Koetle *et al.*, 2017).

The CaMV 35S promoter has been essential in achieving consistent expression of transgenes in plants and has contributed significantly to the understanding of the activity of plant genes. Widely recognised as the most extensively studied cis regulatory element (CRE) in plants, its usage over the decades has generated ample knowledge on the impact which its various domains have on its activity. Besides its extensive use in transgenic expression, its modular structure has led to the development of a variety of transcription control mechanisms (Amack and Antunes, 2020). According to Koetle *et al.* (2017), the E35S promoter displays a higher level of expression in some monocotyledonous plants than the CaMV 35S promoter, and it has also been shown to enhance the expression of GUS in rice plants.

The *Ubi-1* promoter has also been widely used in the genetic engineering of monocotyledonous plants for gene expression characterisation via gene reporter systems (Christensen and Quail, 1996; Rooke, Byrne and Salgueiro, 2000; Schoonbeek *et al.*, 2015), including in rice in both callus and plants via GUS staining (Cornejo *et al.*, 1993). Results from the use of *Ubi-1*p for transgenic expression have however been inconsistent, being highly dependent on the transformation event and thus on the individual cell lines, for example in wheat, where some lines exhibit strong expression in all tissues, while in others the expression is limited and only in a few tissues (Rooke, Byrne and Salgueiro, 2000).

The *Act-1*p has also been used in the transformation of monocotyledonous plants (Schoonbeek *et al.*, 2015), including rice (McElroy *et al.*, 1990), and its activity has been investigated with the GUS reporter gene (Wang *et al.*, 1992). The use of this promoter has

resulted in an increase of GUS expression of several folds in both rice and maize cells, and is thus a good choice for transgene expression (McElroy *et al.*, 1991). The functionality of this promoter appears to rely on the presence of a 5' intron, without which no GUS expression was reported (McElroy *et al.*, 1990).

While these constitutive promoters are a useful tool to generate enhanced expression levels of the gene of interest to all tissues throughout development (Koetle *et al.*, 2017; Jiang *et al.*, 2018), they are a rudimentary, blunt solution to the problem of transgene silencing. Their use could be justified when dealing with a trait at the organismal level (e.g. pest resistance (Xu *et al.*, 1996)), or when the transgene usually demonstrates low expression levels and tissue-specific expression is not crucial (Grefen *et al.*, 2010), but they are not ideal for fine tuning the expression of other traits that need to be confined to specific tissues or situations – in this instance, the nutritional profile of the rice grain. Therefore it is necessary to understand the specificity and expression patterns of promoters, both spatial and temporal (Bandopadhyay *et al.*, 2010), to ensure that a transgene is expressed only in the tissue of interest at a designated time point during development. Reporter genes such as β-glucuronidase (GUS) are especially useful in this endeavour (Bandopadhyay *et al.*, 2010).

An important issue with constitutive promoters is that they can also lead to weaker phenotypes because of the accumulation of excess transgene product or due to excessive energy and nutrient expenditure on its expression (Freeman *et al.*, 2011; Jeong and Jung, 2015), or as has been eloquently put by Zhou *et al.* (2017) about transgenic microorganisms, "Appropriate gene expression level is the basis of high production and lower strain burden", which is also true for transgenic plants.

Other potential problems caused by the use of constitutive promoters include reduced yield, sterility, abnormal morphology, developmental delay, grain composition modification

and even transgene silencing, generating the opposite effect (Elmayan *et al.*, 1998; Ayadi, Brini and Masmoudi, 2019).

It would therefore be beneficial to utilise tissue-specific and inducible promoters in the genetic engineering of plants, as they can be employed to regulate desirable traits with precision, targeting solely the tissues of interest during specific developmental phases, which could lead to the development of crops that exhibit improved yield, tolerance to different stresses, and increased nutritional content (Jeong and Jung, 2015; Ayadi, Brini and Masmoudi, 2019). In particular, the transient expression of transgenes could solve the problems associated with aberrant development (Grefen *et al.*, 2010).

Moreover, transient expression of some traits could ensure that the plant would produce the transgene only when triggered by a stressor (Dhatterwal *et al.*, 2019), mimicking natural plant responses. An example would be a pest resistance gene that is expressed only during an insect attack, when triggered by a lesion (Arnáiz *et al.*, 2019). This would also potentially delay the development of resistance in insects, which has been found to be promoted by the constitutive expression of pest resistance genes (Straub *et al.*, 2020).

Despite the many benefits, the use of tissue-specific and inducible promoters has not become established (Jeong and Jung, 2015), possibly because of a want of a thorough understanding of their workings, as pointed out in Liu *et al.*, 2010, where a promoter thought to be tissue-specific was expressed ectopically in a different host plant, which resulted in the transgene being expressed in undesired tissues. This is but one of the examples that have highlighted the lack of comprehensive knowledge of promoter activity underlying plant biotechnology.

Compared to those found in other eukaryotes, plant promoters might appear puzzling because they do not seem to be governed by the same regulatory elements (Kumari and Ware,

2013), many of which, in fact, have not yet been fully characterised in plants due to a dearth of large-scale functional studies (Jores *et al.*, 2021).

Nevertheless, in recent years there have been substantial advances in the understanding of the different motifs that govern the functioning of promoters. For example, in an attempt to generate a minimal synthetic constitutive plant promoter, Cai *et al.* (2020) showed that some promoters are expressed constitutively because the motifs present in their sequences interact with a large number of transcription factors. This study was also able to identify the functional elements that allow promoters of both plant and pathogen origin to utilize a plant's transcriptional machinery for gene expression. The synthetic promoter created in this study consisted of 19 random nucleotides, a sequence of variable length used for the insertion of CREs, a TATA box, and lastly a promoter core that includes the transcription initiation site. By altering the number of CREs upstream of such promoter, it was possible to modulate the expression levels of the transgene. The creation of synthetic promoters is very promising for plant biotechnology as they could potentially target specific transcription factors and thus exhibit greater tissue specificity.

Promoters therefore play a vital role in regulating gene expression by recruiting transcription factors and are instrumental for transgene expression and thus for biotechnological applications. However, a lack of knowledge about CREs in plants and specifically of the motifs in promoter sequences that contribute to their transcriptional activity has made it difficult to identify tissue-specific and inducible promoters. More research is needed to understand the complexity of promoters in plant physiology so that they can be harnessed for more efficient and diverse biotechnological applications that do not simply rely on constitutive expression.

1.1.3 Investigating the ectopic expression of grain-specific promoters

During his time at Flinders University, PhD candidate Tran developed three transgenic rice lines in an attempt at improving the micro-nutritional profile of this crop by increasing the uptake of zinc and iron in the grain, which could improve health outcomes for those population that rely on rice as their primary food source.

This study was based on previous research that reported a not well understood mechanism by which transgenic rice plants expressing a barley (*Hordeum vulgare* L.) sucrose transporter (*HvSUT1*) not only generated a better yield, but also increased levels of both zinc and iron in their grains (Weichert *et al.*, 2010; Saalbach *et al.*, 2014). Another undiscovered mechanism involving the rice nicotianamine synthase-2 gene (*OsNAS2*) also led to an increase of zinc and iron levels in rice grains (Lee *et al.*, 2012). In these studies, the *Glb-1* and the *GluA2* from promoters from *japonica* rice had been chosen to direct expression of the transgene to the grain because of their tissue specificity.

The first transgenic line was generated by inserting into *indica* rice (cv. IR64) a construct (Single Construct 1, SC1) which fused the *japonica* (cv. Nipponbare) *Glb-1* promoter to *HvSUT1* (*jGlb-1*p*::HvSUT1*) to ensure a grain-specific expression. This construct had been generated by Huynh (2014).

A second construct (SC2) was developed by Tran via the fusion of the *japonica GluA2* promoter to the *japonica nicotianamine synthase-2* gene (*jGluA2*p*::OsNAS2*). Again, this promoter was chosen because of the endosperm-specificity of its gene promoter that would limit the expression of SC2 to the grain. This construct was also transformed into *indica* rice.

Based on an approach to combine these two mechanisms for an even higher uptake of zinc and iron, Tran generated a third construct, named Double Construct 1 (DC1), which included both SC1 and SC2, and was again integrated into *indica* rice.

A diagram of the constructs used by Tran can be found in Fig. 1.1 below, while a summary figure of the different lines mentioned can be seen in the upper section of Fig. 1.2 (A-C) further down.

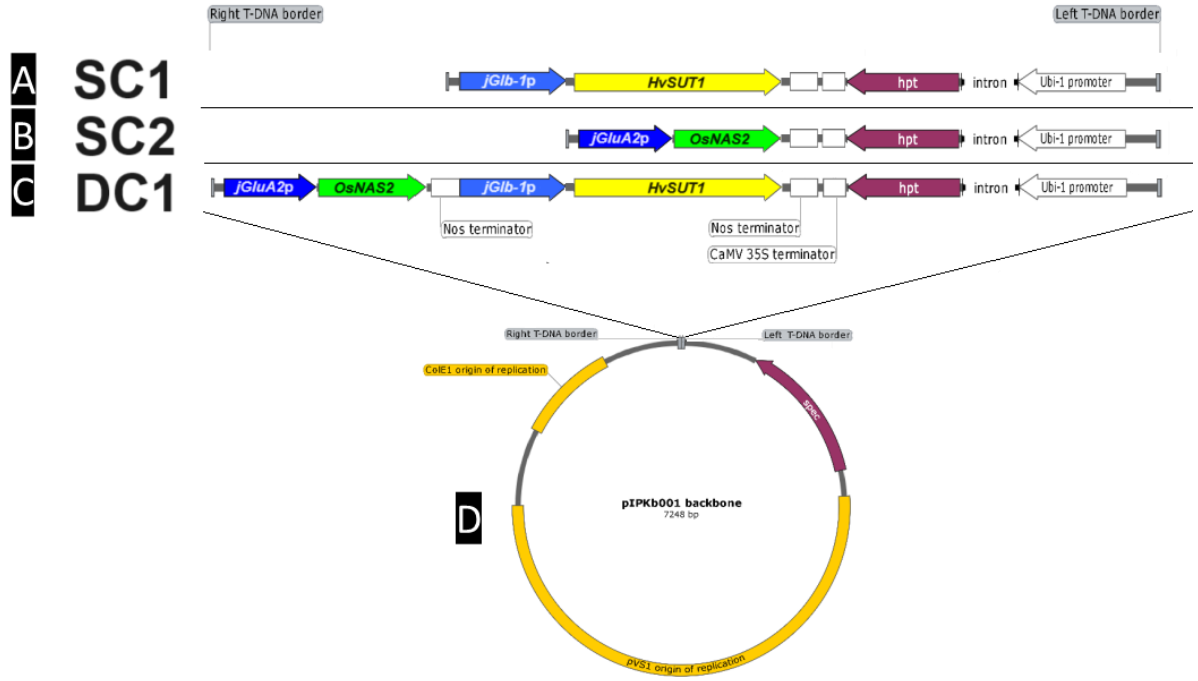

Figure 1.1. Diagram of the three constructs used by Tran (2021). A: construct with *jGlb-1*p driving *HvSUT1* (SC1) (Huynh, 2014); **B**: construct with *jGluA2*p driving *OsNAS2* (SC2) (Tran, 2021); **C**: construct where *jGlb-1*p*::HvSUT1* is combined with j*GluA2*p*::OsNAS2* (DC1); **D**: binary vector backbone. *jGlb-1*p: *japonica globulin-1* promoter; *HvSUT1*: *Hordeum vulgare sucrose transporter 1*; hpt: hygromycin phosphotransferase; Ubi-1: maize *ubiquitin-1*; *jGluA2*p: *japonica glutelin 2A* promoter; *OsNAS2*: *Oryza sativa nicotianamine synthase 2*; Nos: *nopaline synthase*; CaMV 35S: Cauliflower Mosaic Virus 35S; spec: spectinomycin resistance gene. Adapted from Tran (2021).

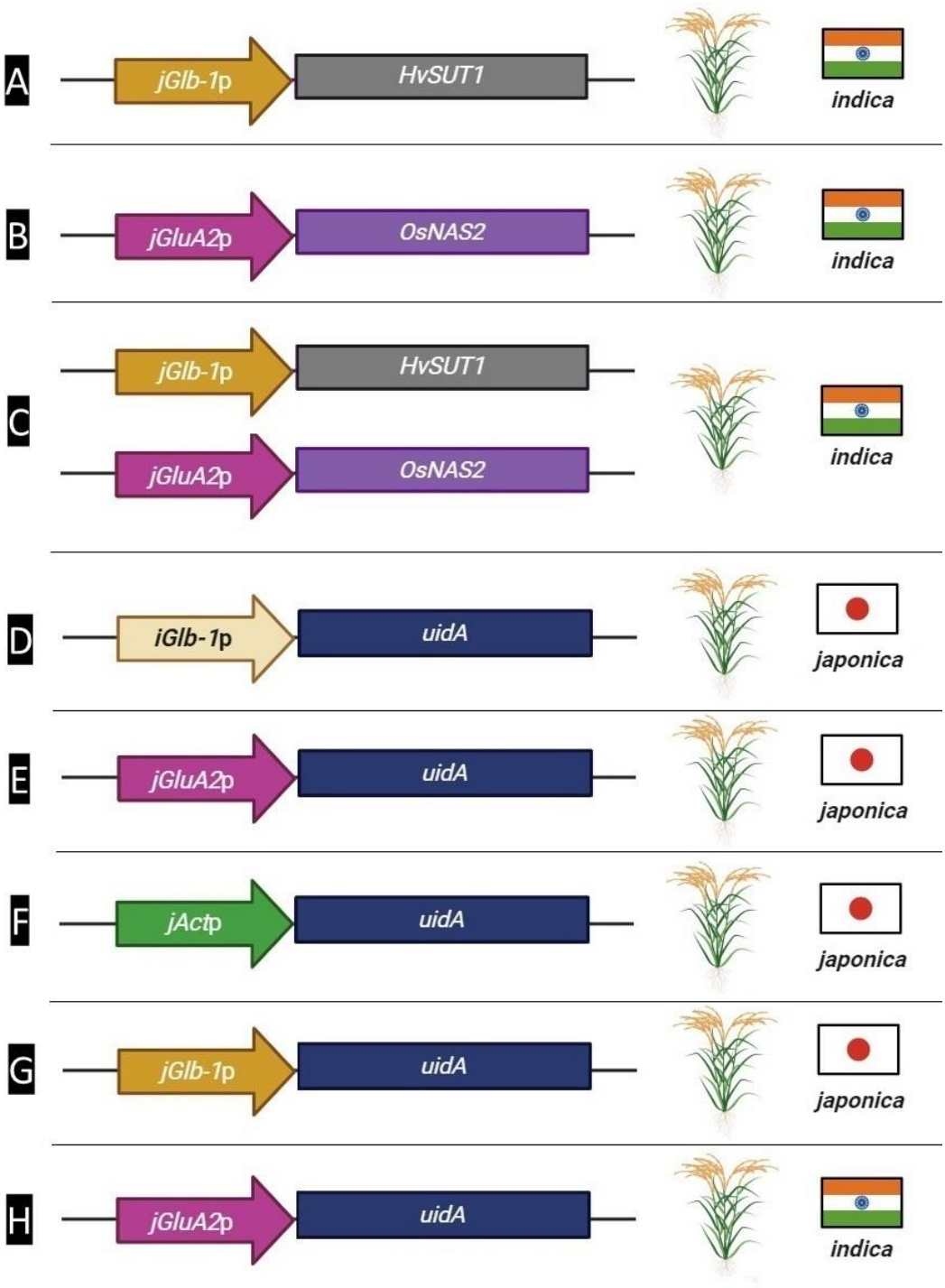

Figure 1.2. The different transgenic lines mentioned in this study. The **upper section** (**A-C**) shows the lines generated by Tran (2021) in *indica* rice; the **middle section** (**D-E**) shows the transgenic *japonica* lines generated in this study; the **lower section** (**F-H**) shows the established transgenic lines (*indica* and *japonica*) grown from seed in this study. Image created with the aid of BioRender.

As expected, Tran reported an enhanced uptake of zinc and iron in the transgenic lines expressing these constructs. However, Tran also noted that the SC1 plants and in particular the DC1 plants exhibited a more vigorous phenotype compared to the non-transgenic controls (Fig. 1.3), which amounted to increased tillering (Fig. 1.4) and biomass (data not shown). Also noted was a decrease in tillering and biomass in the SC2 line.

Figure 1.3. Phenotypic differences between representative specimens of SC2, WT, DC1, and SC1 indica plants three weeks after germination. SC2 (*jGlb-1*p*::OsNAS2 indica*) showed stunted growth compared to WT *indica*, while DC1 (*jGluA2*p*::HvSUT1* and *jGlb-1*p*::OsNAS2 indica*) and SC1 (*jGlb-1*p*::OsNAS2 indica*) showed more vigorous growth. Adapted from Tran (2021).

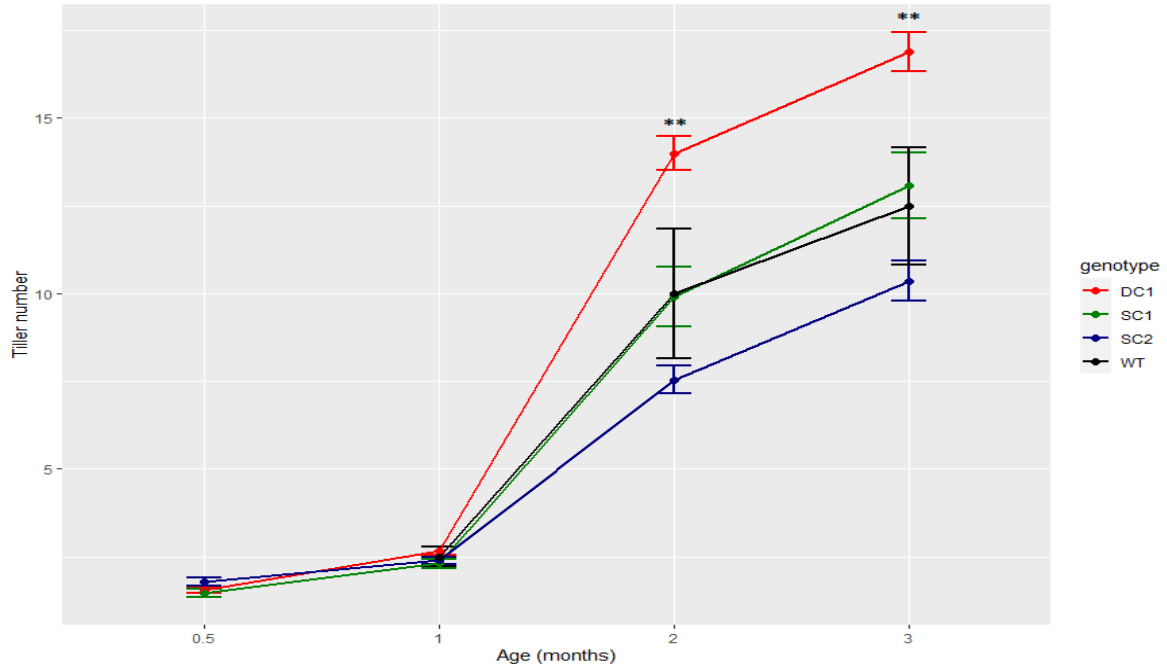

Figure 1.4. Comparison of average tiller number in DC1, SC1, SC2, and WT *indica* plants (T2 generation) between 0.5 to 3 months. DC1 lines are shown in red, SC1 lines in green, SC2 lines in blue, and WT in black. T2-generation transgenic lines were grouped into distinct populations and compared to the WT control. Statistical significance was calculated with one-way ANOVA and is shown with two asterisks (**) for $p \leq 0.01$. *n*=7-10 per line. Adapted from Tran (2021).

Such an effect in tillering and biomass called into question the specificity of the promoters used, the action of which should have been confined to the rice grain. What may have caused this enhanced growth is unclear at this stage, but one possibility is leaky expression of the transgenes leading to higher sucrose uptake, or iron and zinc mobilisation in tissues other than the grain.

SC2 plants exhibited a less vigorous phenotype compared to SC1, DC1, and even WT plants, with reduced tillering and less biomass produced. As reported in Zheng (2010) where

an *OsNAS1* construct was constitutively expressed in *japonica* rice, an increase in nicotianamine might lead to higher iron levels in leaves and thus iron toxicity, stunting SC2 growth. This might point to *jGluA2*p expression occurring in vegetative tissues instead of being confined to the endosperm.

Another reason suggesting the possible 'leakiness' of these promoters is the combined expression of both *jGlb-1*p and *jGluA2*p in DC1, which Tran (2021) speculates might play a role in not only salvaging the DC1 phenotype, but causing it to display more robust growth compared to SC1 despite the impediment of carrying the SC2 construct. This would occur by redirecting the excess metal ions mobilised by higher levels of nicotianamine, and thus by the action of *OsNAS2*, to the tissues where the demand for them was higher due to *HvSUT1* ectopic expression, complementing each other's activity.

Is it therefore possible that the *Glb-1* and *Glu-A2* promoters are directing expression not restricted to the endosperm as thought, but in other tissues as well? This study entertained the possibility that these promoters might lead to the ectopic expression of their driven genes, especially when transformed into a different rice subspecies due to different SNPs that might alter their tissue specificity (see Fig. 1.5 below).

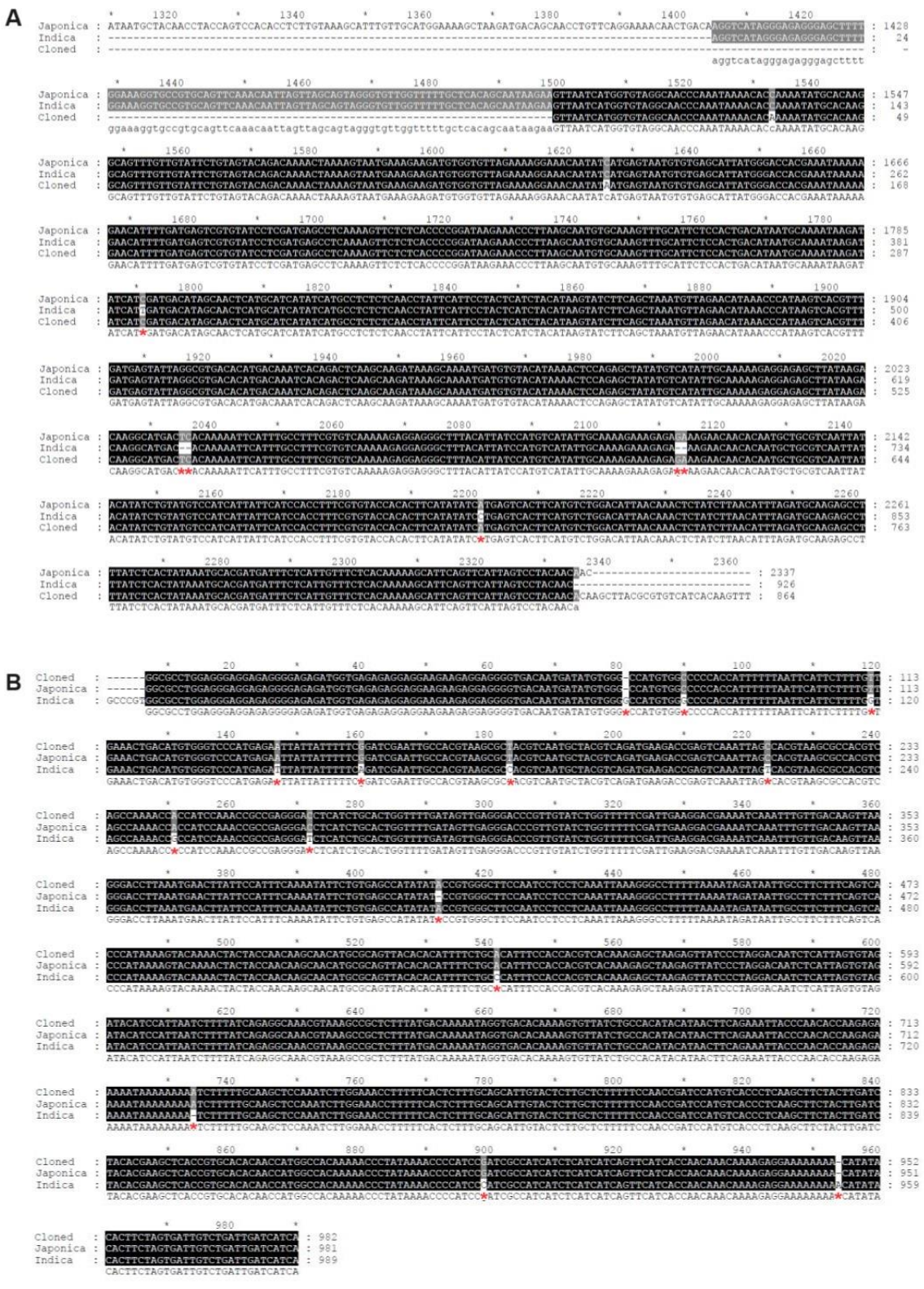

Figure 1.5. Pairwise alignments of *GluA2* and *Glb-1* promoter sequences from *japonica* and *indica* rice. (**A**) *GluA2* – 6 SNPs; (**B**) *Glb-1* – 14 SNPs. Red asterisks denote the different nucleotides between the two subspecies. Adapted from Tran (2021).

This raises the hypothesis that the few SNPs between the sequences are enough for a promoter to lose specificity and be ‘misread’, which would result in ectopic expression of the transgene. It is also possible that any ectopic expression is only confined to specific developmental moments, and to specific tissues other than the grain.

The transgenic vigour exhibited by SC1 and DC1 plants in particular would implicate ectopic expression in the crown, of all non-seed tissues, and is thus considered the most likely candidate as the location of promoter ‘leakiness’. An explanation for the increase of sucrose transporters in the crown resulting in higher tillering can be found in a sugar-mediated bud release mechanism that was only recently discovered involving trehalose-6-phosphate. Typically, tillering is suppressed by the action of auxin, but higher sucrose levels may elevate trehalose-6-phosphate levels, thereby enhancing the crown's sink strength to overcome such inhibition (Bertheloot *et al.*, 2020).

1.1.4 Seed storage proteins and their promoters

In the lines generated by Tran, transgene expression was intended to be targeted to the rice grain with the use of the *Glb-1* and *GluA2* promoters, which are derived from two genes that are only expressed there and encode two seed storage proteins, or SSPs (Kawakatsu and Takaiwa, 2019).

A large supply of nutrients goes into seeds to help with germination and early survival, mostly consisting of starch and oils for energy, as well as proteins, with seeds being the tissue with the largest amount of protein in a plant (Chen *et al.*, 2018). Despite their abundance, the number of proteins in a seed is limited to only a few families, which are commonly referred to as SSPs (Kanai *et al.*, 2023). As such, SSPs are especially important for crops where grain is

the primary tissue that is harvested, playing an important role in determining the nutritional value, as well as other properties (Tang *et al.*, 2020).

According to the traditional fractionation technique developed by Osborne in 1924, rice SSPs can be divided into four categories based on their solubility: albumins (water), globulins (saline), glutelins (acids or bases), and prolamins (aqueous alcohols). Despite the limitations of this technique that did not account for specific conditions necessary to break disulfide bridges and for mixtures of different proteins being present in the fractions, this classification is still useful for protein extraction and analysis (Kawakatsu and Takaiwa, 2019).

The localisation of SSPs to the endosperm is enabled by their promoters, which have distinct motifs, including prolamin boxes, ACGT, AACA, and GCN4 (Wu, Adach, *et al.*, 1998; Mohan, Jayanarayanan and Narayanan, 2017). In particular, the combination of the latter two seems instrumental for endosperm specificity in rice (Yoshihara, Washida and Takaiwa, 1996).

Interestingly, some endosperm-specific promoters do not seem to possess any of these motifs that would direct the expression of their genes to the endosperm. It is possible that one or more motifs are yet to be identified, or that gene expression might be controlled by an as-of-yet still unknown mechanism (Qu *et al.*, 2008).

Along with prolamins, glutelins are the most significant SSPs in rice, as they accumulate in the endosperm and account for approximately 70 to 80% of the total protein content in rice grains (Zhao, Gatehouse and Boulter, 1983). Glutelins can be categorized into four distinct groups based on their specific amino acid sequences, namely, GluA, GluB, GluC, and GluD, with 15 genes responsible for their encoding (Kawakatsu *et al.*, 2008). A well conserved aminoacidic sequence (LVYIIQGRG) ensures the localisation of glutelins to the ER and the proper assembly of proglutelins and formation of protein bodies I (PB-Is) (Tian *et al.*, 2018).

The expression of the *GluA2* gene has been investigated using GUS as a reporter gene, which has resulted in learning that it is expressed mostly in the periphery of the endosperm. Its localisation can be attributed to the several CREs present in the promoter: three prolamin boxes, one ACGT box, one GCN4 motif, and one AACA motif (Qu *et al.*, 2008).

Considered minor SSPs in rice, globulins are encoded by only three genes: *Globulin-1* (*Glb-1*), *RICE EMBRYO GLOBULIN 1* (*REG1*), and *REG2*. While REG1 and REG2, of 35 kDa and 46 kDa respectively, are only localised in the embryo (Sun *et al.*, 1996), *Glb-1* plays the most prominent role as the most abundant of the three in rice grains (Nakase *et al.*, 1996; reviewed in Kawakatsu and Takaiwa, 2019), encoding a 26 kDa α-globulin that is expressed only in the inner endosperm (Qu and Takaiwa, 2004). Based on the tissue-specific expression of *Glb-1*, its promoter has been used to direct the expression of transgenes to the endosperm (Qu and Takaiwa, 2004). As shown in Hwang *et al.* (2002), this might be achieved thanks to several putative regulatory sequences present in *Glb-1*p: 11 AAAG motifs, two rice endosperm Bzip protein binding sites, 4 ACGT motifs, one GCAA motif, and another CRE-like sequence.

The inner-endosperm localisation of *Glb-1* is in contrast to most glutelin genes, which are expressed in the outer endosperm (Qu and Takaiwa, 2004; Kawakatsu *et al.*, 2008; Kawakatsu and Takaiwa, 2019). It appears that the α-globulin stored in the peripheral matrix in protein bodies II (PB-IIs) surrounds the glutelins and prevents them from being digested by proteinases ensuring their accumulation, with studies on *Glb-1* knock-outs showing deformed PB-IIs (Katsube-Tanaka *et al.*, 2016).

Given their common expression pattern, it could be expected that some glutelin promoters and the *Glb-1* promoter share similar motifs that ensure the localisation of their respective products to PB-II in the endosperm. However, by comparing the promoter sequences of a number of glutelin promoters and *Glb-1*p, the only putative CRE they all share, excluding

*GluA1*p and *GluB1*p, is the ACGT box (Wu, Suzuki, *et al.*, 1998; Hwang *et al.*, 2002; Qu *et al.*, 2008). See Fig. 1.6 below for a schematic representation of the different putative CREs in these promoters.

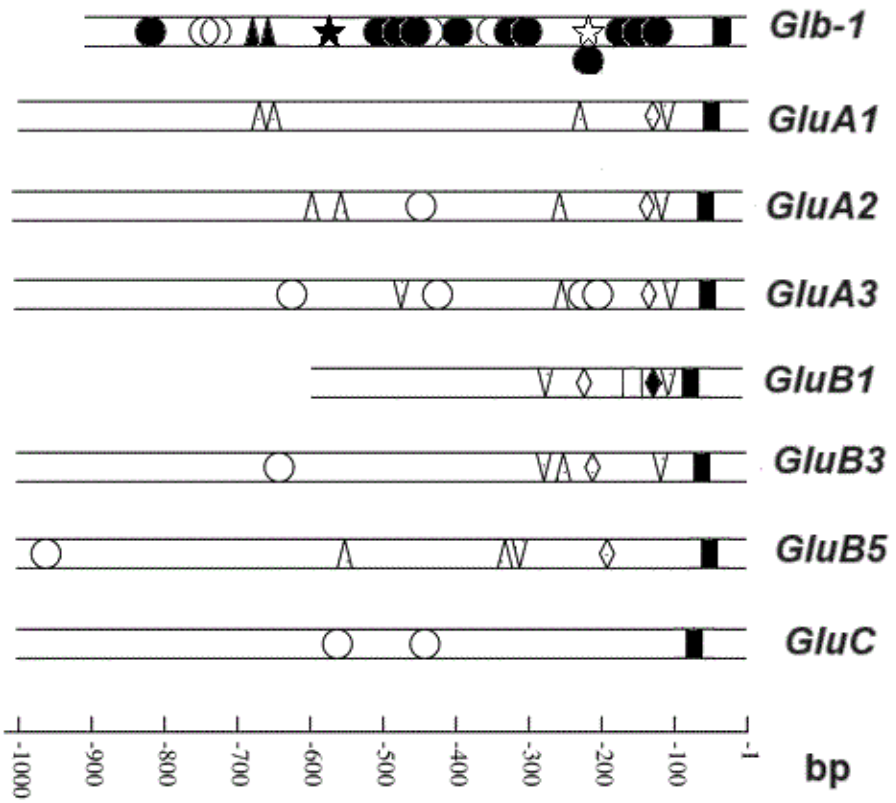

Figure 1.6. Comparison of putative CREs between *Glb-1*p and a number of glutelin promoters. Adapted from Wu, Adach, *et al.* (1998); Hwang *et al.* (2002); and Qu *et al.* (2008). ●: AAAG motif; ○: ACGT box; ▲: rice endosperm Bzip protein binding site; ★: CRE-like sequence; ☆: GCAA motif; ▮: TATA box; △: prolamin box; ◇: GCN4 motif; ▽: AACA motif; □: endosperm motif ; ◆: AG box. The approximate position of the putative CREs is shown with a negative number (bp) relative to the translation start site.

Wu, Suzuki, *et al.* (1998) developed constructs with a glutelin promoter, *GluB1*p, where an increasing number CREs upstream of the TATA box was deleted, as well as some constructs with a mutated GCN4 motif, discovering that the GCN4 motif was essential in directing *GluB1* expression to the grain. However, *Glb-1*p does not possess a GCN4 box or any AACA motif that might direct the expression to the endosperm, therefore the mechanism ensuring *Glb-1* localisation to the endosperm is not fully understood (Hwang *et al.*, 2002). It is likely that the distinctive mechanism behind *Glb-1* expression is the reason it is the most prevalent SSP encoded by a single gene (Hwang *et al.*, 2002). With such a large number of CREs, *Glb-1*p exhibits an increased strength compared to other endosperm-specific promoters, such as *GluB1*p and *GluB4*p, making it a superior choice to drive expression to the endosperm (Hwang *et al.*, 2002; Montesinos *et al.*, 2017).

The fact that there are distinct motifs or mechanisms that direct SSP expression to the grain further complicates things when assessing the potential off-target effects of constructs driven by different SSP promoters.

In general, off-target expression of *Glb-1* or *GluA2* has not been widely reported in literature, unless minimal (Wu, Adach, *et al.*, 1998; Qu and Takaiwa, 2004; Qu *et al.*, 2008). However, it can be said that the endosperm-specific activity of these two promoters has been taken for granted and their expression, despite not having been established, has often been examined only in seeds, where it was expected, leaving the possibility that they might also be expressed in other tissues open to be investigated.

1.1.5 GUS staining

GUS has served as a reporter gene in plants for many decades (Jefferson, Kavanagh and Bevan, 1987), becoming the most widely used reporter gene in plants (Villao-Uzho *et al.*,

2023). It has been employed in the study of promoter activity (Wang *et al.*, 2016), including in the investigation of the role of SSP promoters in rice (Wu, Adach, *et al.*, 1998), and has been noted for its reliability and sensitivity in histochemical analysis of tissues (Chen *et al.*, 2017). The use of GUS can be especially useful when examining the spatial expression of a gene. (Villao-Uzho *et al.*, 2023).

The endosperm-specific activity of seed storage protein (SSPs) in rice was confirmed using β-glucuronidase (GUS) as a reporter gene (Wu, Adach, *et al.*, 1998). Interestingly, the authors reported some, although minimal compared to the endosperm (three orders of magnitude smaller), GUS expression in the leaves of the transgenic lines expressing the *Glb-1* promoter and the *GluA3* promoter (Wu, Adach, *et al.*, 1998). This might point to the leaky expression being linked to the action of the promoter, and not to the effect of the transgenes driven by it that were used in Tran (2021).

1.2 Aims of this work and experimental design

Building on Tran's work (2021), this study set out to investigate the possibility that two SSP promoters that are considered endosperm-specific, specifically *Glb-1*p and *GluA2*p, might exhibit a 'leaky' expression in other tissues, such as leaf, crown, and root, via the use of the reporter gene GUS. In particular, this study considered a scenario where distinct SNPs in these promoters (Fig. 1.5) might modify tissue specificity and induce their unintended expression in a different subspecies.

To assess whether ectopic expression of *Glb-1*p and *GluA2*p is occurring, a histochemical analysis of the GUS staining patterns in leaves, crown, roots, and seeds of both newly generated transgenic rice lines and of transgenic rice lines that had previously been

created was performed. Fig. 1.7 below shows a diagram of the tissue types sampled in a rice plant.

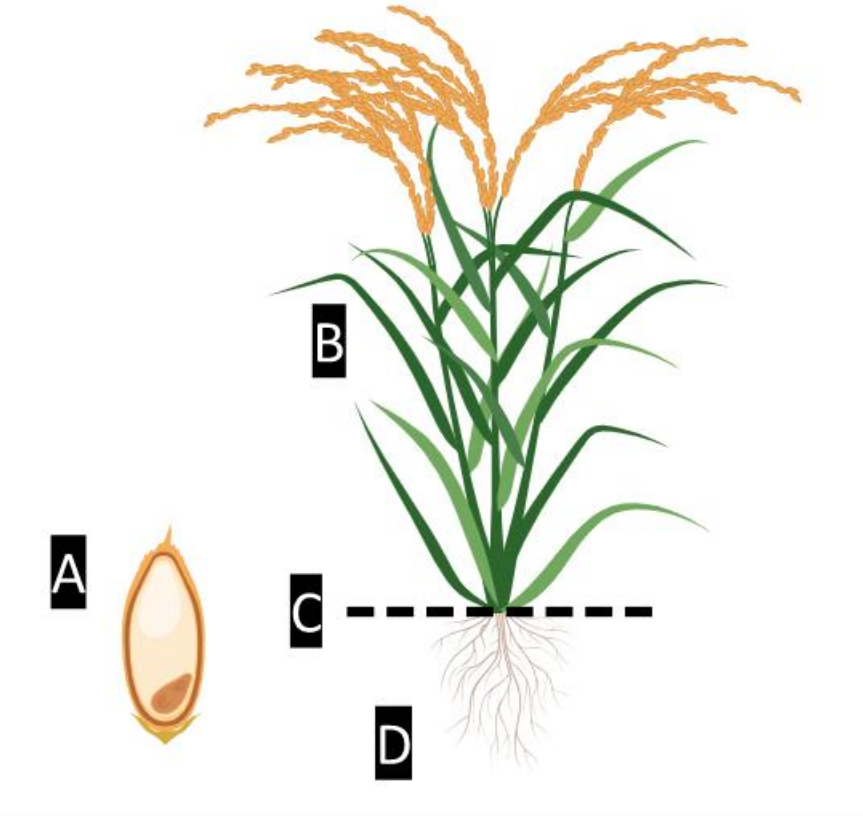

Figure 1.7. The different tissue types sampled in this study. A: rice grain; **B:** leaf; **C:** crown; **D:** root. Image created with the aid of BioRender.

The GUS reporter gene was chosen as it is the most common system to investigate transgene expression in plants (Villao-Uzho *et al.*, 2023). Moreover, a number of established transgenic lines carrying a GUS construct driven by different promoters and some GUS vectors driven by SSP promoters were readily available.

The established transgenic lines used were *jAct*p*::GUS japonica*, *jGlb-1*p*::GUS japonica*, and *jGluA2*p*::GUS indica*. The first two lines had been generated by Huynh (2014), while the third line by Tran (2021) (Fig. 1.2 F-H). *jAct*p*::GUS japonica* had already been

shown to stain different tissues and was expected to stain each tissue, given the constitutive nature of *Act*p (Grunennvaldt *et al.*, 2015; Koetle *et al.*, 2017). While *jGlb-1*p*::GUS japonica* and *jGluA2*p*::GUS indica* were chosen because grain samples from these lines had already been shown to stain, staining in other tissues had not been assessed in these lines.

Two binary vectors, *jGluA2*p*::GUS* and *iGlb-1*p*::GUS*, which were built by Tran and Khalbayeva respectively, were used to generated two novel transgenic lines in *japonica* rice (Fig.1.1 D-E). These vectors included a hygromycin phosphotransferase gene, which was used as a selectable marker for transgenic plants as it grants hygromycin resistance (Hiei and Komari, 2008), and a spectinomycin resistance gene, which was used to eradicate *Agrobacterium* after co-cultivation (McCormac, Elliott and Chen, 1998). The hygromycin phosphotransferase gene was under the control of the constitutive maize *Ubi-1* promoter. *Japonica* rice was chosen as host because, as noted in Tran (2021), the transformation procedure is less tedious and time-consuming compared to *indica* rice (Tie *et al.*, 2012), which would fit the scope and timeframe of this study (8 months).

To characterise transgenic plants, instead of targeting the eukaryotic promoter sequences that might be similar to other eukaryotic sequences in the plant genome, primers were chosen that would allow only for the PCR amplification of the prokaryotic *uidA* gene, which was assumed would reflect the integration of the whole construct, as *uidA* was present in all the transgenic lines utilised, both novel and established. It was thus assumed that no other gene would be amplified and that the construct would be fully integrated based on the presence of a band of the expected size.

As for GUS staining, it was assumed that the staining patterns would accurately reflect the expression patterns of the gene products that were replaced with *uidA*. WT *indica* plants were chosen as a NC as no GUS staining was expected in this line. *jAct*p*::GUS japonica* was

chosen as the PC for GUS staining, as GUS is assumedly expressed in every tissue when driven by the constitutive promoter *Act* (Huynh, 2014). *jAct*p*::GUS japonica* seeds, along with *jGluA2*p*::GUS indica* seeds, were also chosen as PCs for the GUS staining solution, as both lines were shown to produce GUS staining in the rice grain, and GUS staining was expected to occur there. The null seeds from these two lines were used as NCs for the GUS staining solution.

A diagram showing the different constructs utilised, as well as the subspecies they were integrated into, can be found in the middle and lower section of Fig. 1.2. (D-H).

Besides evaluating whether any ectopic GUS staining occurs across all lines, this study focused on comparing two lines with different promoters introduced into the same host (*iGlb-1*p*::GUS japonica* vs *jGlb-1*p*::GUS indica*), as well as comparing two lines with the same promoter introduced into distinct subspecies (*jGluA2*p*::GUS indica* vs *jGluA2*p*::GUS japonica*), to assess whether there are any differences in GUS expression patterns based on distinct SNPs.

In conclusion, the primary aim of this study was to assess 'leaky' promoter expression in different tissues in transgenic rice plants via GUS staining, in particular focussing on the differences in "different construct/same host" and "same construct/different host" scenarios. Any GUS staining occurring outside of the rice grain would be considered evidence of ectopic activity of the SSP promoter driving the GUS construct. The secondary aim was to generate novel transgenic lines with established GUS constructs from callus. In case the secondary aim was unsuccessful, only established lines grown from seed would be assessed for GUS staining.

Chapter 2: Methods

As in the previous experiments this study is based on, the *indica* rice cultivar used was IR64, while the *japonica* rice cultivar was Nipponbare.

2.1 Growth of transgenic plants from seed

2.1.1 Overview

The protocol for the germination and cultivation of transgenic plants was adapted from Huynh (2014) and Tran (2021). The same growth conditions were applied to the novel lines generated from callus. Rice grains were dehusked, surface sterilised, and placed in a cabinet at 28°C in the dark. The seedlings were then grown hydroponically before being moved to soil, with a photoperiod of 14 h.

2.1.2 Seed germination

Transgenic rice grains from three different lines (*jGluA2*p*::GUS indica*, *jAct*p*::GUS japonica*, and *jGlb-1*p*::GUS japonica*) were dehusked and surface sterilised in 70% ethanol for 30 seconds, and then placed in 50% commercial bleach with a drop of Tween-20 for 20 minutes on a rotary shaker. They were rinsed five times with sterile Milli-Q® water before being placed on moist sterile Whatman® filter paper disks and incubated in a cabinet at 28°C in dark conditions for a week. The paper was kept moist with the addition of Milli-Q® water when necessary.

2.1.3 Growth conditions

The seedlings were wrapped in sterile foam and moved to a hydroponics box and maintained in the greenhouse at 28°C with a photoperiod of 14 hours per day. The composition

of the media was adapted from Genc, McDonald and Tester (2007), and Shavrukov, Genc and Hayes (2012), and can be found in Appendix B.

After 12 weeks, a number of seedlings were moved to pots containing BioGro soil and kept under the same conditions in order to promote seed setting.

2.2 Generation of transgenic plants

2.2.1 Overview

The protocol for the generation of transgenic *japonica* rice plants from mature embryo callus was taken from Huynh (2014), which had been adapted based on Hiei *et al.* (1994); Aldemita and Hodges (1996); Toki (1997); Toki *et al.* (2006); and Shrawat and Good (2011).

Mature *japonica* rice grains were first surface sterilised and allowed to germinate. Then the rootlet and the coleoptile were removed to induce the formation of the callus. The callus was allowed to grow and then was co-cultured with the *Agrobacterium* harbouring the respective GUS vector that had previously been built. Callus selection was performed using media with hygromycin, while timentin was included to suppress *Agrobacterium* growth. After spending 4 weeks in dark conditions, the callus was left to incubate in light conditions. Hygromycin-resistant shoots regenerated from the callus and then set root, before the plantlets were moved to soil.

2.2.2 Callus induction

As described in section 2.1.1, *japonica* rice grains were dehusked and surface sterilised and placed on 0.4% GelRite® solid N6D medium (Appendix B) plates, which were then sealed with Micropore™ surgical tape (3M™). The plates were cultured in a cabinet under continuous light at 28°C for a week.

Upon germination, the coleoptiles and rootlets were severed utilising a sterile scalpel to induce callus formation, and the plates were placed back into the cabinet for 4 weeks.

2.2.3 *Agrobacterium* transformation and selection

Two separate electrocompetent *Agrobacterium tumefaciens* (AGL1) cell batches were exposed to electroporation in the Gene Pulser® II (Bio-Rad) device to integrate the two binary vectors: the plasmid containing the *jGluA2*p*::GUS* construct, and the plasmid containing the *iGlb-1*p*::GUS* construct. ~150-300 ng of plasmid DNA of each vector was used every 20 µl aliquots of cell suspension.

The cells were transferred into an electroporation cuvette and placed on an ice bath for 2 min. The device voltage was set to 2.5 kV, resistance to 200Ω, and capacitance to 25 µF. The device delivered a pulse for ~5.00 µs. The cells were suspended in a 1.5 ml Eppendorf tube containing LB broth (Appendix B) and left to recover for 2 hours on a shaker plate at 100rpm.

The cell suspension was plated onto YM media (Appendix B) containing 20 µg/ml rifampicin and 25 µg/ml spectinomycin and left to incubate at 28°C until some large colonies could be observed (approximately 3 days).

A single colony of *Agrobacterium* per construct was spread on a YM plate containing 20 µg/ml rifampicin and 25 µg/ml spectinomycin and grown for 3 days at 28°C in dark conditions. Several loops of bacteria were mixed with 30 ml of 2N6-AS liquid media (Appendix B) containing 20 µg/ml acetosyringone (OD_{600}: 0.1-0.2) and left at room temperature for 2 hrs before incubating with rice callus.

2.2.4 Callus infection and selection

Pieces of healthy callus were washed in the *Agrobacterium* suspension for two minutes before being dried on sterile filter paper disks. The callus was then placed onto 0.4% GelRite® solid 2N6-AS medium (Appendix B) and cultured in a cabinet in dark conditions at 28°C for 5 days.

The callus was then washed 5 times in sterile Milli-Q® water and once in sterile Milli-Q® containing 150 µg/ml timentin. After a rapid drying on sterile filter paper, the callus was placed on solid N6D medium containing 150 µg/ml timentin and 50 µg/ml hygromycin. The callus was kept in the dark for four weeks at a constant temperature of 28°C.

Surviving healthy callus was then moved to fresh solid N6D medium with antibiotics as described above to continue the selection of the transformants for four more weeks.

2.2.5 Plantlet regeneration and growth conditions

Continuously proliferating callus was moved to solid REIII medium (Appendix B) plates containing antibiotics as described in section 2.2.4, and 0.05 µg/ml naphthalene acetic acid (NAA) and 2.5 µg/ml kinetin to favour the formation of shoots. The callus was maintained in constant light conditions.

After four weeks, callus that had produced rootlets and shoots was transferred to Magenta boxes containing solid HF media (Appendix B) with antibiotics as described in section 2.2.4 to continue the selection process. The temperature and the photoperiod were maintained at 28°C and at 24 hours.

Plantlets were then let grow in the cabinet before being transferred into small pots containing BioGro soil, which were placed onto trays filled with water in the greenhouse. Plantlets were kept under upside-down Magenta boxes for a week to harden off. After

three/four weeks, plantlets were moved into larger pots with the base being kept submerged in water. Plants were then allowed to grow under the same conditions described in section 2.1.3.

2.3 Characterisation of transgenic plants

2.3.1 Overview

To confirm the integration of the constructs (*jGluA2*p*::GUS* and *iGlb-1*p*::GUS*), an end-point PCR reaction was set up to amplify the *uidA* gene assumed to be present in all transgenics, The primers chosen were validated against the plasmid DNA used for the transformation, and the bioinformatics tool CLC Genomics Workbench 23 (Qiagen) was used to estimate the size of the PCR product. This was followed by agarose gel electrophoresis. The presence of a band of the expected size of 629 bp on the gel was considered indicative of a successful transformation, based on which the plant was characterised as transgenic.

The established transgenic lines *jAct*p*::GUS japonica*, *jGlb-1*p*::GUS japonica*, and *jGluA2*p*::GUS indica* that were grown from T1 seeds were also characterised in the same manner, ensuring that nulls would not be further considered in the experiment.

2.3.2 Primer validation and fragment size estimate

The oligonucleotides used as GUS primers in this experiment were manufactured by GeneWorks (now Integrated DNA Technologies) and validated via end-point PCR followed by gel electrophoresis (see section 2.3.4 and 2.3.5 below).

CLC Genomics Workbench 23 (Qiagen) was used to estimate the size of the predicted PCR product by aligning the sequences of the forward and reverse primer to the *uidA* gene sequence. The sequence used was taken from *E. coli* str. K-12 substr. MG1655 (accession no.:

NC_000913.3:c1696071-1694260). Please refer to Appendix A for primer sequences and alignment.

2.3.3 DNA extraction and Nanodrop analysis

Leaves collected from putative transgenic rice plants were used to perform genomic DNA extraction with a DNA purification kit (Isolate II Plant DNA, Bioline) according to the manufacturer's instructions. The elution buffer was used twice to ensure an elevated yield of DNA.

A Nanodrop 2000 spectrophotometer (Thermo Scientific) was used to estimate the quantity and purity of isolated DNA.

2.3.4 End-point PCR

To generate DNA fragments of the expected size, a PCR reaction was run using the validated primers to target the *uidA* gene assumed to be present in all transgenics.

The PCR reaction was prepared using 1X GoTaq® Green Reaction Buffer (Promega), 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM each primer, 0.625 U GoTaq® Flexi DNA polymerase (Promega) and 5 ng of genomic DNA of each sample as template, making up to 25 µl with sterile Milli-Q® water. 1 ng of plasmid DNA was used for the positive control.

The protocol used was as follows: initial denaturation step at 95℃ for 4 mins, denaturation at 95℃ for 1 min, annealing at 59℃ for 1 min, extension at 72℃ for 1 min, with a final extension step of 4 mins. Upon completion, the sample was maintained at 4℃. The reaction was run for 35 cycles.

Gradient PCR was used to optimise the protocol by altering the annealing temperature. Based on the melting temperatures of the primers of 49℃ and 51℃, an annealing temperature in a 50.7℃ to 59.2℃ range was investigated that would generate clearer bands on the gel.

2.3.5 Gel electrophoresis

PCR products were visualised on 1.2% agarose gel run for 25 minutes at 95 V. The gel was placed into the Gel Doc™ EZ System (Bio-Rad) and visualised using the Image Lab™ software (Bio-Rad). A Gene Ruler 100 bp ladder (Thermo Scientific) was used to estimate the size of the PCR product and compare it to the expected DNA fragment.

2.4 GUS analysis of transgenic plant tissue

2.4.1 Overview

Seed, leaf, crown, and root tissue was collected, fixed and stained with the GUS solution. Appropriate controls were used for both the GUS staining solution and GUS staining. The samples were processed in an automated tissue processor, embedded in paraffin, cut into sections, placed on slides, dewaxed, and observed under a brightfield microscope to assess any staining.

2.4.2 GUS staining

Root, crown, and leaf tissue was harvested from lines carrying each construct and from PCs and NCs. Seeds, where available, were sectioned longitudinally prior to staining. One sample per tissue for each construct was taken from a confirmed transgenic plant.

Samples were taken at different moments of development: *jGluA2*p*::GUS indica* and WT *indica* at ~7 weeks after germination, *jGluA2*p*::GUS japonica* ~10 weeks after transfer to soil, *jGlb-1*p*::GUS japonica* ~8 weeks after transfer to soil, *jAct*p*::GUS japonica* and *jGlb-1*p*::GUS japonica* ~8 weeks after germination.

Each sample was submerged in 90% cold acetone for two hours, before being placed in a fixative solution (4% formaldehyde) for 30 minutes. They were then transferred to the phosphate buffer solution (Appendix B) for one hour.

The samples were then immersed in the GUS staining solution (Appendix B) and placed in a vacuum pump, where they were exposed to 5 three-minute-long cycles of vacuum infiltration at ~0.8 bar. The samples were then left to incubate at room temperature overnight, before being rinsed with 100% ethanol, and then moved to 70% ethanol for storage. The sample were observed with the naked eye and under a light microscope for any signs of staining.

2.4.3 Sample processing and imaging

The samples were loaded into appropriately labelled cassettes, and then placed in an automated tissue processor (HistoCore PEARL, Leica) where they were dehydrated by subsequent immersions into increasingly higher ethanol solutions (70%, 80%, 90%, 3 × 100%), the whole sequence lasting 2 h 40 mins. Finally, the samples were immersed three times in xylene for a total of 1 h 5 mins. The samples were then immerged in ethanol again, starting another cycle. The ethanol and xylene cycle was run repeatedly over 18 hours.

The samples were then embedded in molten paraffin wax at 62°C. A rotary microtome (Leica RM2135, Leica) was then used to obtain 8 µm thick slices that were placed on microscope slides. The slides were left to dry in an incubator at 37°C for 24 h and then dewaxed in two consecutive xylene baths. DEPEX (Merck) was used as a mounting medium to attach

the coverslips to the slides. After drying for 24 h, the samples were observed under a brightfield microscope (Olympus BX53, Olympus) connected to a colour camera to capture the images using the cellSens Entry software (Olympus). The samples were examined at different magnification settings (4×, 10×, 20×, and 40×) to account for potential minimal GUS staining.

Chapter 3: Results

3.1 Generation of transgenic plants

The transformation method taken from Huynh (2014) was used to generate 56 novel transgenics from mature embryo callus – 41 *jGluA2*p*::GUS japonica* plants and 15 *iGlb-1*p*::GUS japonica* plants. A diagram of the transformation process can be seen in Fig. 3.1 below, where the various stages are shown.

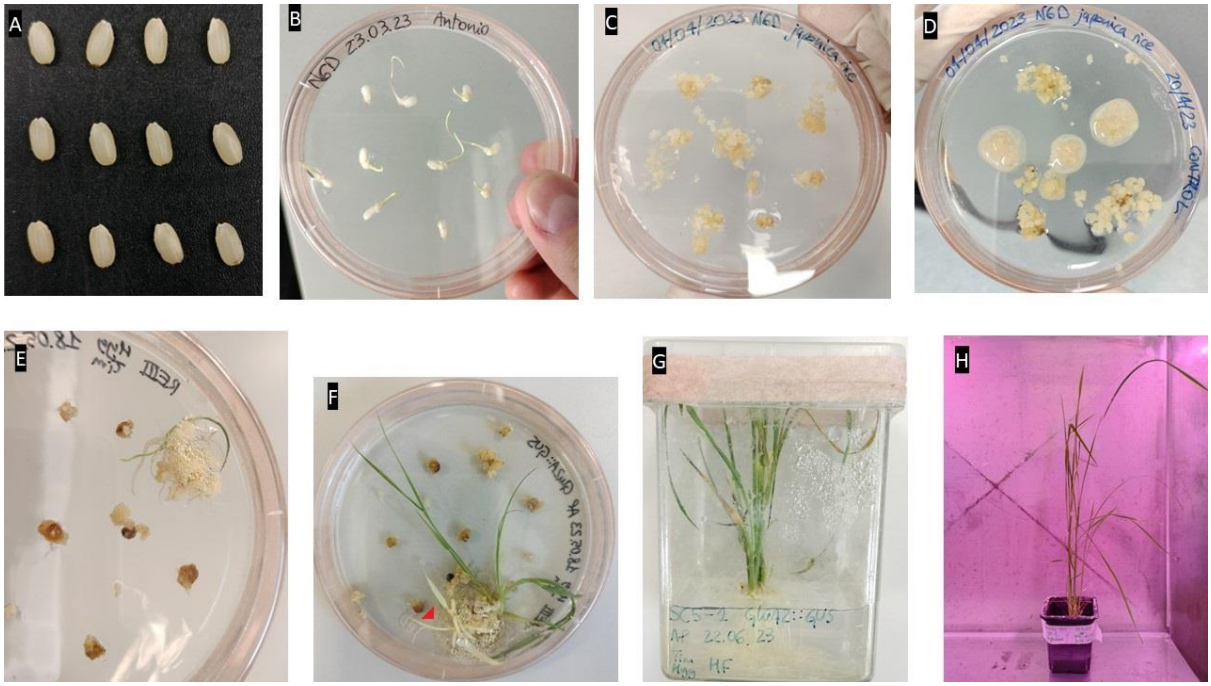

Figure 3.1. The various phases of the generation of a transgenic plant. A: Surface seed sterilisation; **B**: Germination; **C**: Callus culture; **D**: Co-cultivation with *Agrobacterium* containing the GUS vector of interest; **E**: Callus selection with antibiotics; **F**: Regeneration of hygromycin-resistant shoots; **G**: Root induction; **H**: Plantlet transfer to soil. The red arrow in **F** indicates an escape.

After selection with hygromycin, these plantlets were moved to soil. Unfortunately but inevitably, some plantlets could not be characterised as they did not survive long enough after this step.

3.2 Characterisation of transgenic plants

This study produced at least 27 independent *jGluA2*p*::GUS* transformation events and at least 10 independent *jGlb-1*p*::GUS* transformation events in *japonica* rice, with 34

*jGluA2*p*::GUS japonica* rice plants, and 11 *jGlb-1*p*::GUS japonica* confirmed transgenics generated.

3.2.1 PCR optimisation

The specificity of primer annealing was improved by performing a gradient PCR. The same samples were run at different annealing temperatures to assess which temperature would provide the clearest bands on gel. As can be seen in Fig. 3.2 below, an annealing temperature of 56.1°C and above showed clearer bands compared to the other temperatures investigated. Based on this, all subsequent PCR reactions were run at 59°C to generate clearer bands.

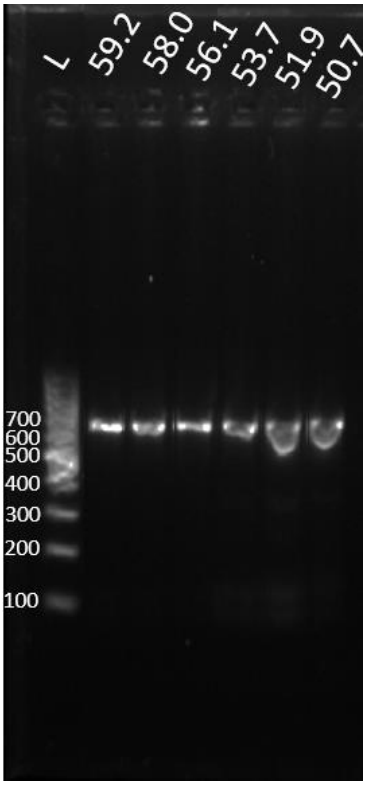

Figure 3.2. Gradient PCR for optimisation of GUS products. The same samples (*jGluA2*p*::GUS indica*) were run at different annealing temperatures between 50.7°C and 59.2°C (50.7°C, 51.9°C, 53.7°C, 56.1°C, 58.0°C, 59.2°C) to assess the temperature that would provide the clearest bands on the gel. L: Gene Ruler 100 bp ladder (Thermo Scientific). DNA length shown in bp on the left-hand side. Expected PCR product size: 629 bp.

3.2.2 Gel electrophoresis of PCR products

The negative control for the PCR reaction was a no-template control (NTC). An additional negative control (NC) was genomic DNA from a WT *japonica* rice plant. The positive control (PC) was *jGlu2::GUS* plasmid DNA.

The NTC showed no bands on the gel, confirming that the PCR reaction was working. In the WT plant genomic DNA used as NC, no PCR amplification was recorded, in line with expectations. GUS fragments were amplified from *jGlu2::GUS* plasmid DNA, confirming that the PC was in order. The PCR products of the putative transgenics visualised on the agarose gel were of the expected size (629 bp), confirming the successful integration of the GUS gene into the plants as the primer pair targeted the fragment of the *uidA* region assumedly present in all transgenics. Only one sample (15-1) did not display a band on the gel and was characterised as negative. A representative example of a gel can be found in Fig. 3.3 below, where the putative rice plants were transformed with the *jGluA2*p*::GUS* plasmid.

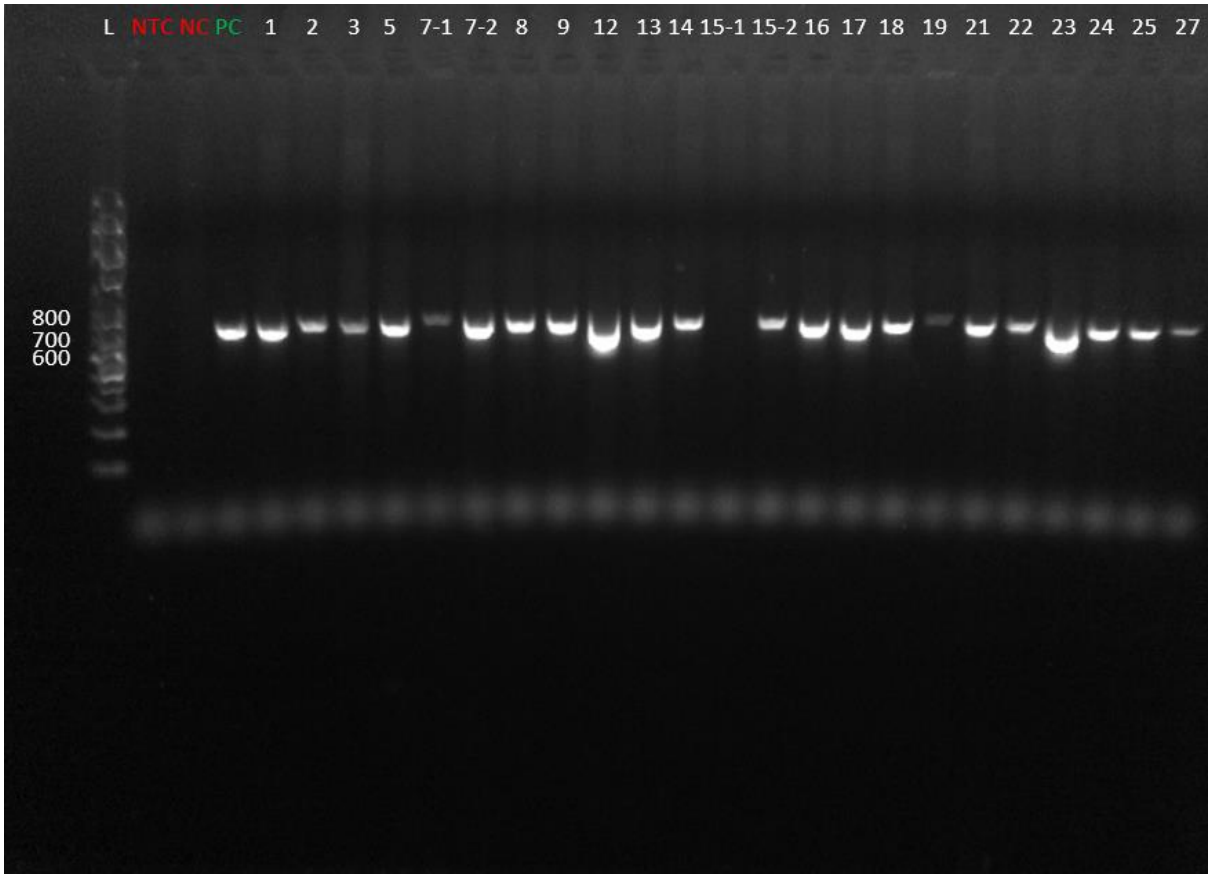

Figure 3.3. Example of a gel for transgenic plant characterisation. An agarose gel confirming the presence of the GUS gene in the transgenic *jGluA2*p*::GUS japonica* line. The amplified DNA fragments in all lanes except one (15-1) are of the expected size (629 bp) and match that of the positive control (PC). No-template control (NTC) and negative control (NC) show no bands. L: Gene Ruler 100 bp ladder (Thermo Scientific); NTC: no-template control; NC: negative control (WT *japonica*); PC: positive control (*jGluA2*p*::GUS* plasmid); lanes 5-14: putative *jGluA2*p*::GUS japonica* transgenics. Sample identification numbers shown at the top. Fragment size shown in bp on the left-hand side.

A summary indicating the number of confirmed transgenic plants and of the potential and confirmed independent transformation event can be found in Table 3.1. Unfortunately, not

all putative transgenics could be characterised as a number of them did not survive long enough for any samples to be taken for analysis.

Table 3.1. Summary table of end-point PCR results for the characterisation of the novel transgenic lines.

Line	No. of putative transgenics moved to soil after selection	No. of potential independent transformation events	No. of confirmed transgenics via end-point PCR	No. of confirmed independent transformation events
*jGluA2*p*::GUS japonica*	41	30	34 (85%)	27 (90%)
*iGlb-1*p*::GUS japonica*	15	13	11 (73%)	10 (66%)
Total	56	43	45 (80%)	37 (86%)

3.3 Histochemical analysis of GUS-stained tissue

The controls for the GUS staining solution produced the expected results, with the NC (null *jAct*p*::GUS japonica* and null *jGluA2*p*::GUS indica* seeds) showing no staining, while the PC (*jAct*p*::GUS japonica* seeds and *jGluA2*p*::GUS indica* seeds) produced staining visible to the naked eye (Fig. 3.4 and 3.7 A). GUS staining was not performed on WT *indica* seeds, as it was not expected to occur. Null *jGluA2*p*::GUS indica* seeds were exposed to the GUS staining solution and did not stain, as expected.

The NC (WT *indica* rice) for GUS staining worked as intended and showed no signs of staining in leaf, crown, or root tissue (Fig. 3.4). The PC (*jAct*p*::*GUS *japonica* rice) also worked

as intended and showed staining in all the tissues, including seed, leaf, crown, and root (Fig. 3.5).

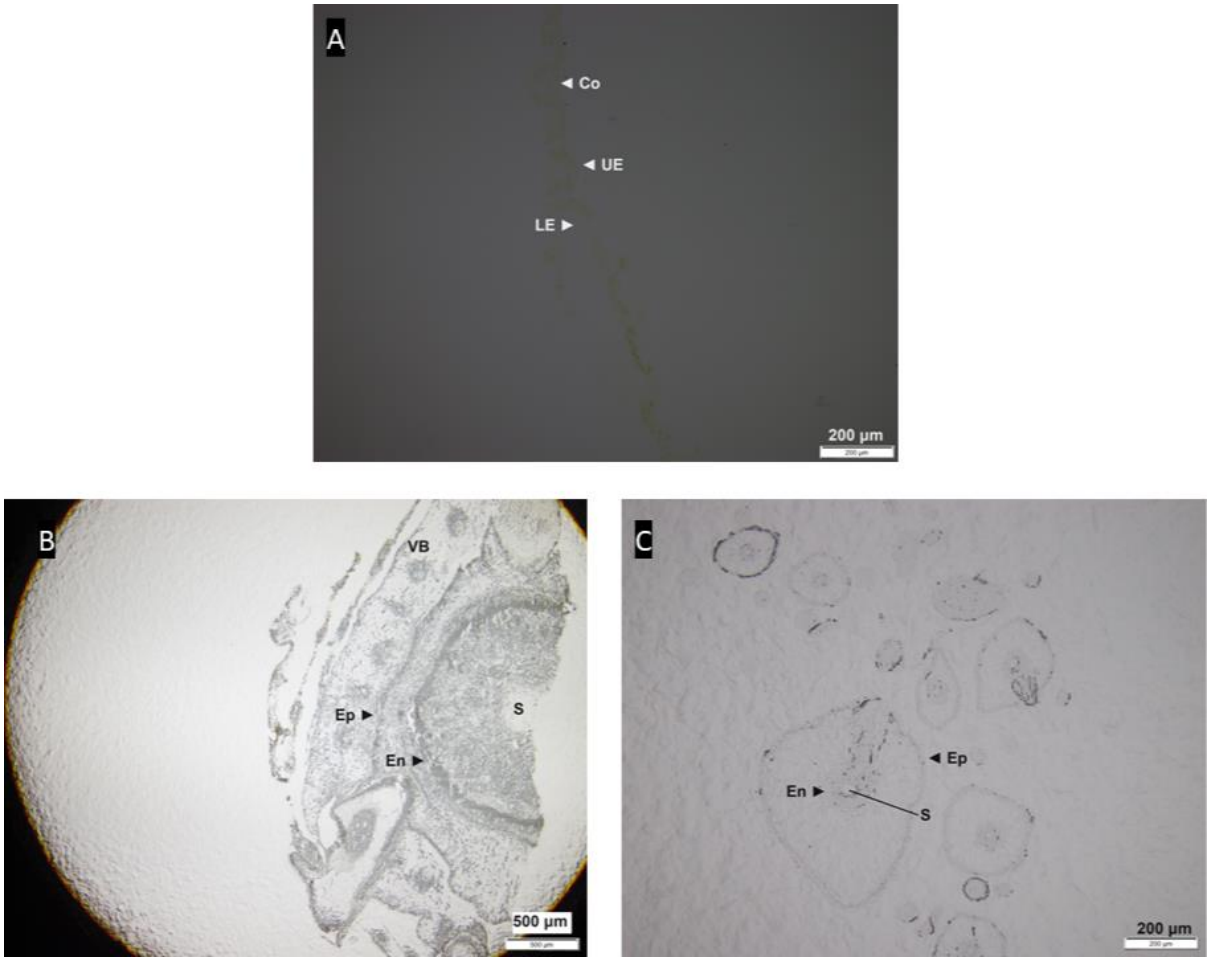

Figure 3.4. GUS staining in WT indica (NC). Cross sections of WT *indica* (NC) tissues observed under a brightfield microscope upon staining with the GUS staining solution. None of tissues exhibits any staining. **A**: leaf; **B**: crown; **C**: root. **Co**: collenchyma; **UE**: Upper epidermis; **LE**: lower epidermis; **VB**: vascular bundle; **Ep**: epidermis; **S**: stele; **En**: endodermis. *n*=1.

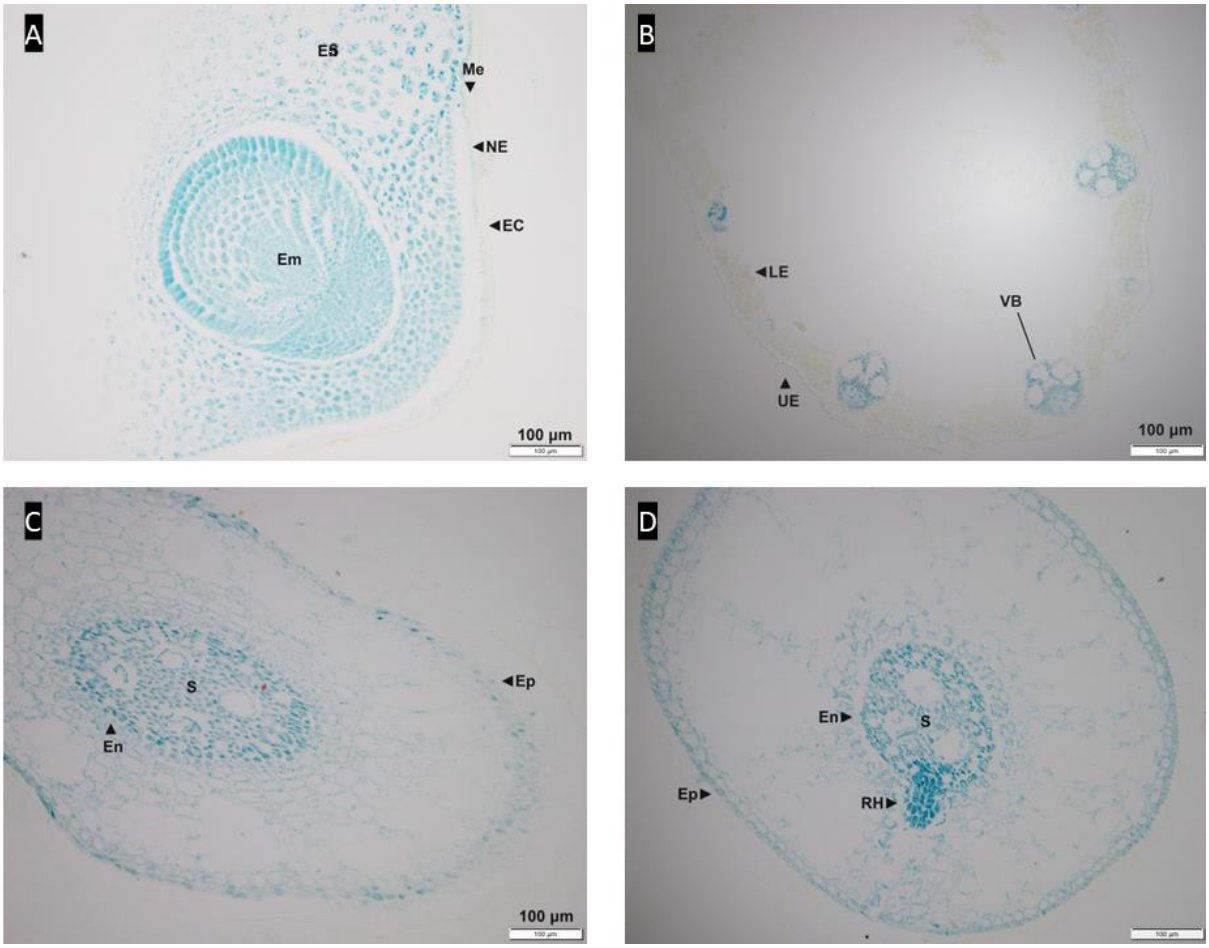

Figure 3.5. GUS staining in *jAct*p*::GUS japonica* rice (PC). Cross sections of *jAct*p*::GUS japonica* (PC) tissues observed under a brightfield microscope upon staining with the GUS staining solution. All tissues exhibit GUS staining. **A**: seed; **B**: leaf; **C**: crown; **D**: root. **ES**: endosperm; **Me**: mesocarp; **NE**: nucellus epidermis; **EC**: epicarp; **Em**: embryo; **LE**: lower epidermis; **VB**: vascular bundle; **UE**: upper epidermis; **S**: stele; **Ep**: epidermis; **En**: endodermis; **RH**: root hair. *n*=1.

Except for the PCs and *jGlb-1*p*::GUS japonica*, which showed GUS staining in the rice grain (Figs. 3.5, 3.6 A, 3.7 A), no other tissue from the novel or established transgenic lines produced any GUS staining detectable to the naked eye (Figs. 3.6 B-D, 3.7 B-D, 3.8, 3.9). One

sample per construct was examined under a brightfield microscope at increasing magnification, confirming the lack of staining. A summary of the results can be found in Table 3.2.

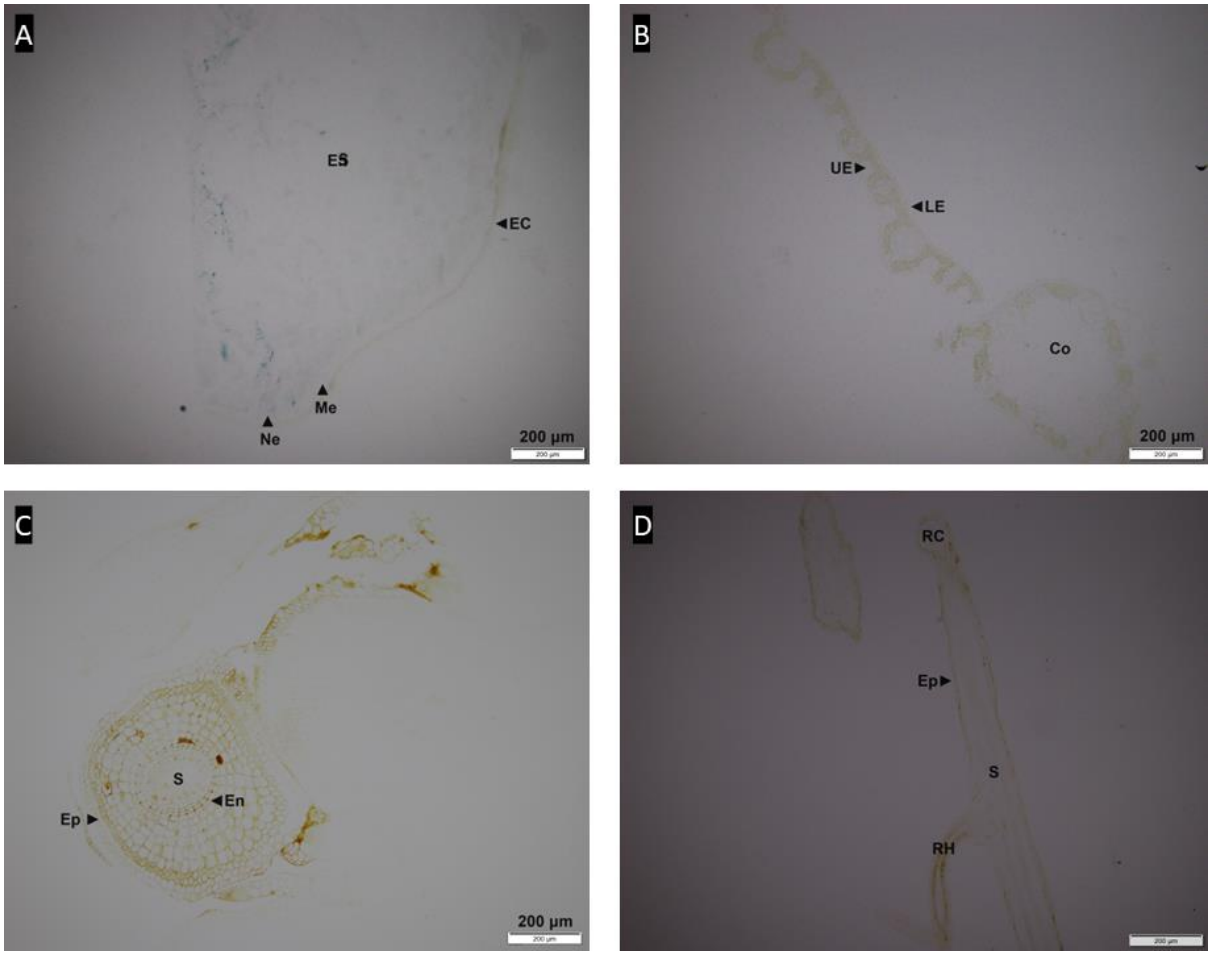

Figure 3.6. GUS staining in *jGlb-1*p*::GUS japonica*. Longitudinal sections (**A, D**) and cross sections (**B, C**) of *jGlb-1*p*::GUS japonica* tissues observed under a brightfield microscope upon staining with the GUS staining solution. Only the seed (**A**) shows staining. **A**: seed; **B**: leaf; **C**: crown; **D**: root. **ES**: endosperm; **EC**: epicarp; **Me**: mesocarp; **Ne**: nucellus epidermis; **UE**: upper epidermis; **LE**: lower epidermis; **Co**: collenchyma; **S**: stele; **En**: endodermis; **Ep**: epidermis; **RC**: root cap; **RH**: root hair. *n*=1.

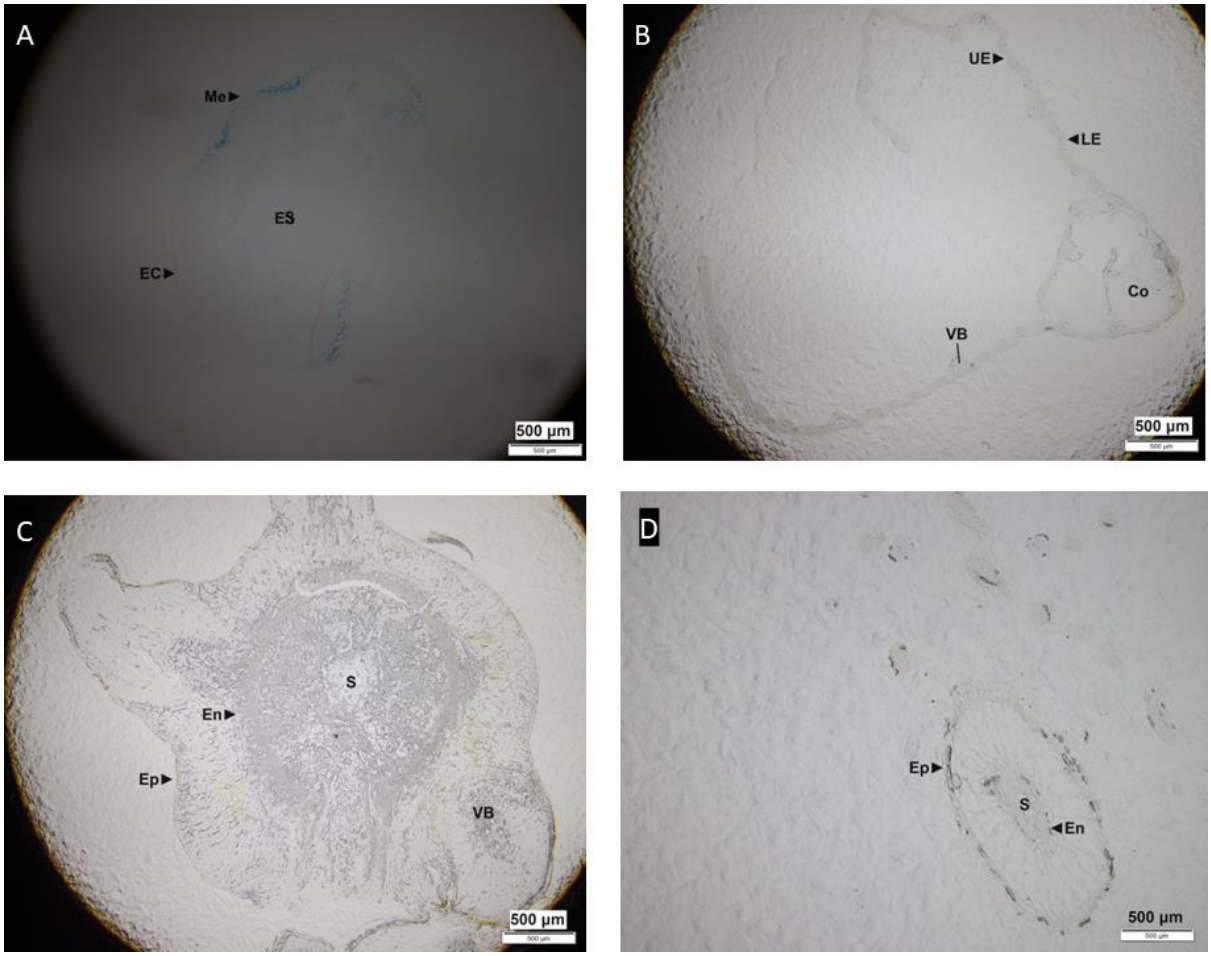

Figure 3.7. GUS staining in *jGluA2*p*::GUS indica*. Longitudinal sections (**A**) and cross sections (**B-D**) of *jGluA2*p*::GUS indica* tissues observed under a brightfield microscope upon staining with the GUS staining solution. Only the seed (**A**) shows staining. **A**: seed; **B**: leaf; **C**: crown; **D**: root. **Me**: mesocarp; **ES**: endosperm; **EC**: epicarp; **UE**: upper epidermis; **LE**: lower epidermis; **Co**: collenchyma; **VB**: vascular bundle; **S**: stele; **En**: endodermis; **Ep**: epidermis. *n*=1.

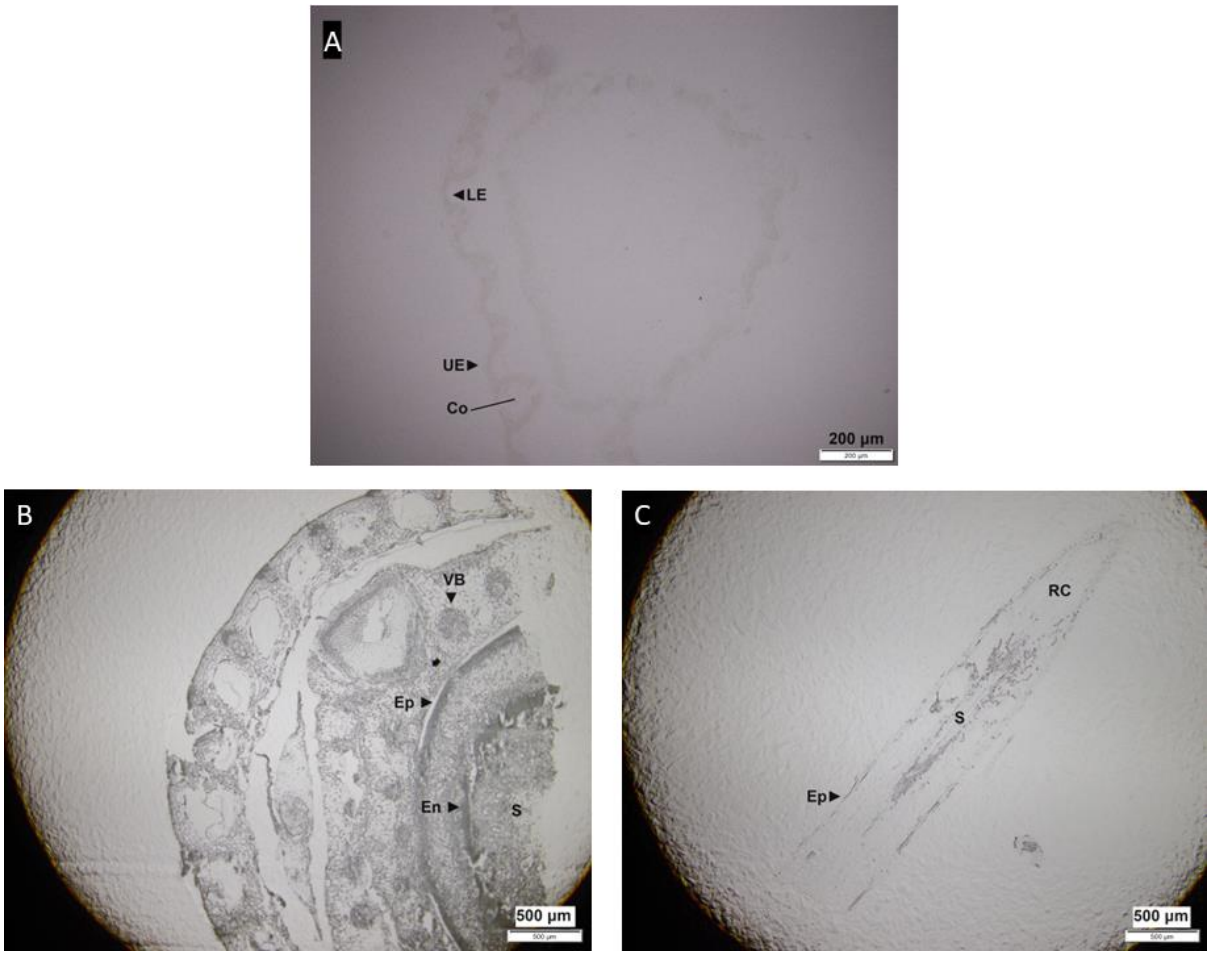

Figure 3.8. GUS staining in *iGlb-1*p*::GUS japonica*. Cross sections (**A, B**) and longitudinal sections (**C**) of *iGlb-1*p*::GUS japonica* tissues observed under a brightfield microscope upon staining with the GUS staining solution. No staining was detected in any of the tissues. **A**: leaf; **B**: crown; **C**: root. **LE**: lower epidermis; **UE**: upper epidermis; **Co**: collenchyma; **VB**: vascular bundle; **Ep**: epidermis; **En**: endodermis; **S**: stele; **RC**: root cap. *n*=1.

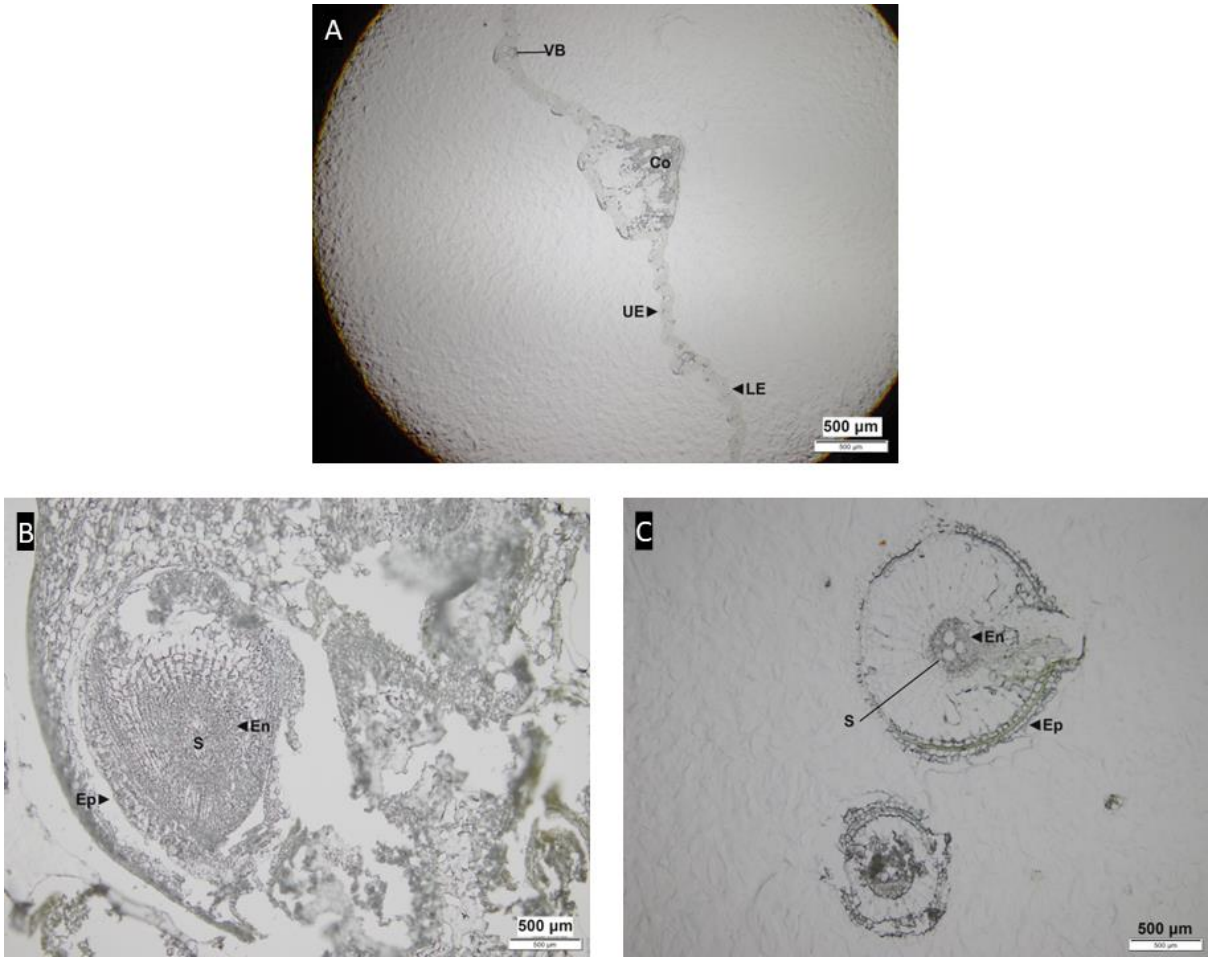

Figure 3.9. GUS staining in *jGluA2*p*::GUS japonica*. Cross sections of *jGluA2*p*::GUS japonica* tissues observed under a brightfield microscope upon staining with the GUS staining solution. No staining was detected in any of the tissues. **A**: leaf; **B**: crown; **C**: root. **VB**: vascular bundle; **Co**: collenchyma; **UE**: upper epidermis; **LE**: lower epidermis; **En**: endodermis; **S**: stele; **Ep**: epidermis. *n*=1.

Table 3.2. GUS-stained tissues in each rice line. Negative control (WT *indica* rice) highlighted in red; positive control (*jAct*p*::GUS japonica* rice) highlighted in green. ×: negative to GUS staining; ✓: positive to GUS staining. ×*: staining was not performed on WT *indica* seeds, null *jGluA2*p*::GUS indica* seeds were utilised instead. N/A: the novel transgenic *iGlb-1*p*::GUS japonica* and *jGluA2*p*::GUS japonica* lines had not set seeds at the time of the experiment. Staining in seed tissue was expected from all lines except for the NC. *n*=1 per tissue for each line, except for seeds (*n*=10).

Donor	Promoter driving GUS	Host	GUS-stained tissue			
			Seed	Leaf	Crown	Root
/	/	***indica***	×*	×	×	×
japonica	***Act*p**	***japonica***	✓	✓	✓	✓
indica	***Glb-1*p**	***japonica***	N/A	×	×	×
japonica	***Glb-1*p**	***japonica***	✓	×	×	×
japonica	***GluA2*p**	***japonica***	N/A	×	×	×
japonica	***GluA2*p**	***indica***	✓	×	×	×

*jAct*p*::GUS japonica* produced the most vivid staining throughout the rice grain, while *jGlb-1*p*::GUS japonica* and *jGluA2*p*::GUS indica* generated fainter staining in fewer areas, mostly in the outer endosperm (Fig. 3.6 A and 3.7 A).

No significant differences in staining were observed between the *indica Glb-1* promoter and the *japonica Glb-1* promoter when expressed in a *japonica* host in the tissues examined.

There were no significant differences in the tissues observed when the *japonica GluA2* promoter was expressed in *indica* compared to when it was expressed in *japonica*.

Chapter 4: Discussion

4.1 Summary of main results

4.1.1 Generation of transgenic plants

The generation of two new transgenic lines, *jGluA2*p*::GUS japonica* rice and *iGlb-1*p*::GUS japonica* rice, was accomplished in this project. After selection with hygromycin, a total of 41 *jGluA2*p*::GUS japonica* plantlets and 15 *iGlb-1*p*::GUS japonica* plantlets were transferred to soil, with a total number of potential independent transformation events of 30 and 13 respectively.

4.1.2 Characterisation of transgenic plants

The generation of the novel transgenic lines was confirmed via end-point PCR followed by gel electrophoresis, with 34 *jGluA2*p*::GUS japonica* plants (85% of those transferred to soil after selection) and 10 *iGlb-1*p*::GUS japonica* plants (90%) surviving long enough to be characterised. The number of independent transformation events confirmed was 27 and 10 respectively for each line.

Except for one escape that was attached to a confirmed transgenic, all the plants transferred to soil that were characterised were confirmed to contain the *uidA* gene.

As for the established transgenic lines, segregating nulls were "weeded out" by performing the same procedure described above.

4.1.3 GUS staining

Although the controls for both the GUS staining solution and GUS staining worked as anticipated, no ectopic GUS staining was detected in any of the novel or established lines.

Only seed tissue from the established lines *jGluA2*p*::GUS indica* and *jGlb-1*p*::GUS japonica* exhibited staining, which was expected.

Novel lines had not yet set seed so staining could not be performed on the rice grains, but it was expected to be positive to staining, in line with literature and previous work.

4.2 Discussion

4.2.1 Generation of novel transgenic lines

The generation of two new transgenic lines, *jGluA2*p*::GUS japonica* rice and *iGlb-1*p*::GUS japonica* rice, was successful.

Out of the several plantlets generated, only one was found to be an escape. As can be seen in Fig. 3.3, the absence of a band in lane 15-1 might indicate that 15-1 is an escape, since a band is showing in lane 15-2. Plantlet 15-1 was attached to a transgenic plantlet that regenerated from the same piece of callus, and as such could have survived the selection with hygromycin. The two were separated when the plantlets were moved to soil, but at the same time antibiotic selection was also stopped, allowing the escape to grow on its own.

Two more escapes were detected prior to the transfer of the plantlets to soil thanks to their bleached appearance (refer to the red arrow in Fig. 3.1 F), and as such were cut off from and disposed of.

As reported by Tran (2021), such a small number of escapes can be explained by the use of hygromycin in the rooting medium compared to previous studies that did not include this selection agent (compare Hiei and Komari (2008)).

4.2.2 Characterisation of transgenic lines

In this study, 45 transgenic *japonica* rice plants, confirmed to contain the *uidA* gene by end-point PCR as shown on electrophoresis gels, were obtained out of a potential total of 56 that survived selection with hygromycin. The PCR products were of the expected size and matched those of the positive control. A number of plantlets did not survive in the soil and could not be characterised.

No substantial differences were noted when promoters derived from either subspecies were expressed in a *japonica* host (i.e. *iGlb-1*p*::GUS japonica* vs *jGlb-1*p*::GUS japonica*), nor when promoters from the same subspecies were expressed in two different hosts (i.e. *jGluA2*p*::GUS indica* vs *jGluA2*p*::GUS japonica*). However, considering that the novel lines had not yet set seed, it will be essential to assess whether there are any differences in GUS staining patterns in their seeds compared to those from the established lines.

4.2.3 GUS staining

The positive controls for the GUS staining solution (*jAct*p*::GUS japonica* seeds) worked as expected. The positive control for GUS staining (*jAct*p*::GUS japonica*) worked as expected showing GUS expression in every tissue examined, i.e. seed, leaf, crown, and root. The negative control (WT *indica* rice) showed no signs of GUS expression in the tissues examined (seed, leaf, crown, root), as expected.

*jGlb-1*p*::GUS japonica* seeds were positive to GUS staining, which was expected based on the positive to GUS staining in Qu *et al.* (2008) and in Huynh (2014). *jGluA2*p*::GUS indica* seeds also showed staining, which reflects the results in Tran (2021).

*jAct*p*::GUS japonica* seed tissue exhibited the most pronounced GUS staining, whereas the stain in *jGlb-1*p*::GUS japonica* and *jGluA2*p*::GUS indica* seeds was weaker, mostly

confined to the outer endosperm. This is reflective of the constitutive nature of the *Act* promoter (Grunennvaldt *et al.*, 2015), and of the usual expression patterns of the *Glb-1* and *GluA2* promoters (Hwang *et al.*, 2002; Qu *et al.*, 2008; Montesinos *et al.*, 2017). None of the other lines, novel or established, showed any staining in any other tissue.

The results obtained show no sign of GUS staining in tissues where it is not expected, i.e. leaves, crown, or roots, in lines *iGlb-1*p*::GUS japonica*, *jGlb-1*p*::GUS japonica*, *jGluA2*p*::GUS japonica,* and *jGluA2*p*::GUS indica*. Therefore, it can be said that there is no evidence, at this stage, of leaky expression of the *Glb-1* or *GluA2* promoter in these conditions.

Given the novel transgenic plants (*iGlb-1*p*::GUS japonica* and *jGluA2*p*::GUS japonica*) did not yet set seed, it was not possible to examine the GUS expression in the grains in those lines. If seeds from these lines do not exhibit staining, it might indicate that some issues occurred during the transformation procedure, such as the construct not having fully been integrated. For example, the promoter and GUS gene construct might have not been integrated along with the antibiotic resistance genes, which would explain the effectiveness of the selection process and the lack of GUS staining. Another possibility is that the promoter might not have been integrated, which could suppress GUS expression, or the GUS gene might have been silenced by other mechanisms. However, given the large number of transformants, this is not considered likely, and GUS staining in these grains is expected to be consistent with prior results and be observed in the endosperm tissue. Therefore, any staining in the seeds from the novel lines would provide further evidence that no ectopic expression of the promoters occurs.

It might be that the sections taken were too thick for the GUS staining solution to penetrate. In this case, it would be beneficial to restain these sections after sectioning to facilitate the infiltration of the staining solution. However, by looking at the positive control, it

appears that the sections were thin enough for the solution to penetrate and stain every tissue (Fig. 3.5), so this is not a likely explanation.

It is possible that the material was collected at a developmental stage where the expression of GUS had faded or had not yet occurred. However, samples were taken at different developmental stages and therefore this scenario appears less likely.

Another possibility is that ectopic expression might have happened elsewhere in other tissues or parts of tissue were not sampled. While examining *jAct*p*::GUS japonica*, older root tissue showed no staining, while rapidly dividing tissue closer to the crown stained well (data not shown). As a constitutive promoter, *Act*p expression is widespread (Grunennvaldt *et al.*, 2015; Koetle *et al.*, 2017), yet its levels in older tissue were still too low to be visible to the naked eye. It is therefore likely that any minimal ectopic expression of *Glb-1*p or *GluA2*p would have gone unnoticed in the older tissue that was sampled.

Another possible explanation for the results of this study is that the GUS expression was minimal in these non-endosperm tissues, and thus too low to be detected visually. This would fit the known expression patterns of *Glb-1* and *GluA2*, which are mostly expressed in the rice grain (Kawakatsu and Takaiwa, 2019).

Minimal *Glb-1* ectopic expression had been noted by Wu *et al.* (1998), and being three orders of magnitude lower might have not been preserved throughout the processing of the sample. In addition, in Wu *et al.* (1998), only a small number of transgenic lines containing a *Glb-1*p-driven GUS construct exhibited staining, which might be the case in this study too. As not all the lines could be investigated, any minimal staining might have been overlooked, since out of the many transgenics generated only one biological replicate per line was assessed.

When it comes to minimal transgene expression, the GUS reporter system has its limitations. Wu *et al.* (1998) detected minimal GUS activity thanks to a sensitive fluorometric

assay rather than a simple colorimetric assay as in this study. As mentioned, since it was not known where exactly ectopic expression might occur, any minimal GUS staining might have been missed due to lower sensitivity. Moreover, the GUS system requires the addition of a staining solution that is toxic to cells, therefore this process cannot be performed *in vivo* (Villao-Uzho *et al.*, 2023). Moreover, a long, multi-step procedure with harsh chemicals is needed to prepare the samples before they can be examined, and this might alter any minimal staining in the tissue to the extent that it could be lost in the process.

It might therefore be useful to consider a more sensitive system that is not destructive, such as green fluorescent protein (GFP). This system can be used *in vivo* and enables whole tissue, or even whole plant, observation, and would allow for the detection of minimal ectopic activity. That being said, using GFP would require the generation of novel constructs and their transformation, which was simply not feasible within the allotted time frame of this project. The availability of established vectors and lines is in fact what allowed this project to materialise.

It would also be interesting to assess GUS staining in the seeds of the novel transgenic lines and in the subsequent T1 generation, a higher copy number of transgenes could increase any minimal GUS expression.

Provided the lack of staining reflects a lack of expression of *Glb-1*p or *GluA2*p in these tissues, other mechanisms might be at play that lead to a more vigorous phenotype in the SC1 and DC1 transgenics generated by Tran (2021).

When investigating the lines generated by Tran, Simonsen (2023) suggested that positional effects might be one of the causes of ‘leaky’ expression of the constructs, and emphasised the role that the genomic landscape, in particular enhancers and permissive domains, might have on a transgene promoter. However, this possibility appears unlikely given

that Tran had used several biological replicates and calculated their average when analysing the vigorous phenotypes. In general, DC1 plants consistently exhibited a more vigorous phenotype, which would point to the effects of the construct itself, rather than the genomic landscape it was integrated into.

Another study reported that SSPs can show transient expression in leaves and stems before the formation of the grain (Scofield *et al.*, 2009). While this study was performed on wheat, it is possible that it could apply to rice as well, as shown in Koller *et al.* (2002), where a proteomic survey detected five different glutelins expressed in rice leaves. Although the protein levels were not reported, and can be assumed to be low, this might indicate that *GluA2*p is 'leakier' than *Glb-1*p, since no globulin was detected in the leaves. This would reflect the differences between glutelin promoters and the *Glb-1* promoter due to their different CREs (Fig. 1.5), and thus their different strength as seed-specific promoters. In fact, the differences in their CREs are the reason they were chosen by Tran (2021) to prevent gene silencing by homology, and it was expected that the two would generate different expression patterns, which could be observed in the different SC1 and SC2 phenotypes. Albeit at a background level, ectopic *GluA2*p expression might be physiological. This however seems unlikely, as a simple colorimetric assay should be sensitive enough to detect expression levels that are high enough to drive *OsNAS2* and cause the significant effects seen in SC2's stunted phenotype.

It is also possible that the off-target expression of the constructs was due to a motif present in *HvSUT1* and *OsNAS2* themselves, rather than their promoters, or to a combination of both promoter and transgene, but this could not have been observed in this study. If that was the case, it might be beneficial to re-assess the original constructs via other methods, for example via RT-qPCR (real-time quantitative PCR).

Chapter 5: Conclusion and future work

This study originally set out to analyse the expression of the *GluA2* and *Glb-1* promoters in different tissues at different times of development.

As for the spatial investigation, the results gathered do not support the hypothesis that the *Glb-1*p and the *GluA2*p are expressed ectopically, in the tissues and at the developmental times examined. Regarding the temporal investigation of ectopic expression, unfortunately it was not possible to assess expression throughout development, therefore future studies might look into the timing of the expression to assess whether any staining occurs at developmental moments other than those examined in this study. This could provide more information on ectopic promoter expression, e.g. the developmental time at which it starts or stops, compared to the results in Tran (2021).

One of the aims of the project, i.e. the generation of two new transgenic lines, *iGlb-1*p*::GUS japonica* and *jGluA2*p*::GUS japonica*, was however successful. The seeds produced by these novel transgenic lines can be an addition to the tools available for future investigations into rice promoters, as the constructs would get fixed in the T1 generation. While staining is expected to occur in the seeds produced by the novel lines, a lack thereof would point to a faulty construct or an improper transformation process.

In summary, as the results of the GUS staining were obtained from limited samples, no final conclusions can be made at this time.

A more extensive investigation on rice SSP promoters will hopefully continue when the new lines generated in this study set seed and the subsequent T1 generations are assessed for ectopic GUS staining at different time points during development, in combination with other methods such as RT-qPCR and fluorometric assays.

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Appendices

Appendix A – DNA Sequences

ATGTTACGTCCTGTAGAAACCCCAACCCGTGAAATCAAAAAACTCGACGGCCTGTGGGCATTCAGTCTGGATCGC
GAAAACTGTGGAATTGATCAGCGTTGGTGGGAAAGCGCGTTACAAGAAAGCCGGGCAATTGCTGTGCCAGGCAGT
TTTAACGATCAGTTCGCCGATGCAGATATTCGTAATTATGCGGGCAACGTCTGGTATCAGCGCGAAGTCTTTATA
CCGAAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGTTTCGATGCGGTCACTCATTACGGCAAAGTGTGGGTCAAT
AATCAGGAAGTGATGGAGCATCAGGGCGGCTATACGCCATTTGAAGCCGATGTCACGCCGTATGTTATTGCCGGG
AAAAGTGTACGTATCACCGTTTGTGTGAACAACGAACTGAACTGGCAGACTATCCCGCCGGGAATGGTGATTACC
GACGAAAACGGCAAGAAAAAGCAGTCTTACTTCCATGATTTCTTTAACTATGCCGGGATCCATCGCAGCGTAATG
CTCTACACCACGCCGAACACCTGGGTGGACGATATCACCGTGGTGACGCATGTCGCGCAAGACTGTAACCACGCG
TCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGTCAGCGTTGAACTGCGTGATGCGGATCAACAGGTGGTTGCA
ACTGGACAAGGCACTAGCGGGACTTTGCAAGTGGTGAATCCGCACCTCTGGCAACCGGGTGAAGGTTATCTCTAT
GAACTGTGCGTCACAGCCAAAAGCCAGACAGAGTGTGATATCTACCCGCTTCGCGTCGGCATCCGGTCAGTGGCA
GTGAAGGGCGAACAGTTCCTGATTAACCACAAACCGTTCTACTTTACTGGCTTTGGTCGTCATGAAGATGCGGAC
TTGCGTGGCAAAGGATTCGATAACGTGCTGATGGTGCACGACCACGCATTAATGGACTGGATTGGGGCCAACTCC
TACCGTACCTCGCATTACCCTTACGCTGAAGAGATGCTCGACTGGGCAGATGAACATGGCATCGTGGTGATTGAT
GAAACTGCTGCTGTCGGCTTTAACCTCTCTTTAGGCATTGGTTTCGAAGCGGGCAACAAGCCGAAAGAACTGTAC
AGCGAAGAGGCAGTCAACGGGGAAACTCAGCAAGCGCACTTACAGGCGATTAAAGAGCTGATAGCGCGTGACAAA
AACCACCCAAGCGTGGTGATGTGGAGTATTGCCAACGAACCGGATACCCGTCCGCAAGGTGCACGGGAATATTTC
GCGCCACTGGCGGAAGCAACGCGTAAACTCGACCCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTGCGAC
GCTCACACCGATACCATCAGCGATCTCTTTGATGTGCTGTGCCTGAACCGTTATTACGGATGGTATGTCCAAAGC
GGCGATTTGGAAACGGCAGAGAAGGTACTGGAAAAAGAACTTCTGGCCTGGCAGGAGAAACTGCATCAGCCGATT
ATCATCACCGAATACGGCGTGGATACGTTAGCCGGGCTGCACTCAATGTACACCGACATGTGGAGTGAAGAGTAT
CAGTGTGCATGGCTGGATATGTATCACCGCGTCTTTGATCGCGTCAGCGCCGTCGTCGGTGAACAGGTATGGAAT
TTCGCCGATTTTGCGACCTCGCAAGGCATATTGCGCGTTGGCGGTAACAAGAAAGGGATCTTCACTCGCGACCGC
AAACCGAAGTCGGCGGCTTTTCTGCTGCAAAAACGCTGGACTGGCATGAACTTCGGTGAAAAACCGCAGCAGGGA
GGCAAACAATGA

Figure A.1. *uidA* gene sequence. Sequence of the *uidA* gene from *E. coli* str. K-12 substr. MG1655 (accession no.: NC_000913.3:c1696071-1694260). The forward primer and the reverse complement of the reverse primer are highlighted in blue.

Table A.1. GUS primers. Forward and reverse GUS primer sequences used in PCR reactions for the characterisation of novel and established transgenic lines and their melting temperatures (Tm).

	Sequence (5'→3')	**Tm**
GUS F	CAGCCAAAAGCCAGACAGA	49°C
GUS R	GGCACAGCACATCAAAGAGA	51°C

Appendix B – Media, stocks, and GUS staining solution

Hydroponics media

Table B.1. Composition of hydroponics media for rice growth. The four macronutrient solutions and one micronutrient solution described below were added to reverse-osmosis water in a 12 L hydroponics box.

Soln	Salts	MW (g/mol)	Mass (g) for stock	Vol (L) of water to dissolve for stock	Vol (ml) of soln to use per 12 L box	Stock concen-tration	Final concentration in 12 L box
Macronutrients						**(M)**	**(mM)**
1	**NH_4NO_3**	80.0	80.0	1	60	1	5.0
	KNO_3	101.1	101.1			1	5.0
2	**$Ca(NO_3)_2{\cdot}4H_20$**	236.1	94.44	1	60	0.4	2.0
3	**$MgSO_4{\cdot}7H_20$**	246.5	98.6	1	60	0.4	2.0
	KH_2PO_4	136.1	2.72			0.02	0.1
4	**NaFe(III)EDTA**	367.1	3.67	0.2	12	0.05	0.05
Micronutrients						**(mM)**	**(µM)**
5	**$ZnSO_4{\cdot}7H_20$**	287.5	0.575	0.2	12	10	10.0
	$MnCl_2{\cdot}4H_20$	197.9	0.198			5.0	5.0
	$CuSO_4{\cdot}7H_2O$	249.7	0.025			0.5	0.5
	$Na_2MoO_4{\cdot}2H_20$	242.0	0.005			0.1	0.1

Tissue culture and transformation media

N6D

- Chu's Basal Salt Mixture with vitamins — 3.99 g
- Casamino acid — 300 mg
- *Myo*-inositol — 100 mg
- L-proline — 2.88 g
- Milli-Q® water — 450 ml

Adjust pH to 5.8 with 1N KOH

- GelRite® — 4 g
- Milli-Q® water — To 750 ml

Autoclave

- Sucrose stock — 250 ml
- 2,4-D (2 mg/ml) — 1 ml

LB

- Yeast extract — 5 g
- Bacto-tryptone — 10 g
- NaCl — 10 g
- Milli-Q® water — To 1 L

Autoclave

YM

- Yeast extract 0.4 g
- D-mannitol 10 g
- K_2HPO_4 1 ml
- KH_2PO_4 4 ml
- NaCl 1 ml
- $MgSO_4.7H_2O$ 2 ml
- Milli-Q® water To 1 L

Adjust pH to 6.8

- Agar 15 g

Autoclave

2N6-AS

- Chu's Basal Salt Mixture with vitamins 2 g
- Casamino acid 150 mg
- *Myo*-inositol 50 mg
- Milli-Q® water

Adjust pH to 5.2 with 1 N KOH

- Milli-Q® water To 250 ml
- (GelRite® 2 g)

Autoclave

- Sucrose + Glucose stock 250 ml
- 2,4-D (2 mg/ml) 0.5 ml
- Acetosyringone (20 mg/ml) 0.5 ml

REIII

- MS Basal Media 4.43 g
- Casamino acid 2 g
- Milli-Q® water

Adjust pH to 5.8 with 1N KOH

- Milli-Q® water To 500 ml
- GelRite® 4 g

Autoclave

- Sucrose + Sorbitol stock 500 ml
- NAA (1 mg/ml) 50 µl
- Kinetin (2 mg/ml) 1.25 ml
- Hygromycin (50 ug/ml)
- Timentin (150 ug/ml)

HF

- MS Basal Media 4.43 g
- Milli-Q® water

Adjust pH to 5.8 with 1N KOH

- Milli-Q® water To 750 ml
- GelRite® 4 g

Autoclave

- Sucrose stock 250 ml
- Hygromycin (50 µg/ml)
- Timentin (150 µg/ml)

Sucrose stock

- Sucrose 30 g
- Milli-Q® water To 250 ml

Autoclave

Sucrose + Glucose stock

- Sucrose 15 g
- Glucose 5 g
- Milli-Q® water To 250 ml

Autoclave

Sucrose + Sorbitol stock

- Sucrose 30 g
- Sorbitol 30 g
- Milli-Q® water To 500 ml

Autoclave

Antibiotic stocks

- Rifampicin 20 mg/ml
- Spectinomycin 200 mg/ml
- Hygromycin 50 mg/ml
- Timentin 150 mg/ml

Phytohormone stocks

- 2,4-D 2 mg/ml
- Kinetin 2 mg/ml
- NAA 1 mg/ml
- Acetosyringone 20 mg/ml

2,4-D stock

• 2,4-D	20 mg
• 1 N KOH	Few drops
• Milli-Q® water	To 10 ml

Filter sterilise

1 ml aliquots

Store at -20°C

Kinetin stock

• Kinetin	20 mg
• 1 N KOH	Few drops
• Milli-Q® water	To 10 ml

Filter sterilise

1 ml aliquots

Store at -20°C

NAA stock

• NAA	10 mg
• 1 N KOH	Few drops
• Milli-Q® water	To 10 ml

Filter sterilise

1 ml aliquots

Store at -20°C

Acetosyringone stock

- Acetosyringone stock 200 mg
- DMSO To 10 ml

Filter sterilise

1 ml aliquots

Store at -20°C

GUS staining solution

Phosphate buffer

- 50 mM NaH_2PO_4
- 50 mM Na_2HPO_4

GUS staining solution

- 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, sodium salt)
- 1 mg/ml DMSO
- 1 mM potassium ferricyanide
- 1 mM potassium ferrocyanide
- 0.05% (w/v) Triton X-100 at pH 7.0
- Phosphate buffer