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ORIGINAL ARTICLE

Amino acids induce high seed-specific expression driven by a soybean (*Glycine max*) glycinin seed storage protein promoter

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Abstract

Key message **We characterize GFP expression driven by a soybean glycinin promoter in transgenic soybean. We demonstrate specific amino acid-mediated induction of this promoter in developing soybean seeds in vitro.**

Abstract In plants, gene expression is primarily regulated by promoter regions which are located upstream of gene coding sequences. Promoters allow transcription in certain tissues and respond to environmental stimuli as well as other inductive phenomena. In soybean, seed storage proteins (SSPs) accumulate during seed development and account for most of the monetary and nutritional value of this crop. To better study the regulatory functions of a SSP promoter, we developed a cotyledon culture system where media and media addenda were evaluated for their effects on cotyledon development and promoter activity. Stably transformed soybean events containing a glycinin SSP promoter regulating the green fluorescent protein (GFP) were generated. Promoter activity, as visualized by GFP expression, was only observed in developing *in planta* seeds and in vitro-cultured isolated embryos and cotyledons from developing seeds when specific media addenda were included. Asparagine, proline, and especially glutamine induced glycinin promoter activity in cultured cotyledons from developing seeds. Other amino acids did not induce the glycinin promoter. Here, we report, for the first time, induction of a reintroduced glycinin SSP promoter by specific amino acids in cotyledon tissues during seed development.

Keywords Glycinin · Promoter · GFP · Amino acids · Soybean

Introduction

In soybean (*Glycine max*), the glycinin (11 s) and β-conglycinin (7 s) seed storage protein (SSP) families constitute the majority of seed protein content, the most abundant being glycinin at 33% of total seed protein (Krishnan 2001; Schmidt et al. 2011). The glycinin family is composed of five closely related genes, along with two pseudogenes. Glycinin proteins are cleaved post-translationally to form oligomers of acidic and basic subunits connected by a disulfide bond, and accumulate as protein bodies in cotyledon tissues during embryo development (Nielsen et al. 1989; Staswick et al. 1984). Both glycinin and β-conglycinin are the main nutritive sources of protein in soybean and are present in increased levels in high-protein soybean lines (Yaklich 2001). Expression of both major SSP families is highly regulated throughout seed development (Goldberg et al. 1981).

Gene expression in soybean seeds has been studied using RNA-seq, microarray analysis and RT-qPCR. These tools have provided an extensive framework of gene expression data to increase our understanding of transcriptional regulation in a variety of soybean tissues. However, these methods of measuring gene expression require extraction of RNA from plant tissues and continual monitoring is therefore not possible. In addition, detection of tissue-specific expression is difficult, as analyses are typically performed using extractions from whole tissues or organs. In order to continually monitor promoter-regulated gene expression in soybean, isolated promoters from genes of interest have been

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fused to *GFP* and introduced into soybean using transgenic approaches (De La Torre and Finer 2015; Gunadi et al. 2016; Hernandez-Garcia et al. 2010; Zhang et al. 2015). Unfortunately, very few soybean promoters have been analyzed using this approach (Hernandez-Garcia and Finer 2014), and there have been few efforts to evaluate soybean embryo-specific promoters in transgenic soybean following introduction of a promoter:*gfp* fusion (Zhang and Finer 2015). Transgenic approaches have been used for characterization of constitutive (Zhang et al. 2015) and wound-inducible (Hernandez-Garcia and Finer 2016) soybean promoters in soybean but, most often, soybean promoters are evaluated in heterologous systems, as transformation of soybean remains consistent but inefficient (Finer 2016).

For studies of soybean seed-specific promoters, the β subunit of the β-conglycinin promoter was fused to the GUS coding region and introduced into *Arabidopsis* (Hirai et al 1995). Heterologous systems have also been used to study promoter variants and specific elements within soybean promoters. Two legumin box elements within the legumin α' subunit promoter from soybean were shown to be responsible for expression strength in mature *Nicotiana* seeds, and isolated CCAAT elements could drive low-level expression specific in tobacco seed tissues (Chamberland et al 1992). An isolated soybean *GH3* promoter was characterized in *Nicotiana* using β-glucuronidase (GUS), where it was inducible upon auxin application up to 50-fold, with otherwise low expression in roots and floral tissues (Hagen et al. 1991). A soybean SRS1 promoter, also evaluated in *Nicotiana* with the luciferase reporter, regulated expression in both a tissue-specific and light-inducible manner (Quandt et al. 1992). These heterologous systems are useful for rapid analysis of promoters and promoter variants when stable transformation of source species may not be readily available.

Although use of heterologous systems for promoter analysis has proven useful, homologous environments are preferred as they contain the full native components needed for promoter activity. Due to inefficiencies in soybean transformation (Widholm et al. 2010), few studies have been done on native promoter activity in transgenic soybean plants, even though this approach provides the most accurate method for characterization of promoter activity (Hernandez-Garcia et al. 2009; Zhang et al. 2015). Within these studies, stable transformation in soybean tissues has been employed to further characterize isolated promoter regions, specific promoter elements cloned in tandem upstream of core promoters, and the effects of leader introns on gene expression (Grant et al. 2017; Hernandez-Garcia and Finer 2016; Zhang et al. 2016). Soybean glycinin promoters have been utilized to drive seed-specific expression of synthetic transcription factors within a chemically inducible system (Semenyuk et al. 2010), as well as to demonstrate proteome rebalancing upon the knockdown of native SSP genes that resulted in enhanced accumulation of foreign proteins in a seed-specific manner (Schmidt and Herman 2008). Although glycinin promoters were successfully utilized in each case, there was no further investigation of potential tissue-specific or inducible properties of the glycinin promoters themselves in soybean.

To best study the complexities of highly regulated promoters, it is necessary to adopt systems in which possible inducers of promoter activity can be tested in specific tissue types and at precise developmental stages. For embryo- or seed-specific promoters, embryo and seed culture systems would be useful, as continual monitoring of gene expression in developing embryos directly in soybean pods would be exceptionally challenging. In vitro culture of soybean seed tissues has been used to better understand embryo development, and to test the effects of media addenda on growth and development (Dyer et al. 1987; Rainbird et al. 1984; Thompson et al. 1977). Immature seed tissues develop in vitro, mimicking seed development in the pods (Obendorf et al. 1983; Millerd et al. 1975), although in vitro tissues tended to mature more rapidly (Thompson et al. 1977). Because immature seeds can be developed in vitro, the effects of individual media components and environmental cues can be precisely controlled and used as a model to understand the factors that might influence seed development *in planta*. Soybean in vitro seed development systems have been used to monitor single-time point gene expression and accumulation of specific proteins during embryo development (Fujiwara et al. 1997; Holowach et al. 1984; Horta and Sodek 1997; Schmidt et al. 2005). Seed protein and oil content (Hayati et al. 1996; Pipolo et al. 2004), storage protein degradation (Shuttuck-Eidens and Beachy 1985), mRNA stability (Cheng et al. 1999), and sucrose uptake (Patrick 1981) studies exemplify the tremendous potential of developing seed tissues in vitro for basic research.

Since soybean seeds accumulate high levels of protein, amino acids are a critical part of the nutrients shuttled to the seed during development. Glutamine (Gln) and asparagine (Asn) are the most transported form of nitrogen to the seeds *in planta* (Rainbird et al. 1984) and in vitro development studies showed that Gln supply constitutes a large fraction (10–23%) of carbon used for biomass production (Allen and Young 2013) while also supporting the highest increases in both dry weight and protein deposition (Haga and Sodek 1987; Rainbird et al. 1984; Thompson et al. 1977). Like in vitro and *in planta* development of zygotic embryo tissues, specific amino acids, such as Gln and Asn, have been included for somatic embryo development to generate large embryos that germinate readily to form whole plants (Finer and McMullen 1991; Schmidt et al 2005).

In this work, we optimized a cotyledon culture system to study the expression of a glycinin promoter (Gunadi et al. 2016) driving expression of GFP during development in

stably transformed soybean seed tissue. In addition to tissue-specific expression in seed tissues late in development, the glycinin promoter was induced by specific amino acids in vitro, with the highest induction from Gln addition.

Methods

RNA-seq analysis

The native gene expression profile of a soybean glycinin was mined from the RNA-seq atlas (Severin et al. 2010) available through soybase.org (http://www.soybase.org) (Grant et al. 2010). Expression data from the following 14 different tissues were evaluated: young leaf, flower, pod shell 10 days after flowering (DAF), pod shell 14 DAF, seed 10 DAF, seed 14 DAF, seed 21 DAF, seed 25 DAF, seed 28 DAF, seed 35 DAF, seed 42 DAF, root, and nodule.

Plant transformation

A 1478 bp promoter from the glycinin gene *Gy5* (A3B4 subunit; Glyma13G18450, Wm82.a1.v1.1; Glyma.13G123500, Wm82.a2.v1) was previously cloned upstream of *smRS* (soluble-modified red-shifted) *gfp* in a promoter cloning vector, pFLEV (Finer Lab Expression Vector, GenBank Accession Number KX156843.1) (Gunadi et al. 2016; Hernandez-Garcia et al. 2010). Soybean embryogenic tissue cv. Jack was transformed via particle bombardment, and stably transformed promoter:*gfp* plants were recovered according to Santarém and Finer (1999). In brief, embryogenic tissues were induced from immature soybean cotyledons on a medium containing 40 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and transferred to and maintained on a medium containing 20 mg/l 2,4-D. Rapidly growing, proliferative embryogenic tissues were co-bombarded with a plasmid conferring hygromycin resistance (pHytru, Chiera et al. 2004) and a second plasmid containing the glycinin promoter regulating GFP (Gunadi et al. 2016). After selection of embryogenic tissue using hygromycin, resistant embryogenic tissues were recovered and proliferated, prior to placement on embryo development medium. After 2 months, developed embryos were desiccated and placed on a solid growth regulator-free Murashige and Skoog-based medium (0MS) containing Murashige and Skoog salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 3% sucrose, and 0.2% Gelrite™ (Aceto Corporation, Lake Success, NY) for germination and plant recovery. Regenerated plants were initially grown under fluorescent lights and in high humidity, with a gradual reduction in humidity prior to transfer to the greenhouse. After flowering and pod formation, GFP-expressing T1 seeds were identified using a Leica MZFLIII stereomicroscope (Leica, Heerbrugg, Switzerland) equipped with an LED illuminator. Homozygous T1 plants were identified based on the presence of GFP expression in all developing T2 seeds. Homozygous T2 developing embryos and seeds were used for tracking GFP expression in all experiments.

Characterization of transformants

Tissues from seed coats, pollen, floral tissues, leaves, roots, nodules, and stems of T2 homozygous transformed plants were collected and observed for GFP expression, along with a non-transformed control plant. Embryos from developing transgenic T2 seeds containing the glycinin promoter regulating GFP were also evaluated for GFP expression throughout seed development.

Media evaluation for embryo growth and GFP induction

Isolated immature cotyledons (cotyledons), immature intact zygotic embryos with the seed coat removed (embryo), and immature zygotic embryos, including the seed coat (ovules), were taken from 4 mm seeds of cv Jack, which was the same variety used for transformation. Immature pods were collected 15–25 DAF and surface sterilized in 70% ethanol for 30 s. Immature seeds were then aseptically removed from the pods and a shallow incision was made along the length of the seed coat to facilitate removal of the embryo. The embryo axis was either removed for the cotyledon explants or left intact for culture of the intact embryo. For GFP expression analysis, 4 mm homozygous T2 transgenic seeds of a low expressing event were selected.

To monitor GFP expression and surface area in developing cotyledons, an automated image collection system (Buenrostro-Nava et al. 2006; Chiera et al. 2007) was used. Images were collected every 2 h for 150 h using both blue light illumination for GFP capture (Buenrostro-Nava et al. 2006; Chiera et al. 2007) and white light illumination for surface area determination. Image sets were aligned using Adobe ImageReady, and both the cotyledon surface area and mean GFP expression intensity were quantified using ImageJ. For GFP expression, individual images were separated into the red, green, and blue channels and any background fluorescence was subtracted. Greyscale values representing GFP expression intensity were extracted from the green channel after the threshold values were set. GFP expression and area at each time point consisted of a mean of the two time points immediately preceding and following, to reduce the variation caused by the image collection system. White light images were split into RGB channels, and area values of tissue extracted from the green channel after applying the appropriate threshold. Tissue growth was represented as average fold change in area, using a Tukey's Studentized Range test to determine statistical significance.

Three different media (MSM6, SHaM, B5) were initially evaluated for both growth and GFP expression in the three different explant types. MSM6 medium (Finer and McMullen 1991) consisted of Murashige and Skoog (MS) salts (Murashige and Skoog 1962), Gamborg's B5 vitamins (Gamborg et al. 1968), 6% maltose, and 0.2% Gelrite™. SHaM (Soybean Histodifferentiation and Maturation) medium (Schmidt et al. 2005) consisted of FN Lite macro salts (Samoylov et al. 1998), MS micro salts, B5 vitamins, 3% sucrose, 3% sorbitol, 30 mM glutamine (Gln), 2 mM methionine (Met), and 0.2% Gelrite™. The B5-based medium (Gamborg et al. 1968) contained B5 salts and vitamins along with 0.1 mg/l indole-3-butyric acid (IBA) and 0.2% Gelrite™ (Tilton and Russell 1984). All media contained 0.5% activated charcoal and were adjusted to pH 5.7. For all media where amino acids were added, the amino acids were prepared at 5X concentration, filter sterilized, and added to an appropriate amount of autoclaved, warm medium to make the final amino acid concentration. Each explant type was placed on all three media. After the first basal media comparison, all media addenda were subsequently evaluated using only SHaM medium.

For evaluation of the effects of amino acids, various salts, and sorbitol on induction of the glycinin promoter, cotyledons were isolated as described previously and place on SHaM without sorbitol (SHaM-No Sorb), SHaM without Gln and Met (SHaM-No AA), and SHaM with MS macro salts in place of FN Lite macro salts (SHaM-MS Salt). Image capture for both GFP expression and cotyledon growth was initiated within one hour after explant culture.

To evaluate the effects of specific amino acids on both growth of isolated cotyledons and induction of the glycinin promoter, asparagine (Asn), glutamine (Gln), methionine (Met), proline (Pro), and serine (Ser) were separately added at 30 mM to SHaM medium containing no other amino acids (SHaM-No AA), and both cotyledon area and GFP expression were captured for image analysis.

To determine the concentration of Gln for optimal induction of the glycinin promoter in transgenic, developing soybean cotyledons, Gln was added to SHaM-No AA at 0, 1.5, 5, 15, 30, 50, and 150 mM. After cotyledons were isolated as previously described and placed on each medium, tissue growth and GFP expression in the cultured cotyledons were quantified.

To determine if specific carbon sources or osmoticum were inducing expression of the glycinin promoter in developing transgenic soybean cotyledons, cotyledons were isolated and cultured as previously described on SHaM-No AA medium containing 30 mM Gln made with either 30 g/l sucrose, sorbitol or maltose singly and in pairwise combination. Both tissue growth and GFP expression in the cultured cotyledons were quantified.

To confirm that amino acids specifically were inducing activity of the glycinin promoter, SHaM-No AA containing 30 mM Gln was made and compared with two SHaM-No AA variant media formulations, each containing equimolar nitrogen salts. One medium contained 60 mM KNO_3, and the other contained 30 mM NH_4NO_3.

Results

Glycinin promoter-driven expression is developmentally regulated in seeds

Expression of the native glycinin seed protein gene, mined from the Soybase RNA-seq atlas online database (Severin et al. 2010), was strongly seed specific, beginning at roughly 25 DAF (Fig. 1a). There was no expression in non-seed tissues (Fig. 1a).

Glycinin promoter:*gfp* transgenic event recovery

Three glycinin promoter:*gfp* expressing events were successfully recovered. During somatic embryo development in these events, low levels of GFP expression from the glycinin promoter were observed. In transgenic soybean plants, GFP expression closely mimicked native expression across all events (Fig. 1) and was restricted to seed tissues during mid- to late seed development. No other GFP expression was observed in the seed coat, pollen, floral tissues, leaves, roots, nodules, or stems (data not shown). GFP expression was not observed in early embryo development, but gradually increased beginning around the 4–5 mm stage corresponding to roughly 25 days after flowering (Fig. 1b). GFP expression remained high until seed maturity, and was visible through the seed coats in dry, mature seeds for all transgenic events. T1 seeds showed a normal segregation distribution of 3:1 for high levels of GFP expression, and homozygous seeds could not be distinguished from hemizygous seeds based solely on GFP expression. All transgenic events displayed the same seed-specific expression, and the lowest expressing event was chosen for in vitro development and promoter induction experiments. The selected event still displayed high levels of GFP expression in the seed compared to non-transformed seeds (Fig. 1c) even though it was the lowest expressing of the three recovered events. The higher expressing events were difficult to evaluate using our automated GFP detection system as the high expression levels saturated the detection field, during early cotyledon development.

Seed and cotyledon development

Transgenic embryos and cotyledons cultured on SHaM medium showed statistically significant tissue growth at the

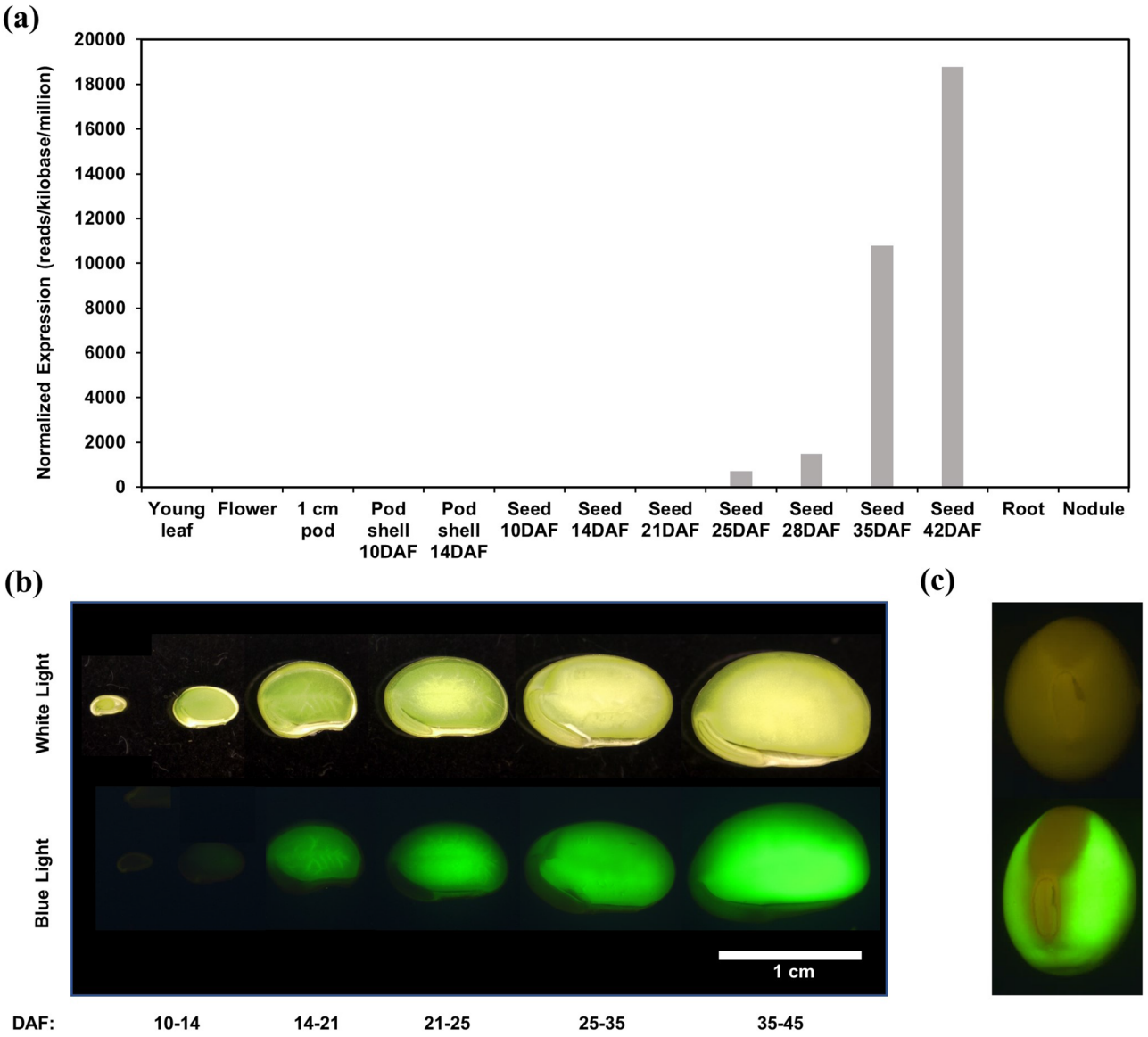

Fig. 1 **a** RNA-seq expression data of a glycinin gene in many tissues mined from Soy Base, **b** GFP expression driven by the glycinin promoter at various stages of seed development in stably transformed glycinin promoter:*gfp* soybean (DAF = days after flowering), and **c** a mature transformed glycinin promoter:*gfp* seed (bottom) and a non-transformed 'Jack' seed (top)

end of the development period, while embryos and cotyledons cultured on MSM6 and B5 media did not grow as much (Fig. 2a). Both cotyledons and intact embryos began to enlarge within two hours of placement onto each medium, while ovules displayed no appreciable increase in size over 150 h of development on SHaM medium (Fig. 3). Isolated cotyledons were initially smaller due to removal of the embryo axis and seed coat but displayed similar rates of area increase compared to intact embryos with no seed coat (Figs. 2, 3). Growth rates between cotyledons and embryos within each of the three media were statistically similar (Figs. 2, 3).

GFP expression in transgenic seed tissues

Although cotyledons grew in size on all three media (Figs. 2, 4), increases in GFP expression of transformed cotyledons were only observed when the cotyledons were cultured on SHaM when compared with MSM6 and B5 media (Fig. 4b). Expression in cotyledons cultured on MSM6 and B5 media decreased slightly over the course of 150 h of image capture, as tissues were selected for in vitro culture just as or slightly after GFP expression began to increase *in planta*. Expression in cotyledons cultured on SHaM increased dramatically over the 150 h time course.

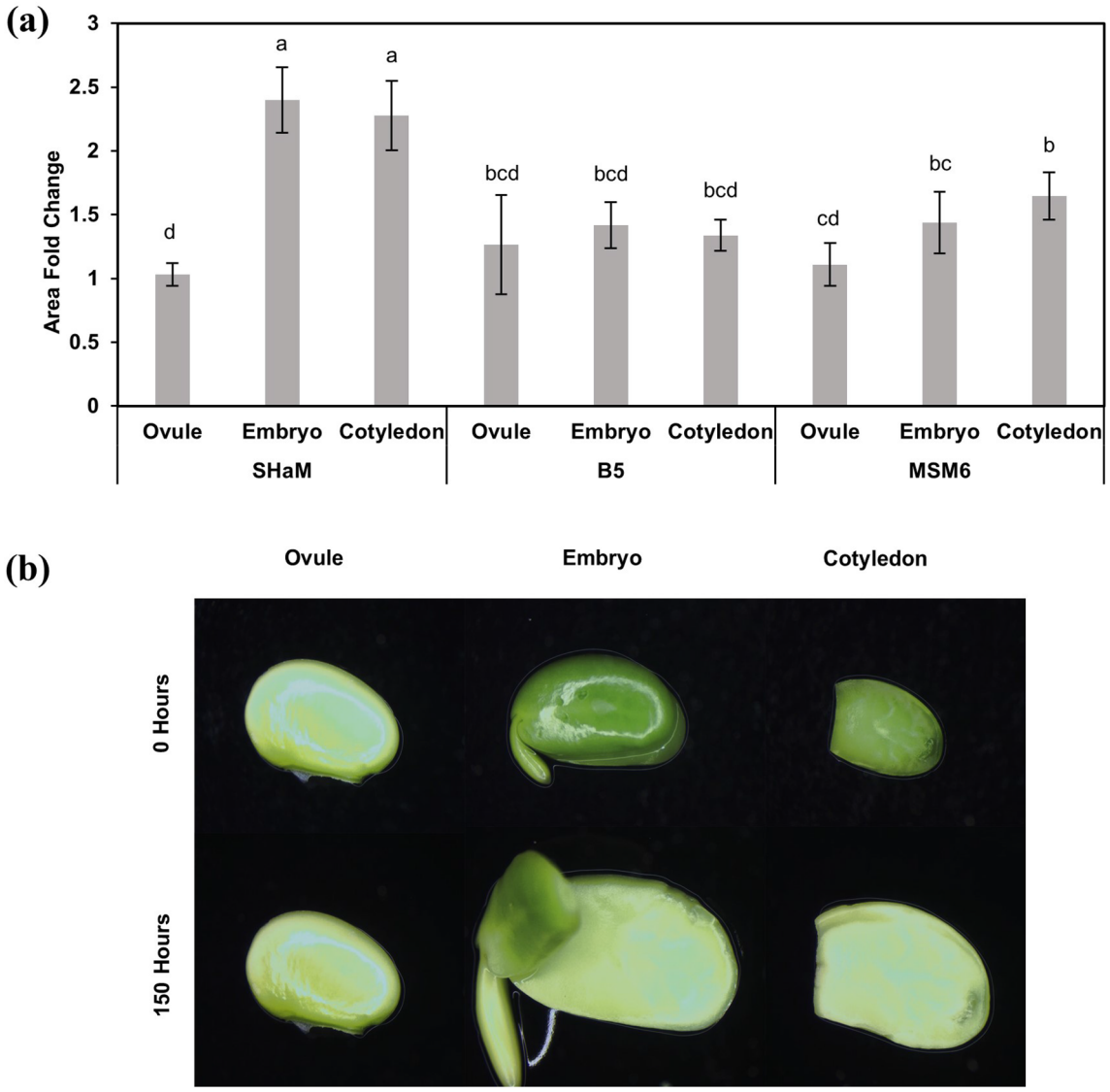

Fig. 2 **a** Fold change in area over 150 h of three 'Jack' tissues (cotyledon, ovule, intact embryo) on three development media and **b** tissues developed on SHaM at 0 and 150 h. Between 6 and 7 replicates of each tissue type were tested on each medium. Area fold change values are expressed as mean ± SD. Columns followed by the same letter are not significantly different at $p < 0.05$ using Tukey's Studentized Range (HSD) test

Amino acids induce expression of the glycinin promoter in developing seed tissues

Although there were no *significant* differences in cotyledon growth between variants of the original SHaM formulation, use of SHaM-No Sorb resulted in the highest average cotyledon growth compared with all three initial media variants (Fig. 5a). Average growth of cotyledons cultured on SHaM-No AA was the lowest of all three media (Fig. 5a), although this difference was not significant. Large increases in GFP expression were observed when the cotyledons were cultured on SHaM-MS Salts and SHaM-No Sorb, similar to the expression pattern observed in cotyledons developed on the original SHaM formulation. Cotyledons developed on SHaM-No AA did not show the same large increase in GFP expression (Fig. 5b). Cotyledons cultured on the two media containing amino acids showed increased GFP expression within two hours after culture that peaked at around 120 h.

When cotyledons were cultured on media containing varying amino acids, average cotyledon growth was the highest on media containing Gln, Asn, Pro, and No AA (Fig. 6a). Media containing Met and Ser promoted less cotyledon growth than the SHaM-No AA control (Fig. 6a). Media containing Gln, Pro, and Asn all led to increased GFP expression in cotyledon tissues (Fig. 6b). Although cotyledons developed on No AA showed a decrease in GFP expression,

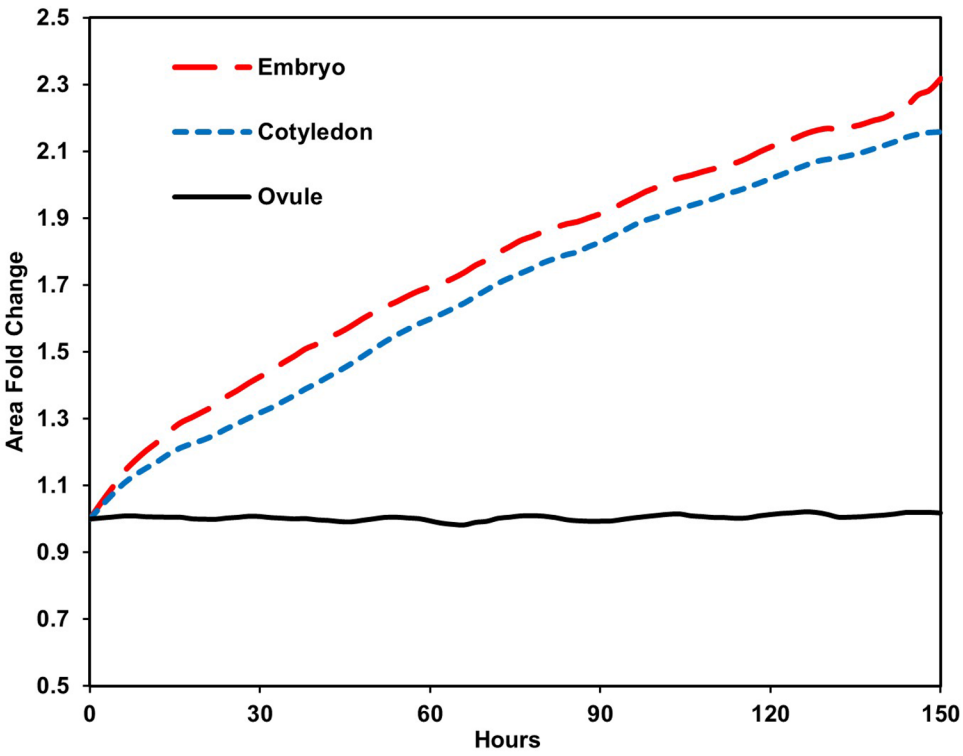

Fig. 3 Fold change in area over 150 h of three 'Jack' tissues (cotyledon, ovule, intact embryo), cultured on SHaM. Each series represents an average fold change in area of 6 to 7 replicates of each tissue type developed on SHaM for each time point collected at 2 h intervals

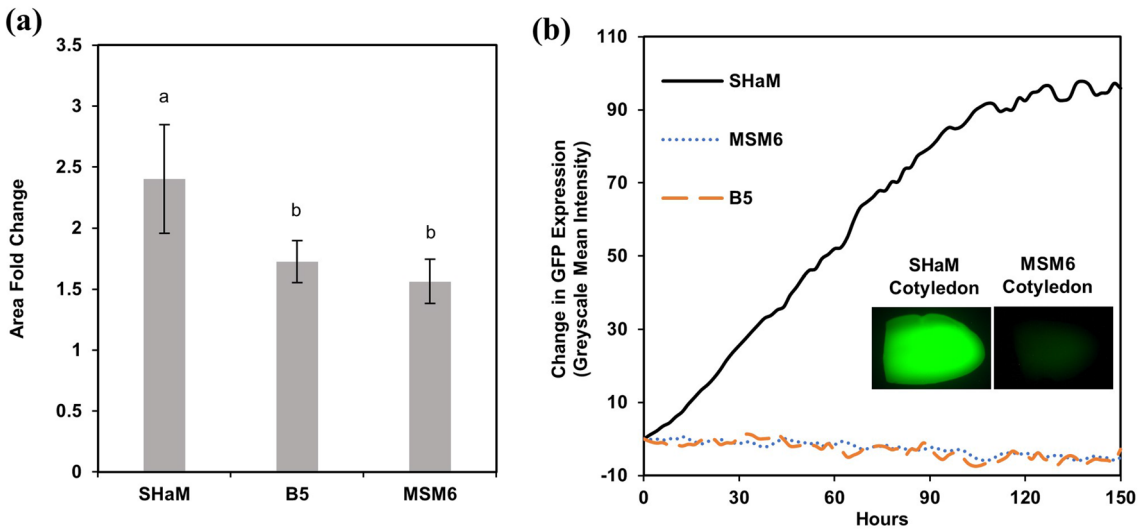

Fig. 4 **a** Fold change in area over 150 h of 'Jack' cotyledons on three development media and **b** expression of transformed glycinin promoter:*gfp* cotyledons on each media. Area fold change values are expressed as mean of 13 individual cotyledons developed on each of the three media ± SD. Expression values are an average of the change in expression of the same 13 cotyledons over the time course. Columns followed by the same letter are not significantly different at $p < 0.05$ using Tukey's Studentized Range (HSD) test. Inset images in **b** are two representative cotyledons from two media treatments

both Met and Ser addenda caused a greater decrease in GFP expression (Fig. 6b). Culture of transgenic cotyledons on media containing Gln led to the earliest and highest induction of GFP expression, while the media containing Pro and Asn promoted expression in cotyledons at an intermediate level.

Growth of cotyledons was similar on media containing Gln at all concentrations; however, cotyledons cultured on medium containing 30 mM Gln had the highest average

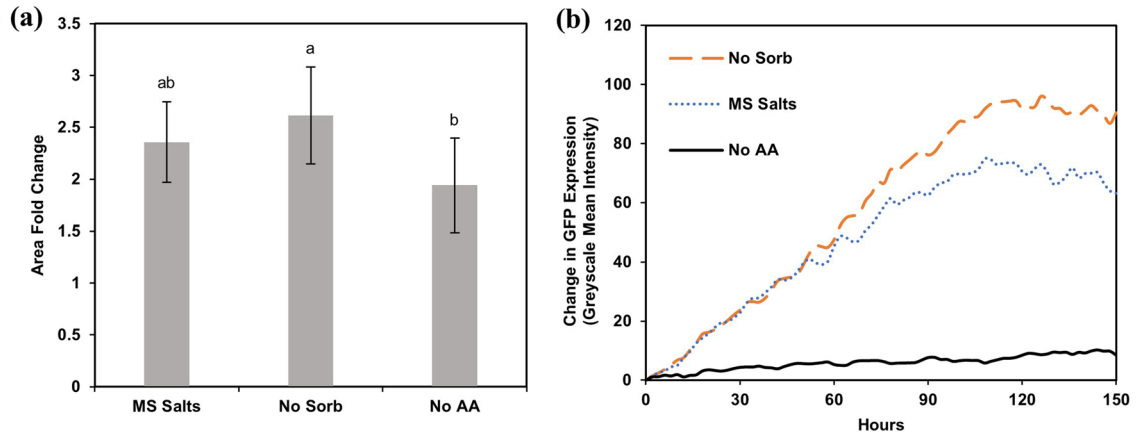

Fig. 5 **a** Fold change in area of glycinin promoter:*gfp* transformed immature cotyledons on three different SHaM variants and **b** changes in expression on each variant medium over 150 h. Area fold change values are expressed as mean of 7–12 individual cotyledons developed on each of the three media ± SD. Expression values are an average of the change in expression of the same 7–12 cotyledons over the time course. Columns followed by the same letter are not significantly different at $p < 0.05$ using Tukey's Studentized Range (HSD) test

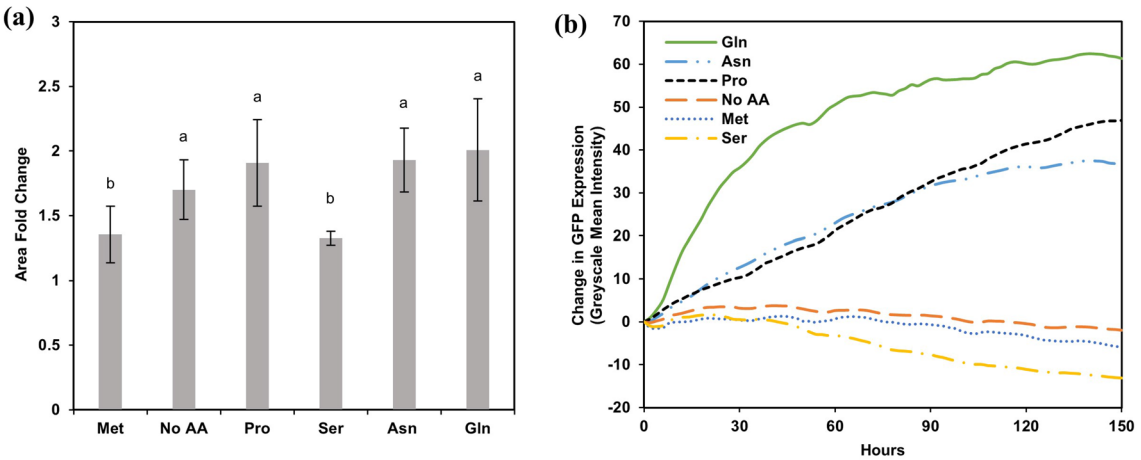

Fig. 6 **a** Fold change in area of the glycinin promoter:*gfp* transformed immature cotyledons on SHaM containing varying amino acids at 30 mM over 150 h, and **b** changes in expression on each variant medium. Area fold change values are expressed as mean of 11–13 individual cotyledons developed on each of the three media ± SD. Expression values are an average of the change in expression of the same 11–13 cotyledons over the time course. Columns followed by the same letter are not significantly different at $p < 0.05$ using Tukey's Studentized Range (HSD) test

increase in area (Fig. 7a). Cotyledons developed on the medium with no Gln showed an initial slight increase in GFP expression, followed by a decrease in GFP expression over the 150 h image collection time (Fig. 7b). Cotyledons cultured on the medium containing 1.5 mM Gln also displayed an initial increase in GFP expression, followed by reduced expression but not to the same level as with no Gln. GFP expression in cotyledons cultured on the medium containing 5 mM Gln steadily increased to a moderate level over the time course and represented a threshold increase in GFP expression as a result of Gln concentration (Fig. 7b). Cotyledons, cultured on medium containing 15 mM Gln, showed increases in GFP expression at almost twice the level of cotyledons developed on medium containing 5 mM Gln, and GFP expression steadily increased throughout the image collection time course (Fig. 7b). Cotyledons developed on the 30 mM and 50 mM Gln media showed a similar increase in GFP expression that peaked around roughly 100 h and sustained at a high level for the remainder of the image capture (Fig. 7b). Cotyledons developed on a medium containing 150 mM Gln showed a rapid initial increase in GFP expression that peaked early around 50 h and was followed by decreasing GPF expression for the remainder of the time course experiment (Fig. 7b). Gln addition at 30 mM

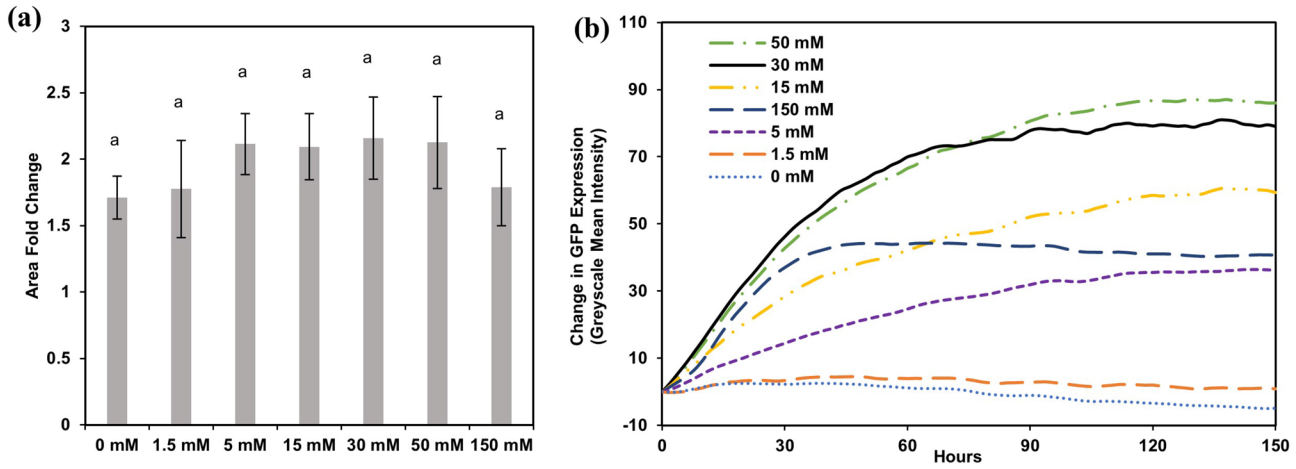

Fig. 7 **a** Fold change in area of glycinin promoter:*gfp* transformed immature cotyledons on SHaM containing varying levels of glutamine over 150 h, and **b** changes in expression on each variant medium. Area fold change values are expressed as mean of 6–8 individual cotyledons developed on each of the three media ± SD. Expression values are an average of the change in expression of the same 6–8 cotyledons over the time course. Columns followed by the same letter are not significantly different at $p < 0.05$ using Tukey's Studentized Range (HSD) test

was optimal for further studies, as media containing 50 and 150 mM Gln promoted aberrant expression patterns and inconsistency of expressing cells within each cotyledon. After the 150 h time course, cotyledons developed on media containing 50 and 150 mM Gln showed reduced expression in the middle of the cotyledon surfaces, greatly increased expression at the cut edges of each cotyledon, and cotyledons on the 150 mM Gln media began to brown with continued culture (unpublished observation).

Of the carbon source SHaM variants tested, maltose appeared to be the best promoter of cotyledon tissue growth, while sorbitol alone promoted the lowest amount of cotyledon growth (Fig. 8a). All carbon variant media combinations promoted cotyledon GFP expression to a similarly high level, except for those containing sorbitol (Fig. 8b). Each of the sorbitol-containing media caused lower GFP expression than the other treatments. Expression in cotyledons developed on media supplemented with sorbitol alone initially

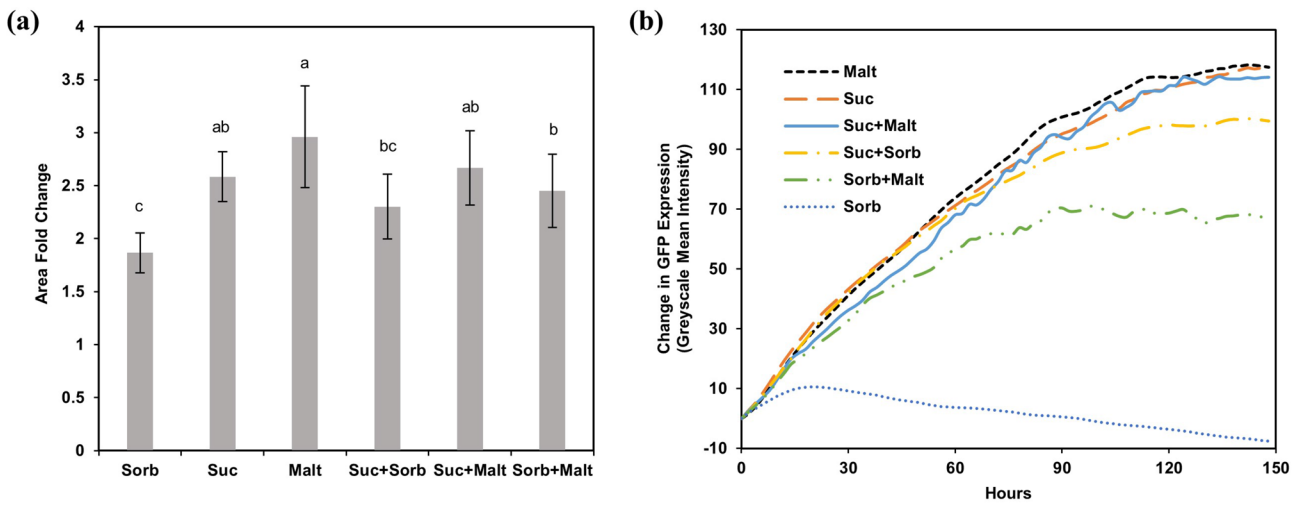

Fig. 8 **a** Fold change in area of glycinin promoter:*gfp* transformed immature cotyledons on SHaM with 30 mM glutamine, containing varying carbon sources (maltose, sucrose, and sorbitol alone or in combination) over 150 h, and **b** changes in expression on each variant medium. Area fold change values are expressed as mean of 9 individual cotyledons developed on each of the three media ± SD. Expression values are an average of the change in expression of the same 9 cotyledons over the time course. Columns followed by the same letter are not significantly different at $p < 0.05$ using Tukey's Studentized Range (HSD) test

increased, but steadily decreased throughout the remainder of the image capture time course (Fig. 8b).

SHaM-No AA media variants containing molar equivalent amounts of salt-based N showed statistically dissimilar promotion of GFP expression in transformed cotyledons compared to SHaM-No AA supplemented with 30 mM Gln (Fig. 9).

Discussion

The expression pattern of the glycinin promoter in seeds of transgenic plants was generally consistent with the expression of the native *glycinin* gene RNA-seq data mined from the Soybase 'SoySeq' RNA-Seq database (Fig. 1) (Severin et al. 2010). Each of the three recovered transgenic events showed extremely high expression in mature seeds with no observable expression in any other plant parts, indicating very stringent seed-specific expression. In this research, the transgenic glycinin promoter regulating the *gfp* gene seemed to display the same expression patterns as the native gene in soybean plants and seeds (Goldberg et al. 1981; Neilsen et al. 1989). Using the same glycinin promoter:*gfp* construction, our earlier results showed low GFP expression in stably transformed soybean hairy roots (Gunadi et al. 2016), but hairy roots show altered morphologies and the gene expression profile is not the same as in non-transformed roots. Transgenic expression may not always accurately reflect native expression patterns or profiles, as promoters removed from their native context may be influenced by surrounding regulatory regions, epigenetic regulation, or loss of regulatory elements in the promoter cloning process (Hernandez-Garcia and Finer, 2014). For further characterization of the glycinin promoter in this research, the weakest expressing transgenic event was selected for further characterization as the higher expressing events led to complete early pixel saturation in the collected GFP images. When the images were saturated, higher expression of gfp later in seed development was difficult to detect. With the lower expressing event, seed-specific expression was distinctly observed and easier to track and quantify, allowing further characterization of promoter inducibility in cotyledonary tissues.

Although in vitro culture of intact developing soybean zygotic embryos has been previously used to determine the effects of media addenda on protein and oil accumulation (Thompson et al. 1977; Hsu and Obendorf 1982; Holowach et al. 1984; Tilton and Russell 1984), our results clearly showed that culture of intact embryos led to disparities in growth between each cotyledon pair (Fig. 2, 150 h, intact embryo). The cotyledon that was in direct contact with the medium showed much more growth that the other cotyledon that was not touching the medium surface. These results indicate that the two cotyledons from the cultured, isolated zygotic embryos were not physiologically similar over time in culture, as only one was in contact with the solid medium. Isolated cotyledons from the zygotic embryo were then evaluated to provide a flat unimpeded surface for image capture and allow more similar exposure of the pair of cotyledons

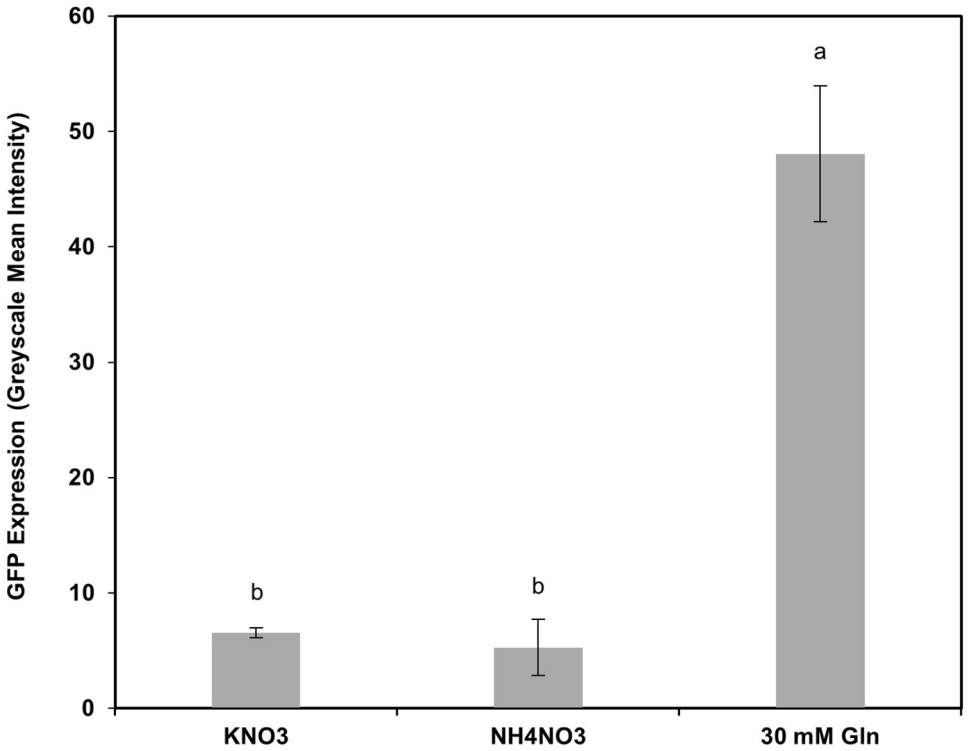

Fig. 9 GFP intensity of the glycinin promoter:*gfp* cotyledons after one week of development on SHaM using variable sources of molar equivalent nitrogen. GFP expression values are expressed as mean expression of 5 cotyledons ± SD. Columns followed by the same letter are not significantly different at $p < 0.05$ using Tukey's Studentized Range (HSD) test

to the medium. Isolated, cultured cotyledons provided uniform and consistent media contact, which was essential for evaluating tissue growth and GFP expression in developing seed tissues. Isolated cotyledons of lima bean have been previously used for evaluation of transient GFP expression because the flat adaxial surface of the cotyledon was ideally suitable for automated image capture and GFP quantification (Chiera et al. 2007). In our experiments, direct media contact was beneficial for development of cotyledonary tissues in vitro using solid media, and culture of ovules with intact seed coats inhibited tissue growth (Figs. 2, 3). In previous studies of protein and oil production in cultured embryos (Thompson et al. 1977; Hsu and Obendorf 1982; Holowach et al. 1984; Tilton and Russell 1984), differential growth of cotyledons was not reported and the cotyledons were not separately analyzed, based on medium contact. Our results here clearly showed that the cotyledons from isolated zygotic embryos grew differentially, depending on contact point with the medium. The cotyledon culture system reported here was advantageous for studying seed characterization because cotyledon growth was not impeded, GFP quantification was simplified, and variation in cotyledon response to media was eliminated.

Using isolated cotyledon culture, each of the three initial media (MSM6, SHaM, B5) promoted tissue growth (Fig. 2). MSM6 and SHaM media were previously formulated specifically for soybean somatic embryo development (Finer and McMullen 1991; Schmidt et al. 2005), while the B5-based medium was used for zygotic embryo rescue after wide hybridization in soybean (Tilton and Russell 1984). SHaM medium promoted the best overall tissue growth when compared to MSM6 and the B5-based medium (Fig. 2a), although all media supported growth of both intact embryos and cotyledons. Although use of SHaM medium previously promoted the development of large somatic embryos (Schmidt et al. 2005), GFP expression in the transgenic cotyledons cultured on SHaM was much higher than transgenic cotyledons developed on the other two media. While all media promoted growth, only SHaM-developed cotyledons displayed a large increase in GFP expression intensity, indicating physiological growth and gene expression states of seed tissues may not be entirely correlated. This phenomenon was further validated, as varying levels of supplemental Gln showed no significant impact on tissue growth, while exposure of the developing cotyledons to this amino acid led to a tremendous increase in promoter induction (Fig. 4).

Further evaluation of promoter induction on different media demonstrated that the use of the MS salt formulation and/or elimination of sorbitol did not eliminate the high levels of promoter induction observed on SHaM medium (Fig. 5). However, use of a SHaM variant without amino acids (glutamine and methionine) gave no increase in GFP expression (Fig. 5), clearly demonstrating that one or both amino acids were responsible for induction of the transgenic promoter.

Both Gln and Asn are transported to seeds during development, are major components of soybean seed storage proteins, and promote increases in seed dry weight and total protein content (Thorne and Rainbird 1983; Rainbird et al. 1984; Haga and Sodek 1986). By comparing different amino acids at 30 mM in a SHaM base medium, we determined that Gln was responsible for the large increases in promoter activity observed in tissues during development on SHaM. Although uptake of Asn was greater than Gln in in vitro-cultured seed tissues (Haga and Sodek 1987), our results showed that Gln was a stronger inducer of glycinin promoter activity. We also identified Pro as a suitable amino acid for inducing the glycinin promoter to similar levels as Asn, but at a lower expression level compared to Gln (Fig. 6). It has been suggested that SSP levels in soybean are closely linked to either Gln supply directly or metabolites downstream of Gln (Ohtake et al. 2002), and it may be that these 3 amino acids, abundant in key seed storage proteins, are interconverted after uptake into seed tissues.

Although Hernandez-Sebastia et al. (2005) showed that 60 mM Gln promoted much higher accumulation of SSPs in soybean compared to 6 mM Gln, we found that Gln concentrations higher than 30 mM were detrimental to both glycinin promoter induction and cotyledon growth. Induction of promoter activity and cotyledon size increased when using up to 30 mM Gln, but higher concentrations had a negative impact on cotyledon size (Fig. 7). Like previous reports of the effect of nitrogen sources on SSP accumulation (Haga and Sodek 1987; Thompson et al. 1977), our results confirmed that supplemental nitrogenous salts were unable to stimulate promoter activity when compared to Gln (Fig. 9), indicating the importance of specific forms of nitrogen to glycinin promoter activity. Surprisingly, inclusion of specific maturation/desiccation inducers (Sorbitol alone or in combination) had a detrimental effect on the high level of promoter expression normally observed on media containing 30 mM Gln (Fig. 8). Media addenda used to promote tissue desiccation seem to be detrimental to glycinin promoter induction, and may be more beneficially added later in the maturation process.

Ribosome profiling in soybean has suggested that regulation of the major native SSP genes occurs primarily at the transcriptional level (Shamimuzzaman and Vodkin 2018), and our observed strong induction of the isolated glycinin promoter by amino acids supports that conclusion. These results suggest a model whereby transcriptional regulation of the sink (SSPs) is modulated by promoters based on the availability of the source supply (amino acids), implying either a direct interaction between the amino acid and the promoter region or a master regulator capable of sensing

the availability of specific free amino acids in the tissue and adjusting sink strength accordingly. In plants, amino acid-regulated induction of gene expression has not been reported, and the role of amino acids as signaling molecules remains largely unknown (Hildebrandt et al. 2015).

In summary, we present evidence that the glycinin SSP promoter from soybean regulates both seed-specific and inducible expression by amino acids in a transgenic setting. These data suggest a mechanism of regulation involved in amino acid sensing and downstream signaling in plants that mediates gene expression via the promoter region. The work presented here adds to the available toolbox for synthetic biology and lays groundwork for future study involving soybean SSP regulatory components.

Author contributions EAD and JJF conceived and designed the research. EAD conducted experiments. EAD analyzed data. EAD and JJF wrote the manuscript. All the authors read and approved the manuscript. The authors thank Casey Baik for assistance with media and tissue preparation.

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Declarations

Conflict of interest The authors declare no competing interests.

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