Plant Biotechnology 23, 413–417 (2006)

Technical Note

An efficient transient gene expression system using aleurones of diploid wheat seeds

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Received December 6, 2005; accepted April 21, 2006 (Edited by S. Ogita)

Abstract To establish a transient gene expression system for investigating the molecular function of genes in mature wheat seeds, we bombarded embryos or aleurones of three hexaploid wheat (*Triticum aestivum* L.) varieties Chinese Spring, RL4137 and OS21-5, two diploid wheat (*T. monococcum* L.) varieties KT3-1 and KT3-5 and a barley (*Hordeum vulgare*, L.) variety Himalaya with fusion constructs containing the GUS reporter gene driven by the *Actin1* promoter or *α-amylase* promoter using a particle gun, and compared the efficiency of gene expression for the transient assay. The efficiency of transient expression was high in aleurone tissues of OS21-5, KT3-1, KT3-5 and Himalaya. In the aleurone tissues of KT3-5, the *α-amylase* promoter was especially markedly activated by gibberellic acid (GA) and by a GA-inducible MYB transcription activator of *T. monococcum*, TmGAMYB. This assay system using the aleurone tissues of KT3-5 may be especially useful for investigating which genes are expressed and how they are regulated in germinating wheat seeds.

Key words: Aleurone, α-amylase, GA, transient assay, wheat.

Transient gene expression systems are important tools for understanding the functions of genes in specific organs of plants, and are especially useful for analyzing various gene functions in the aleurones of cereal seeds. In wheat, however, there have been no efficient transient gene expression systems using mature seeds so far.

Aleurones of cereal seeds are known to have various functions in relation to their response to plant hormones such as GA and ABA, signal transduction and transport of various substances (Gilroy and Jones 1994; Lovegrove and Hooley 2000). Thus, aleurone tissues are suitable materials for investigating regulation mechanisms of certain genes that respond to GA and ABA, which control seed germination and dormancy. Aleurone cells have important roles in signal transduction triggered by GA that is synthesized in embryos and secreted from embryos to aleurones. Transient assay systems using aleurones of cereals were crucial for investigating regulations of some specific genes, *Em*, *dehydrin* and *α-amylase* etc., which are involved in seed dormancy and germination in cereals (Jacobsen and Close 1991; Robertson et al. 1995; Vasil et al. 1995). In barley, a direct transient assay system for aleurone tissues has been used by several groups (Shen et al. 2001; Gomez-Cadenasm et al. 2001; Zentella et al. 2002).

One of the keys to improving grain quality is the control of seed germination and dormancy especially in wheat. We conducted this study to construct an efficient transient assay system using mature wheat seeds for molecular biological studies mainly on seed germination. We used three hexaploid wheat varieties Chinese Spring, RL4137 and OS21-5, and two diploid wheat varieties KT3-1 and KT3-5. Chinese spring is a standard line for genetic studies in common wheat. RL4137 and OS21-5 are independent lines that have been used for some physiological experiments in our laboratory. KT3-1 and KT3-5 are varieties of the diploid wheat, *T. monococcum*, the latter being an early flowering mutant line induced by EMS-treatment of the former line. As a control, we also used a barley variety Himalaya, often used for studies on seed germination, due to its high sensitivity to GA (Shen et al. 2001; Gomez-Cadenasm et al. 2001; Zentella et al. 2002).

In the first experiment, we performed a transient expression analysis using mature seeds of a hexaploid wheat variety Chinese Spring, a diploid wheat variety KT3-5 and a barley variety Himalaya. Mature seeds sterilized in 2% sodium hypochlorite solution were either longitudinally cut into half-grains or processed to isolate the embryos. The half-grains or isolated embryos were

Abbreviations: ABA, abscisic acid: CaMV, cauliflower mosaic virus: cDNA, complementary DNA: DAP, day after pollination: GA, gibberellic acid: GFP, green fluorescent protein: GUS, β-glucuronidase: LUC, luciferase: MS medium, Murashige-Skoog medium: NOS, nopaline synthase.
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incubated on Murashige-Skoog medium (MS, Sigma) agar plates without sucrose at 4°C for 3 days for imbibition. For bombardment by a particle gun (Bio-Rad biolistic PDS-1000/ He particle delivery system), two μg of a rice *Actin1* promoter-β-glucuronidase (GUS) reporter fusion construct (*pAct1*-F) (McElroy et al. 1990) purified by Quiagan tip (Quiagan) was precipitated onto 0.75 mg of 1.6 μm gold particles. The gold particles coated with the DNA were spread onto rupture discs. The half-grains or isolated embryos were then bombarded with the DNA-coated gold particles from a distance of 5 cm under a pressure of 1100 psi. The distance and pressure used here have been previously determined as optimum in some experiments using some materials including protoplasts and cultured cells derived from wheat aleurone tissues (data not shown). The tissues were subsequently incubated at 24°C for 2 days and used for the histochemical GUS assay. GUS staining was performed by incubating the tissues in phosphate buffer containing 0.06% Triton X-100, 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc), 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 10 mM EDTA and 10 g l^{-1} cycloheximide for 24 h at 37°C. The stained tissues were treated with ethanol several times for bleaching.

Patterns of GUS expression differed with the material as described below. Only a few GUS spots were observed in the embryos and seeds of Chinese Spring (Figure 1A and D), while high levels of GUS activity were detected in tissues other than the endosperms, i.e., in the embryos and aleurone layers, of KT3-5 (Figure 1B and E). GUS expression was also observed in the embryos of Himalaya (Figure 1C).

To examine whether the wheat aleurone tissues are suitable for the assay system, the tissues of certain wheat varieties were bombarded with a reporter construct containing a GUS gene driven by the high-pI α-*amylase* promoter, Am (−877) IGN (Jacobsen and Close 1991), and the transient expressions of the reporter construct were compared. This expression system is important for studying the functions of α-*amylase*, a gene encoding a key enzyme involved in GA-regulated seed germination. GA is not synthesized endogenously in aleurones, but synthesized in embryos and secreted into them during seed germination. Aleurone tissues, from which embryos have been removed, are thus ideal materials for examining the effect of exogenous GA.

For the transient assay described below, half-grains without embryos were incubated on MS plates without sucrose at 4°C for 3 days. Then, the half-grains were cut longitudinally, and the aleurone tissues were isolated by removing the starchy endosperms. Twenty pieces of aleurone tissues (approximately 25 mm^2) were placed on each plate and bombarded with DNA-coated particles. The expression of α-*amylase* was analyzed by

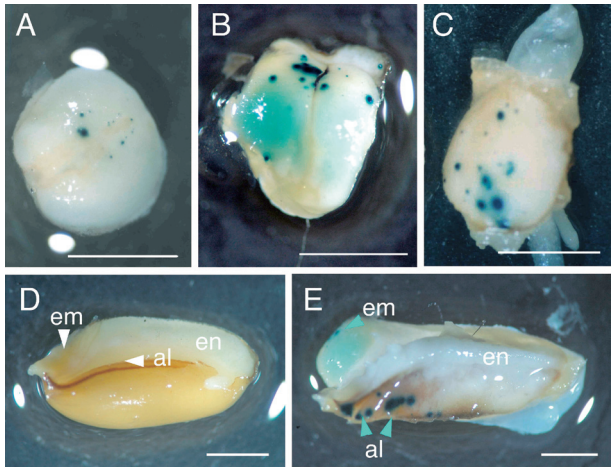

Figure 1. Histochemical staining of wheat and barley seeds and embryos bombarded with the *pAct1*-F construct. Longitudinally cut seeds or isolated embryos were bombarded, incubated at 24 °C for 48 h and subjected to GUS staining. GUS expressions in embryos of Chinese Spring (A), KT3-5 (B) and barley (C) carrying the *pAct1*-F, respectively. GUS expression in longitudinally cut seeds of Chinese Spring (D) and KT3-5 (E) carrying the *pAct1*-F, respectively. Blue arrowheads indicate GUS spots. em: embryo, en: endosperm, al: aleurone layer. Bar: 2 mm.

bombardment using 2 μg of Am (−877) IGN. For normalization of GUS activity, an internal control for transformation (Ubi1-LUC) was added in a ratio of 1 : 1 to the reporter plasmid. After bombardment, 10 pieces of aleurone tissues were incubated as controls on filter paper impregnated with a solution containing 10 mM $CaCl_2$, 50 U ml^{-1} nystatin and 150 mg l^{-1} cefotaxime. The remaining 10 pieces of aleurone tissues were incubated on filter paper impregnated with the above solution further containing 10^{-6} M GA_3. After incubation at 24°C for 48 h, the cultured tissues were harvested. Preparation of extracts and GUS assays were conducted according to the procedure described by Lanahan et al. (1992). For the quantitative GUS assay, each bombarded aleurone tissue was homogenized in 200 μl lysis buffer. One hundred microliters of each extract from the aleurone tissues were added to 400 μl lysis buffer containing 1 mM 4-methyl-umbelliferyl β-D-glucuronide (MUG, Sigma) and incubated at 37°C. At 0, 2 and 4 h after the start of incubation, a 100 μl aliquot was taken out from the reaction mixture and the reaction was terminated by the addition of 400 μl of 0.2 M Na_2CO_3. The GUS activity was measured as intensity of the fluorescence excited at 365 nm and emitted at 455 nm using a spectrofluorophotometer (RF-5300PC, Shimadzu). The GUS activity was normalized against the LUC activity of the internal control that was measured using 50 μl of each extract by a luciferase assay system (Promega). Normalized GUS activity was expressed by multiplying the ratio of GUS activity/LUC activity by 1000 (Figures 2 and 3C). Each experiment was replicated using at least 6 samples (n>6).

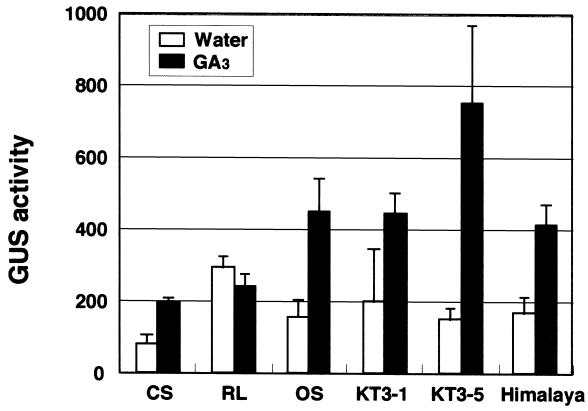

Figure 2. GUS expression in aleurone tissues of wheat and barley. An *α-amylase* promoter-GUS reporter construct Am (−877) IGN was used to test the efficiency of transformation by particle bombardment in aleurone tissues of three hexaploid wheat varieties Chinese Spring (CS), RL4137 (RL) and OS21-5 (OS), two diploid varieties KT3-1 and KT3-5, and a barley variety Himalaya. Isolated aleurone tissues were bombarded and incubated at 24°C for 48 h, and GUS activity in the tissue extracts was fluorophotometrically measured. The GUS activity was normalized against the activity of the LUC internal control (1 μM 4-MU h^{-1} per LUC1000). The means and standard errors are shown for bombardment experiments repeated at least six times.

Figure 2 shows the normalized GUS activity in the aleurone tissues of three hexaploid varieties Chinese Spring, RL4137 and OS21-5, two diploid wheat varieties KT3-1 and KT3-5, and a barley variety Himalaya, each bombarded with Am (−877) IGN. In Chinese Spring, the GUS activity was very low both in GA-treated and non-treated aleurone tissues although the activity was increased two- to three-fold by GA treatment. As mentioned above, the GUS activity was also low in Chinese Spring embryos and seeds bombarded with *pAct1*-F (Figure 1A and D). The GUS activity in the aleurone tissues of RL4137 was three- to four-fold that of Chinese Spring in non-GA-treated cells and apparently not much affected by GA treatment (Figure 2). Such properties of RL4137 were repeatedly observed in some other experiments (data not shown). However, it remains unknown why the GUS activity in RL4137 aleurone tissues is relatively high without GA and less influenced by added GA. In OS21-5, KT3-1, KT3-5 and Himalaya, GUS activity was low in non-GA-treated cells and significantly increased by GA treatment. Among these varieties, KT3-5 aleurone tissues showed the most dramatic increase of GUS activity by addition of GA. The activity was about five-fold of that without GA. An eight- to ten-fold increase was observed in some other experiments (data not shown). The GUS activity in KT3-1, the parent line of KT3-5, was significantly lower than that of KT3-5 for an unknown reason. However, it might have been due to the difference in the maturity of seeds. The early-flowering variety KT3-5 set completely mature seeds in our field, whereas the field conditions here of high temperature and high humidity during the flowering season of the parent variety KT3-1 hinder the development of its seeds, resulting in poor seeds having immature embryos and almost lacking endosperms.

Efficient transient expression was seen in both the embryos and the aleurone layers of KT3-5 seeds (Figure 1B and E). The activity of the *α-amylase* promoter introduced into the aleurone tissues was dramatically enhanced by the addition of GA (Figure 2). The α-amylase activity was unstable in the aleurone tissues of immature seeds with water contents of over 20%, while it was stably high in those of fully mature seeds with water contents of 20% or lower (data not shown). Thus, aleurone tissues of mature dry seeds (60 DAP) of KT3-5 were used for the following transient assay for investigating the regulation of *α-amylase* expression by GA.

In the aleurones, GA induces the transcription of genes encoding *α-amylase* and other hydrolytic enzymes responsible for mobilizing endosperm reserves (Fincher, 1989). The induction of *α-amylase* expression is controlled through interactions of regulatory proteins with cis-acting elements such as Gibberellic Acid Response Element (GARE) in the *α-amylase* promoter (Rogers et al. 1994). Recently, a GA-inducible MYB transcription factor GAMYB, which specifically binds to a TAACAAA box in GARE in the *α-amylase* promoter and activates the gene expression, has been isolated from barley (Gubler et al. 1995). To confirm the validity of KT3-5 in the amylase expression system, we cloned a cDNA derived from *TmGAMyb* of *T. monococcum*, which corresponds to *GAMyb* encoding the barley GAMYB, based on its homology to barley *GAMyb*. A cDNA library was constructed using embryos derived from immature seeds (10 DAP) of KT3-5 (Clonetech). Rapid amplification of the 5′ and 3′ ends of the cDNA was performed according to the manufacturer's instruction using 5′ or 3′ gene-specific primers, *TmGAMyb*-A6 (5′-GTTGGGCCTGAGGTGGTTCGCCC-3′) or *TmGAMyb*-S2 (5′-GCGGCTCCTGCGATGCA-3′), based on the sequence of the barley *GAMyb*, and an adapter primer. PCR fragments were cloned into pBluescript SK^+ (Stratagene) by the TA-cloning method. The cloned cDNA fragment was sequenced by the DyeDeoxy terminator cycle sequencing method using an Applied Biosystems Genetic analyzer 310 DNA sequencer. Then, to test whether GA controls the expression of the *TmGAMyb* homolog corresponding to the obtained cDNA and *α-amylase*, Northern hybridization analysis was carried out by using each cDNA as a probe. Total RNA was extracted from embryos isolated from seeds, which had been incubated in 10^{-6} M GA_3 solution containing 10 mM $CaCl_2$. Twenty μg of the total RNA was electrophoresed on a 1.2% formaldehyde agarose gel. The RNAs were blotted

onto a nylon membrane (Hybond-N^+, Amersham Pharmacia biotech) and hybridized with a DIG-labeled cDNA derived from a *TmGAMyb* homolog or α-amylase. Northern hybridization was performed as described by Engler-Blum et al. (1993).

The result of the sequence analysis showed that the obtained cDNA (accession no. AB214883) contained an ORF of 1659 bp encoding 552 amino acids and showing 96.6% and 86.2% identities to the barley and rice GAMYBs, respectively. As shown in Figure 3A, the expression of the gene corresponding to the cDNA was first detected at 12 h after the start of incubation with GA_3, while the expression of *α-amylase* started at 24 h. These results show that the expression of the gene corresponding to the cDNA was induced by GA prior to the induction of the *α-amylase* expression. The homology of the sequence and the expression pattern suggest that the obtained cDNA was derived from *TmGAMyb*. The Genomic Southern hybridization analysis indicated that the genome of *T. monococcum* contains only a single copy of this gene (data not shown).

The Act1-TmGAmyb effector construct used in the transient assays was synthesized by cloning the rice *Actin1* promoter (MacElroy et al. 1990) and fragments containing the coding regions of *TmGAMyb* into a pUC19 vector. The rice *Actin1* promoter and the 5′ untranslated leader sequence (including intron 1) in pDM302 (Cao et al. 1992) were excised as a *Hin*dIII fragment and cloned into the *Hin*dIII site of the pUC19-based pTH-2. The PCR fragment containing the *TmGAMyb* coding region was cloned into the pBluescript SK^+ (Stratagene) T-vector. The entire insert was excised as a *Sal*I-*Not*I fragment and cloned into the *Sal*I-*Not*I site of the pTH-2-fused *Actin1* promoter. Then, the *Sph*I-*Sal*I fragment was excised to remove the CaMV 35S promoter from pTH-2. For the expression analysis of *α-amylase* regulated by TmGAMYB, 2 μg of the reporter construct Am (−877) IGN and 0.1 to 2 μg of the effector construct were mixed to prepare bombardment mixtures with different ratios of the two constructs.

To confirm the validity of KT3-5 in the direct assay using aleurone tissues of seeds, we examined whether the transcript from the Act1-TmGAmyb effector construct could transactivate a reporter construct containing a GUS gene driven by Am (−877) IGN (Figure 3B). The aleurone tissues were bombarded with the reporter construct or its mixture with the effector construct and were incubated in water (control) or GA_3 solution at 24°C for 48 h. As shown in Figure 3C, GA_3 induced the GUS expression in the Am (−877) IGN-transformed tissues. In the absence of GA_3, the TmGAMYB effector also induced the expression. The highest expression level was achieved in the tissues co-transformed with 1/4 volume of the TmGAMYB effector, and the activity was

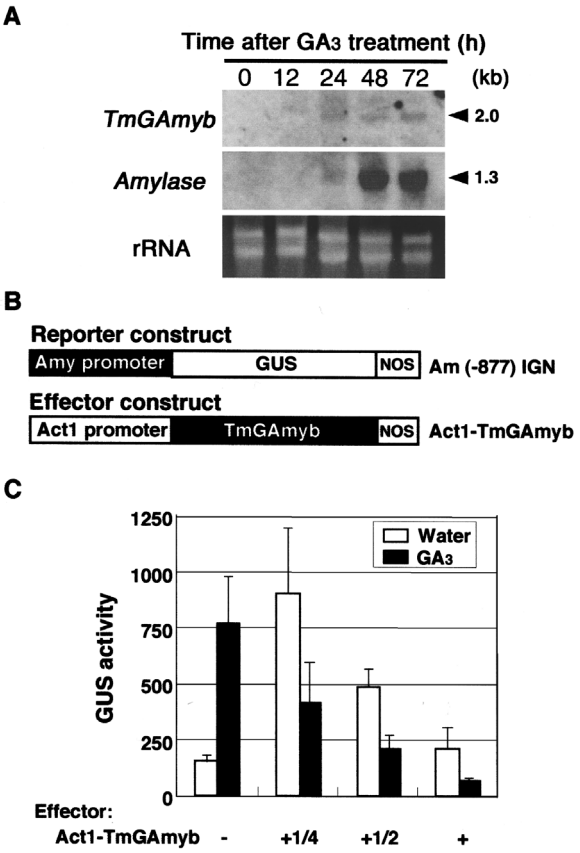

Figure 3. Regulation of the expression of *α-amylase* and *TmGAMyb*. (A) Effect of GA_3 on *TmGAMyb* and *α-amylase* expression in wheat embryos. Twenty μg of total RNA per lane was separated on formaldehyde agarose gel, blotted onto a nylon membrane and hybridized with either DIG-labeled *TmGAMyb* or *α-amylase* probe as indicated on the left. The time after the GA_3 treatment is indicated at the top. The size of the mRNA detected from each blot is indicated on the right. (B) Structures of the effector and reporter constructs. Am (−877) IGN is a reporter construct with a barley high-pI *α-amylase* promoter driving the intron-GUS-Nos reporter cassette. Act1-TmGAmyb is an effector construct with *TmGAMyb* cDNA inserted downstream of a rice *Actin1* promoter. Nos, nopaline synthase. (C) Quantitative analysis of the GUS activity in the aleurone tissues of KT3-5 bombarded with the Am (−877) IGN and the Act1-TmGAmyb, and incubated at 24°C for 48 h. The effector construct used in the co-bombardment experiments in the presence of the reporter construct is indicated below the graph: + and − indicate the presence and absence of each construct, respectively. −, +1/4, +1/2 and + represent 0, 1/4, 1/2 and 1 volume of DNA of the effector construct, respectively, relative to 1 volume of Am (−877) IGN. The closed and open columns show the GUS activity in the aleurone tissues incubated in the solution with GA_3 and without it, respectively. The GUS activity was normalized against the activity of the LUC internal control (1 μM 4-MU h^{-1} per LUC1000). The means and standard errors are shown for bombardment experiments repeated at least eight times.

eight-fold of that in the tissues transformed with Am (−877) IGN alone without GA_3-treatment. These results suggest that the obtained cDNA contains a gene encoding TmGAMYB, an orthologue of barley GAMYB that functions as an activator for *α-amylase*. The tissues co-transformed with Am (−877) IGN and 1/2 or 1 volume of Act1-TmGAmyb showed lower GUS activity.

In addition, GUS activity was lower in GA_3-treated tissues co-transformed with Am (−877) IGN and Act1-TmGAmyb than in H_2O-treated tissues. The overexpression of *TmGAMYB* may have reduced the effect of the gene through either co-suppression between the introduced *TmGAMYB* and the preexisting *TmGAMYB* induced by GA_3 treatments or squelching transcription of the *TmGAMyb* by sequestering some components such as co-activators necessary for the transcription.

Since mature seeds are physiologically more stable than immature seeds, and have fully differentiated mature embryos and starchy endosperms, the assay system using mature seeds is more suitable than that using immature seeds for investigating the roles of aleurone layers and their interactions with endosperms and embryos. Mature seeds are much easier to handle than immature seeds that often vary in their developmental stages. Moreover, mature seeds can be easily stored for a long time without any change in their quality, and can be used at any time of the year. This study showed highly efficient transient expression in the aleurone layers and embryos in the mature seeds of KT3-5, and the promoter of *α-amylase* was markedly activated by GA. The transient expression system using the aleurone tissues of KT3-5, a diploid wheat variety having simpler genomic construction than hexaploid common wheat, provides a convenient tool for investigating the molecular mechanisms of gene expression and regulation by plant hormones in seeds.

Acknowledgements

The authors would like to thank Dr. J. Jacobsen for kindly providing the Am (−877) IGN. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant no. 17780007).

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