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Isolation of *Nucellin* gene promoter from *Hordeum vulgare* and its characterization in *Arabidopsis thaliana*

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ABSTRACT

Towards genetic manipulation of plant reproductive processes, tissue-specific promoters are highly essential. Using ovule-specific promoter, it is possible to express desired genes in the ovules. In the present study, 993bp 5' upstream region of *Nucellin* gene (*HvNUC* promoter) was isolated from *Hordeum vulgare* (Gene bank accession number UB 7149) and its activity was characterized in *Arabidopsis thaliana*. The *HvNUC* promoter was cloned into pGEM-T Easy vector and sequenced. The promoter sequence was analyzed in *silico* using various bio-informatic softwares such as Plant CARE, PLACE and Plant PAN indicated several *cis*-regulatory elements which could be transcription binding factors. *HvNUC* promoter::*GUS* (*β-glucuronidase*) cassette was developed by fusing promoter with *uidA* gene through triple ligation and was subcloned in the pZP211 binary vector in proper orientation. The plant transformation vector pZP211 containing *HvNUC::GUS* cassette was mobilized into *Agrobacterium tumefaciens* strain GV3101. *Arabidopsis thaliana* Columbia 0 plants were transformed with the modified binary vector through floral dip method. The transgenic T_1, T_2, and T_3 plants were analyzed for the β-glucuronidase (GUS) activity and for the presence of *HvNuc::GUS* cassette using PCR. The GUS activity was observed in 300 T_3 plants analysed. The promoter activity could be observed in the ovules of Arabidopsis flower while it was absent in petal, sepal, stamen and leaf tissues. All the *GUS* positive T_3 plants showed the presence of 2.3 Kb long *HvNUC*promoter*::GUS* cassette when amplified by PCR. GUS activity was observed after anthesis till mature seed stage. The non-transformed control plants did not show any GUS activity. Our study has indicated *Nucellin* gene promoter from *Hordeum vulgare* as ovule-specific in *Arabidopsis thaliana.* Therefore, this promoter may be employed in targeting various pathways and developmental processes taking place in the ovules of *Arabidopsis thaliana*.

Key words : *Nucellin*, ovule-specific, gene expression, promoter activity, *cis*-regulatory elements

Plant reproduction takes place in the ovules which develop into seeds. During ovule development, megaspore mother cell differentiates and undergoes meiosis to produce haploid megaspores. One of the megaspores develops into an eight nucleated embryo sac which hosts female gametes, egg cell and central cell (Schneitz *et al.* 1995). Both the gametes participate in double fertilization resulting in embryo and endosperm.

Towards understanding differentiation of various tissues and organs, promoters play an important role. Cell or tissue-specific gene expression in response to various developmental clues depends on presence or absence of specific promoter elements (Sharma *et al.* 2015). Various tissue-specific promoters have been isolated and characterized in plants (Chen *et al.* 1989, Ellerstrom *et al.* 1996, Keddie *et al.* 1994, Aine *et al.* 1994) which can be used to precisely control transgenic expression in these tissues. Further, identification of regulatory elements of a tissue-specific promoter could help in regulating the level of gene expression in targeted tissue. The core promoter region contains *cis*-regulatory elements which comprise 6-10 nucleotides (Odell *et al.* 1985, Kenichi *et al.* 1998). Transcription factors bind to these *cis*-regulatory elements and regulate transcription independently or synergistically (Carlos *et al.* 2014, Potenza *et al.* 2004). These regulatory elements may be found in the 5' or 3' untranslated region (UTR) and introns (Vedel *et al.* 2011).

In order to understand various mechanisms controlling plant reproduction, putative genes associated with developmental events need to be either over-expressed or silenced in any model plant using specific cell or tissue-specific promoter. Ovule-specific promters are highly desirable for engineering ovule development. So far, only few ovule-specific promoters have been isolated and characterized in *Arabidopsis* (Dwivedi *et al*. 2010, Nain *et al*. 2008 and Sharma *et al*. 2015). Towards this, *Nucellin* gene promoter which was earlier reported from *Hordeum vulgare* (Chen and Foolad 1997) was further characterized in our study. Earlier, *Nucellin* gene from barley showed expression in nucellar cells and functioned as aspartic protease during programmed cell death after fertilization. *Nucellin* gene expression was found to be ovary specific and developmentally regulated as its expression increased after fertilization. Its expression before pollination was low and was limited to a few nucellar cells close to the embryo sac of the chalazal end and not in other nucellar cells. Since this pattern of expression was similar to the pattern of nucellar cell degeneration after fertilization its role in PCD of nucellar cells is implicated (Chen and Foolad 1997). Besides ovules, *Nucellin* gene expression in other cells/tissues of barley was not characterized. To find out its ovule-specific expression, characterization of its promoter in different plant parts/ tissues of a model plant, *Arabidopsis* is highly desirable.

By understanding the activity of *Nucellin* promoter it could be suitably employed to manipulate related cells/ tissues/organs using genetic engineering tools. Before attempting genetic engineering to advance our knowledge on plant development, tissue-specific promoters to drive expression of any desired gene to targeted cell/ tissue/ organ are required to be characterized using Promoter::*GUS* fusion construct. Recombinant vectors are so developed to make chimeric Promoter::*GUS* fusion construct, which on transformation facilitate localization of promoter activity through GUS assay. The present investigation was undertaken with the objective of isolating *Nucellin* promoter from *Hordeum vulgare* and assays its activity in different tissues/ organs of *Arabidopsis thaliana*, at different stages of development. The information generated from this study could be used to manipulate ovule development processes in *Arabidopsis*.

MATERIALS AND METHODS

Isolation of *Nucellin* gene promoter — The 5' flanking sequence (993bp) upstream to ATG of *Hordeum vulgare Nucellin* gene was isolated from *H. vulgare* DNA (cultivar K329) (Gene Bank accession number UB7149). The promoter was isolated by using sequence-specific primers (Forward primer HVNF: 5'- GGTTGCTTGATGGAGTGGAGATT-3' and Reverse primer HVNR: 5'- ACAGGGTACACATT GCCTTCGAG-3') of *Nucellin* gene. The amplified fragment of 993bp size was cloned in pGEM-T Easy vector and sequenced for confirmation by BLAST analysis. The analysis of promoter elements was done using Plant CARE (Wen-Chi Chang *et al.* 2008) PLACE and Plant PAN softwares (Kenichi *et al.* 1998).

Construct preparation and transformation—The *HvNUC*promoter*: GUS* cassette was prepared by cloning and subcloning between different vectors. For the directional subcloning, the required restriction enzyme sites of *Hind III* and *EcoRI* were generated by adding recognition site sequences at the 5' end of primer sequences used for amplification of promoter fragment. The amplified fragment was trimmed and directionally ligated to 5' upstream of *GUS* fragment that was obtained by restriction digestion of another vector pBI121with *HindIII* and *EcoRI*. The triple ligation was performed with *HindIII* digested binary vector pZP211, purified and trimmed promoter and *GUS* fragment that resulted in the formation of *HvNUC::GUS* cassette in pZP211 binary vector in the proper orientation (Fig. 1). After confirmation, this construct was mobilized into *Agrobacterium tumefaciens* strain GV3101 by $CaCl_2$ chemical method (An *et al.* 1988).

The *Agrobacterium* strain GV3101 harboring *pZP211* binary vector containing *HvNUC::GUS* cassette was used for *in-planta* transformation of *Arabidopsis thaliana* (Columbia 0) by floral dip method (Clough and Bent 1998). The transformed plants were grown in the growth room under controlled condition (22º C with 16 hours of light) till the seed set. Kanamycin resistance was used at the T_1, T_2, and T_3 seed germination stages to identify segregant/transgenic plants. The integration of *HvNUC::GUS* cassette into *Arabidopsis thaliana* was confirmed by amplifying a 2.3 Kb fragment including promoter and *GUS*. The PCR was performed with promoter-specific and *GUS* specific primers (Forward primer from *Nucellin* promoter region: 5'-GGTTGCTTGATGGAG TGGAGATT-3' and Reverse primer from *GUS* region: 5'-AGTTTAGGCGTTGCTTCCGCCAGT-3) and amplified 2.3Kb fragment using genomic DNA from leaf tissue of transgenic plants. The untransformed wild types did not show any amplification with the above primer pair.

Histology-Flower, leaf, anther, petal, and siliques of transgenic plants of T_1, T_2 and T_3 generations were used for biochemical GUS assay along with untransformed wild-type as the control. Tissues were stained for GUS activity (Jefferson *et al.* 1987). After GUS assay the material was cleared by chloral hydrate method (Berlteh and Jugens *et al.* 1993). The specimens were fixed in Ethanol: Acetic acid (9: 1) solution and vacuum was applied for 2 hours at room temperature. Tissues were cleared by clearing solution (2.5 gm chloral hydrate in 30% glycerol) overnight at room temperature. The samples were mounted in chloral hydrate solution and examined for GUS staining under the stereomicroscope (Zeiss Axio zoom V16 stereo-microscope).

RESULTS AND DISCUSSION

Isolation of *Nucellin* gene promoter-The promoter fragment of 993bp was isolated from the genomic DNA extracted from the young leaf tissue of *Hordeum vulgare* and cloned in the pGEM-T Easy vector. The insertion of *HvNUC* promoter in the pGEM-T Easy vector was confirmed by colony PCR and restriction analysis followed by sequencing.

Bioinformatic analysis of *Nucellin* promoter-The gene expression is regulated by the combinatorial interaction of transcription factors and specific *cis*-regulatory DNA elements of the promoter. The identification of potential regulatory elements in promoter could be a useful tool for understanding regulatory networks (Junhua *et al.* 2012). The in *silico* analysis was done using candidate promoter sequence. The analysis was done by using several online tools available in public domain such as PLACE, TSSP, Plant PAN, Plant CARE etc. A number of *cis*-acting elements, transcription factor binding sites, core motifs and other regulatory elements were localized in the sequence. The PLACE analysis showed that around 60 different types of *cis*-acting regulatory elements (CREs) were broadly distributed throughout the length of promoter sequences (Fig. 2). Similarly, the Plant PAN analysis showed around 250 different types of transcription factor binding motifs (TFs) from different plant species distributed along the length of the promoter sequence (Table 1). Few motifs were universal and

were a part of the core promoter and other motifs were responsive to environmental conditions and common physiological functions. In spite of this, some unique motifs associated with ovule and seed development were also found. The presence of these motif sequences strongly implicated it as a tissue-specific promoter.

The SKN motif with sequence GTCAT is required for expression in endosperm in co-operation with GCN4, AACA, and ACCGT. GATA box found in multiple copies are required for light regulation and tissue- specific expression (Gidoni *et al.* 1989). The motif 300MOTIFZMZEIN having sequence ATGAGTCAT is ZEIN motif of alpha-zein genes of maize. The transacting factors of AP-1, fos, jun or yeast hisS bind to this motif. The relation of this motif with the endosperm has been reported earlier (Thomas *et al.* 1990). CACGTG motif is a G box and acts as a binding site for Arabidopsis GBF4 as well as for *C. roseus* G box binding factor 1 and 2. This motif is necessary for the expression of beta-phaseolin gene when embryogenesis takes place in bean, tobacco, and *Arabidopsis* (Chandrasekharan *et al.* 2003, Hudson *et al.* 2003). The motif GCN4OSGLUB1 with the sequence TGAGTCA is similar to GCN4 like motif. GCN4 is involved in the expression of seed-specific activity in *Brassica napus* (Albani *et al.* 1997, Ericson *et al.* 1991, Washida *et al.* 1999). The motif DPBFCOREDCDC3 with sequence ACACGTG is a core binding site for DC3 promoter binding factors in carrot. The expression of DC3 is generally embryo specific and is also triggered in the presence of abscisic acid (Kim *et al.* 1997). The SEF1 motif with the sequence ATATTTATT is a soybean embryogenesis factor involved in embryo development in Soybean (Lessard *et al.* 1991, Allen *et al.* 1989).

The presence of the above mentioned regulatory elements associated with expression in developing ovule, embryo, seed and endosperm indicate *HvNUC* promoter as an ovule-relevant promoter. The ovule specificity could be the result of evolutionary assemblage of regulatory elements (Dwivedi *et al.* 2010).

Preparation of *HvNUC::GUS* construct-The *HvNUC* promoter was subcloned in the upstream region of *GUS* gene in binary vector pZP211. The right orientation of *HvNUC*::*GUS* gene cassette in the pZP211 vector was confirmed by PCR and restriction analysis (Fig. 3). *HvNUC::GUS* fusion construct was transferred to *Agrobacterium tumefaciens* strain GV3101.

Plant transformation and production of transgenics-The *Agrobacterium* strain GV3101 harboring *HvNUC::GUS* cassette in the pZP211 binary vector was used for *in planta* transformation of *Arabidopsis thaliana* by floral-dip method and the transformed plants were grown in the growth room under controlled condition till seed setting. The seeds of T_1 generation were collected and screened on MS Agar plate supplemented with kanamycin antibiotic for the selection of transgenics. One transgenic plant in a plate having 200 seeds of T_1 generation was found surviving (Fig. 4). The transgenic plant was transferred into the plastic pot with soilrite and allowed to grow in the growth room under controlled condition till seed setting. The seeds of T_2 generation were collected and stored for further analysis. About 500 sterilized seeds of T_2 generation were grown as 100 seeds on each plate for selection on MS agar plate supplemented with kanamycin (50ug/ml). Out of these, 300 healthy kanamycin resistant T_2 plants (Fig. 5A) were transferred to pots at five plants per pot and a total of 60 pots were used. None of the transgenic plants analyzed by histochemical assay of GUS showed any GUS expression. For PCR based analysis the genomic DNA was isolated from randomly selected 5 transgenic plants.The promoters and *GUS* gene region were amplified from 5 transgenic plants (Fig. 5B) by using the forward primer from *HvNUC* promoter region and reverse primer from *GUS* gene region. All the plants were grown till seed setting and seeds of T_3 generation were collected and stored for further analysis.

Analysis of T_3 generation transgenic plants—For the transgenic analysis, 500 sterilized T_3 generation seeds were grown on MS agar plate supplemented with antibiotic kanamycin (50ug/ml). 100 seeds were germinated on each plate. All the seeds had germinated (Fig. 5C) which showed resistance against the kanamycin in selection medium. From

Table 1— Distribution of the total number of transcription factor binding sites present in *HvNUC* promoter indicated by PlantPAN Promoter analysis software.

Name of *Species*	No of Transcription factor binding motifs	Data bases used in prediction of TF binding sites
Arabidopsis thaliana	107	PLACE, JASPER, TRANSFAC, AGRIS
Barley	11	PLACE, JASPER,TRANSFAC
Maize	46	JASPER, PLACE, TRANSFAC
Petunia	7	PLACE, TRANSFAC
Pea	22	PLACE, JASPER,TRANSFAC
Tobacco	22	PLACE,TRANSFAC,
Rape	15	PLACE, TRANSFAC, AGRIS, JASPER
Others	105	PLACE, TRANSFAC, AGRIS, JASPER

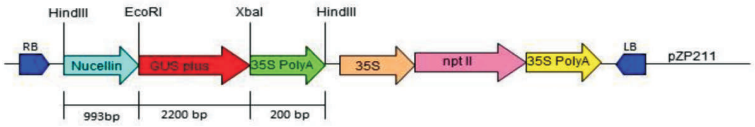
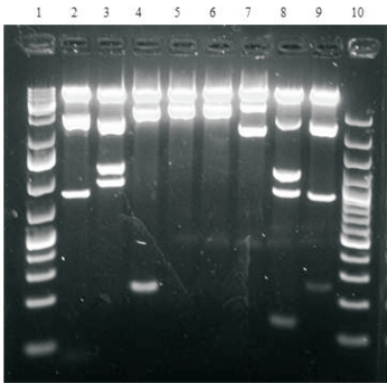


Fig. 1—T-DNA region of pZP211 vector showing *HvNUC::GUS* cassette

Fig. 2—Bioinformatic analysis of Nucellin promoter showing initiation site, TATA box and other cis regulatory elements.



Lane 1 : 1kb ladder
Lane 2 : Digested with Hind III + EcoRI
Lane 3 : Digested with Hind III + NooI
Lane 4 : Digested with Hind III + XbaI
Lane 5 : Digested with Hind III + PstI
Lane 6 : Digested with Hind III
Lane 7 : Digested with NcoI
Lane 8 : Digested with Eco RI-NcoI
Lane 9 : Digested with Eco RI - XbaI
Lane 10 : 100bp ladder

Fig.3 —Restriction analysis of pZP211 vector containing *HvNUC::GUS* cassette with multiple restriction enzymes

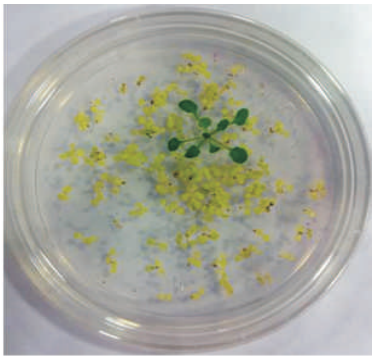


Fig.4 – T_1 transgenic plant of *Arabidopsis thaliana* harbouring *HvNUC::GUS* cassette selected on kanamycin

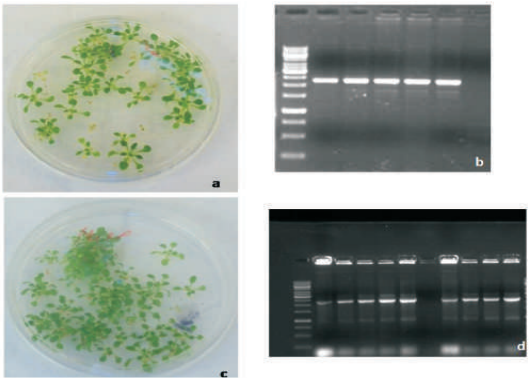


Fig. 5 (A) – T_2 transgenic plants of *Arabidopsis thaliana* selected on MS agar plate supplemented with kanamycin (B) PCR analysis of T_2 transgenic plants with forward primer from promoter and reverse primer from GUS (C)T_3 Transgenic plants of *Arobidopsis thaliana* selected on MS agar plate supplemented with kanarnycin (D) PCR analysis of T_3 transgenic plants with forward primer from promoter and reverse primer from GUS gene.

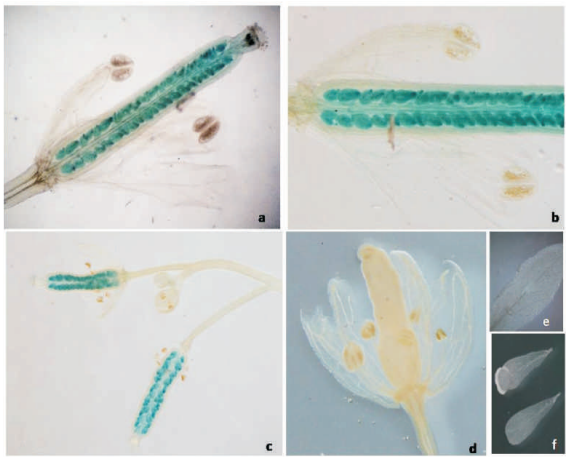


Fig. 6 Histochemical localization of GUS activity in transgenic plants of *Arabidopsis thaliana* (A-C) Siliques containing ovules (D) Control plant showing absence of GUS activity (E and F) Leaf and sepals of transgenic plants not showing GUS activity.

these plants, healthy plants were transferred to pots at 5 plants per pot and a total of 60 pots were used. The plants were grown under controlled conditions in growth room till the flowering stage along with negative control, wild-type untransformed plant. The integration of *HvNUC::GUS* cassette into T_3 generation plants of *Arabidopsis thaliana* was also confirmed by amplification of *Nucellin* promoter and *GUS* gene fragments (2.3 Kb) in selected transgenic plants (Fig. 5D) while the absence of above fragment in the untransformed wild type was confirmed. The flowers (immature inflorescence) before pollination stage were selected for GUS histochemical assay. The pistils were stained with GUS solution and cleared by chloral hydrate method and visualized in the microscope. The histo-chemical GUS assay was carried out in the siliques and leaves of all the 300 plants and in all of the plants it was found that only ovules in pistil showed GUS activity (Fig. 6A, B, C) and GUS expression was not observed in any other tissue of transgenic or wild-type plants (Fig. 6D, E & F). The intensity of GUS activity was found almost equally distributed throughout ovular tissue including integuments, funicle and embryo sac. GUS activity was localized in the ovules immediately after anthesis and it was observed till mature seed stage. *GUS* was not expressed in any part of the non-transformed plant. Assay of T_3 plants showed *GUS* expression only in the young ovary and ovule tissues. Similar experimental results were observed by Dwivedi *et al.* 2010, in which the construct *WM403::GUS* showed GUS expression in T_3 generation plants and was restricted to the pre-meiotic ovule, nucellus, embryo sac and early embryo. They used promoter of watermelon nucellus-specific gene fused with *GUS* gene and transformed *A. thaliana*. GUS expression was observed only in T_3 plants which could be due to homozygosity of *HvNUC* promoter in the T_3 generation. In T_2 plants, hemizygosity of the *HvNUC* promoter or the failure of transcription factors of *A.thaliana* binding to specific motifs in *HvNUC* promoter could have resulted in the absence of GUS expression. GUS expression was also reported in the center of young ovaries of *Arabidopsis* when plants were transformed with AGL11::*GUS* fusion construct (Nain *et al.* 2008).

Our study attempted isolation, cloning, and characterization of *HvNUC* promoter which showed its activity in the ovules of *A. thaliana. In silico* characterization of this promoter indicated several regulatory elements functionally relevant to ovular tissues. Functional characterization of the *HvNUC* promoter in *Arabidopsis* has indicated that it can be employed in genetic engineering of ovules in *Arabidopsis*. By using this promoter, any gene of interest can be expressed in the ovules of *A. thaliana*. *HvNUC* promoter can also be employed as a tool in understanding the function of ovule-specific genes in the developmental process. Being specific to ovule and developing seeds, this promoter could also be used for developing seedless fruit formation and in improving the nutritional quality of seeds through engineering of metabolic pathways.

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