BIOLOGIA PLANTARUM 52 (2): 275-280, 2008

Location and prokaryotic expression of low molecular mass glutanin subunit gene *177-21* from *Triticum aestivum*

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Abstract

To characterize the low molecular mass glutenin subunit gene *177-21* (AY994364) in wheat (*Triticum aestivum* L. cv. Jinan 177), we developed a specific PCR primer set to decide its locus with nullisomic-tetrasomic lines of Chinese spring wheat. The result showed that it was assigned to *Glu-D3*. The DNA fragment of *177-21* was then subcloned into the pGEX-4T-1 expression vector and expressed in *E. coli* with isopropyl-1-thio-β-D-galactoside induction. The result indicated that this gene encodes about 30 kD polypeptide and deduced amino acid sequence consists of eight cysteine residues. Of the eight, six may be related with the formation of intra-molecular disulfide bonds, the last two with the formation of inter-molecular disulfide bonds, which could be a potential extender in "glutenin polymer" to have positive influence on quality of wheat flour.

Additional key words: *Escherichia coli,* gene location, nullisomic-tetrasomic lines, prokaryotic expression.

Introduction

In wheat, seed storage proteins consist of two major components: glutenins and gliadins. The former are aggregates of high molecular mass (HMM) subunits and low molecular mass (LMM) subunits held together by disulphide bonds (Payne 1987). The role in wheat quality and their encoded gene loci of HMM glutenin subunits were well known (Payne 1987, Shewry *et al.* 2000). However, the exact role or their contribution and loci of many individual LMM glutenin subunits were not fully clear. This is, at least, partly because of the complexity of LMM glutenin subunit gene family (Johal *et al.* 2004).

It is known that LMM glutenin subunits have been classified into LMM-s, LMM-m and LMM-i types based on the first amino acid of mature peptide (Lew *et al*. 1992, Cloutier *et al*. 2001). Studies also showed that LMM glutenin subunit contains more cysteine (Cys) residues than HMM glutenin subunit, playing a significant role in the formation of large polymers (Masci *et al.* 1998).

In this study, we constructed the specific PCR primer set of *177-21* to determine its locus and further expressed in *E. coli*. Through checking its protein of prokaryotic expression and function in wheat further, we could use it for the quality improvement of wheat *via* gene engineering and marker-assistant selection (MAS) in the future.

Materials and methods

Plants and strains: The wheats used in this study were euploid wheat (*Triticum aestivum* L. cv. Chinese Spring and *Triticum aestivum* L. cv. Jinan 177) and aneuploid stocks of Chinese Spring: three nullisomic-tetrasomic lines, nulli1A-tetra1D, nulli1B-tetra1D, nulli1D-tetra1A. *Escherichia coli* DH10B and BL21, pGEX-4T-1, and plasmid *177-21* were kept in our laboratory.

Received 29 May 2006, *accepted* 17 January 2007.

Abbreviations: Cys - cysteine; HMM - high molecular mass; IPTG - isopropyl-1-thio-β-D-galactoside; LMM - low molecular mass; MAS - marker-assistant selection; ORF - open reading frame; PCR - polymerase chain reaction; SDS-PAGE - sodium dodecylsulfate polyacrylamide gel electrophoresis.

Acknowledgements: National Natural Science Foundation of China No. 30370857 and the National 863 High Technology Research and Development Project No. 2006AA10Z173 supported this study.

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Prokaryotic expression of 177-21: Primers were synthesized with the following nucleotide sequences: P1 5'-CTAGAATTCCTAACCAATTTCACAGCAA-3' (*Eco*RI site was underlined) and P2 5'-GCAGTCGAC TTATCAGTAGGCACCACC-3' (*Sal*I site was underined). 3 ng of DNA of plasmid *177-21* were used as template in the 0.02 cm^3 PCR reaction system, in which also consists of 0.2 mM dNTPs, 0.2 pmol of each primer and 5 units LA GC Taq enzyme. This reaction mixture were incubated at 95 °C for 5 min, followed by 10 cycles of 94 °C for 1 min, 68 °C for 1 min, 72 °C for 1 min (for each turn, the second temperature is 1 °C lower than its previous turn); and then 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR product of *177-21* was digested with S*al*I/E*co*RI and inserted into the two same enzyme sites of pGEX-4T-1. The recombinant plasmid was transformed into *E. coli* DH10B cells. The recombinant clones were identified using PCR and restriction endonuclease analysis. *E. coli* BL21 cells were transformed with pGEX-4T-1-177-21. The cells were induced by different IPTG concentration (0.4 and 0.7 mM) at 37 °C for at least 4 h, collected by centrifugation at 10 000 *g* for 30 s, then resuspended in lysis buffer and incubated at 100 °C for 10 min with occasional agitation. The resulting supernatant was collected at 12 000 *g* for 15 min for later use. 0.007 cm^3 of extracts of induced cells and controls was used for SDS-PAGE analysis.

Sequence analysis: Sequence analysis was performed according to DNAMAN and program from the NCBI.

Location of *177-21*: The DNA amplification of the three Chinese Spring nullisomic-tetrasomic lines were done by using specific PCR primer set of the following nucleotide sequences: P3:5'-CTGCATCTCTGGTTTGGAGA and P4:5'-GCTGTTGCACAAGTTGTTGT. PCR reaction mixture was according to above. Then it was incubated at 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Chromosomal location of gene *177-21* was then accomplished by comparing PCR product of *177-21* with that of nullisomic-tetrasomic lines of Chinese Spring using 1.5 % agarose gel electrophoresis.

Results

Prokaryotic expression and deduced amino acid sequence analysis of *177-21*: The PCR production of *177-21* and the pGEX-4T-1 were digested with the same enzymes, E*co*RI/*Sal*I (Fig. 1). *Lane* 1 indicated that the length of digested fragment is about 4 900 bp, which is identical to pGEX-4T-1. *Lane* 2 indicated that the length of digested fragment is about 900 bp, which also is accordance with *177-21*. Then the DNA fragment of *177-21* was inserted down-stream of the glutathione S-transferase (GST) gene of pGEX-4T-1. The recombinant plasmid was named pGEX-4T-1-177-21. Recombinants were identified by PCR and enzyme digestion (Fig. 2*A*,*B*).

Plasmids pGEX-4T-1-177-21 from the positive cells were isolated and then transformed into competent *E. coli*. BL21. Cells harbouring this recombinant were analyzed for expression after induction with 0, 0.4, and 0.7 mM IPTG by SDS-PAGE on 12.5 % gel (Fig. 3). A protein band of predicated molecular mass (60 kD), which presumably comprises GST and LMM glutenin subunit of *177-21*, was observed in samples induced by IPTG, but not in the samples without IPTG induction.

The open reading frame (ORF) and deduced amino acid sequence of 177-21 were shown below (Fig. 4). It has a high similar sequence with other LMM glutenin subunit genes reported previously. Comparison of the deduced amino acid sequence between 177-21 and those published from wheat shows that they have similar structures: signal peptide domain, N-terminal repetitive domain and C-terminal domain. The different-sized polypeptides among them are due to the presence of various deletions/insertions within repetitive and glutamine-rich domains. 177-21 contains eight Cys residues, which are conservative in most of the previously published LMM glutenin subunit sequences. Of the eight, six may be related with the formation of intra-molecular disulfide bonds, the last two with the formation of inter-molecular disulfide bonds.

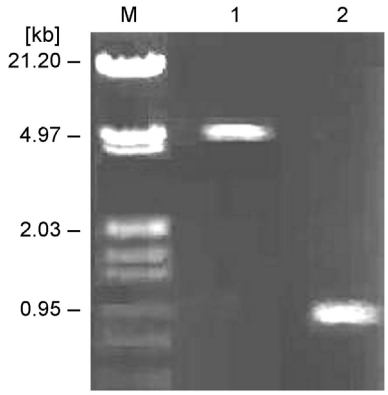

Fig. 1. Amplifying *177-21* and pGEX-4T-1 vector digested with *EcoR*I/*Sal*I. M - DNA marker, 1 - pGEX-4T-1 vector digested with *Eco*RI/*Sal*I, 2 - amplifying *177-21* digested with *EcoR*I/*Sal*I.

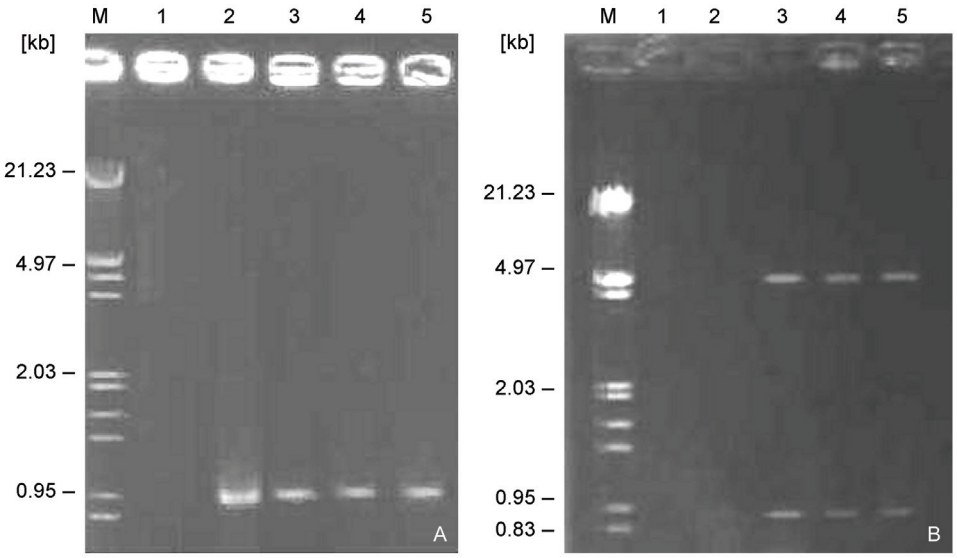

Fig. 2. *A* - Screening of *E.coli* DH10B clones transformed by pGEX-4T-1-177-21 *via* PCR. M - DNA marker, 1 - negative control, 2 - positive control, 3 to 5 - different recombinant clones. *B* - Restriction enzyme digestion analysis of recombinant plasmid. M - DNA marker, 1 to 5 - different clones digested with *Eco*RI/*Sal*I.

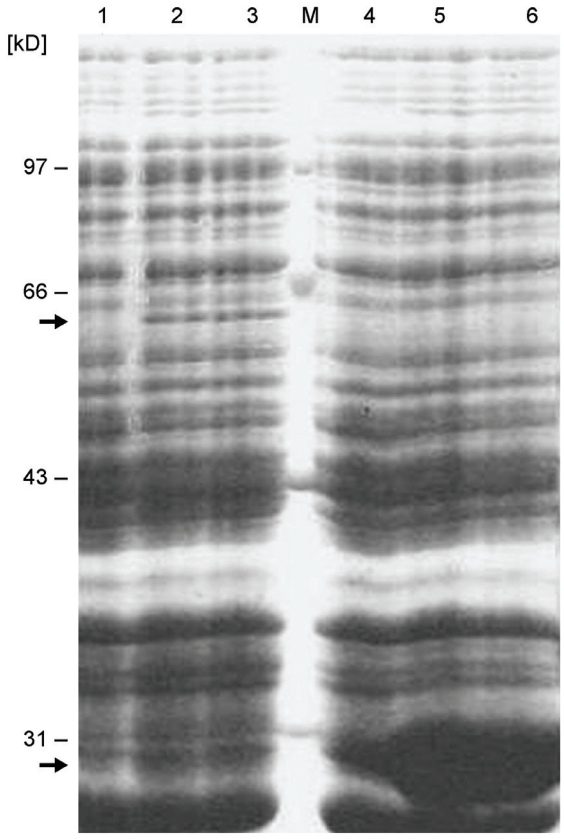

Fig. 3. SDS-PAGE analysis of expression of fusion protein. M - standard protein marker, 1 - induced BL21 by 0.4 mM IPTG in control, 2 - non-induced pGEX-4T-1-177-21 in transformed BL21, 3 - induced pGEX-4T-1-177-21 in transformed BL21 by 0.4 mM IPTG, 4 - induced pGEX-4T-1-177-21 in transformed BL21 by 0.7 mM IPTG, 5 - non-induced pGEX-4T-1 in transformed BL21, 6 - induced pGEX-4T-1 in transformed BL21 by 0.4 mM IPTG, 7 - induced pGEX-4T-1 in transformed BL21 by 0.7 mM IPTG. The *upper arrow* indicates the fusion protein; the *lower arrow* indicates the tag protein.

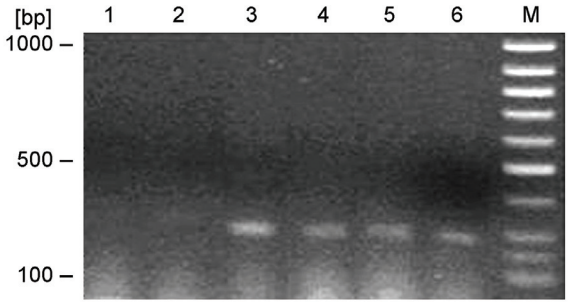

Fig. 5. The PCR product using the special primer set of *177-21*. M - 100 bp DNA ladder, 1 to 2 - N1DT1A, 3 - Jinan177, 4 - N1AT1D, 5 - N1BT1D, 6 - Chinese Spring. The ORF of *177-21* was used to produce a dendrogram together with some other previously characterized LMM-GS genes from *T. aestivum*. It is also related with one (AB062872) of *Glu-D3* (Fig. 6).

Location of *177-21*: To identify the locus of *177-21*, a specific primer set was designed based on the published nucleotide sequence of *177-21* (Fig. 4). This primer set gave a single PCR product using nullisomic-tetrasomic lines of Chinese spring lacking group 1 chromosomes (Fig. 5). The PCR products were sequenced and the results indicated that these products were accordance with the proper part of *177-21* (data not shown). With the

specific primer set of *177-21*, we failed to amplify PCR products with N1DT1A, but succeeded with N1AT1D and N1BT1D. The result suggests that *177-21* is located on 1D chromosome.

1
*ATG*AAGACCTTCCTCGTCTTTGCCCTCATCGCCGTTGTGGCGACAAGTGCCATTGCACAGATGGAGACTAGCTGCATCTC
TGGTTTGGAG
1 M K T F L V F A L I A V V A T S A I A Q M E T S C I S G L E
91
AGACCATGGCAGCAGCAACCATTACCACCACAACAGTCATTTTCACAACAACCACCATTTTCACAACAACAACAACAAC
CATTACCTCAA
31 R P W Q Q Q P L P P Q Q S F S Q Q P P F S Q Q Q Q Q P L P Q
181
CAACCATCATTTTCGCAGCAACAACCACCATTTTCGCAGCAACAACCAATTCTATCGCAGCAACCACCATTTTCACAGCAA
CAACAACCA
61 Q P S F S Q Q Q P P F S Q Q Q P I L S Q Q P P F S Q Q Q Q P
271
GTTCTACCGCAACAATCACCATTTTCGCAGCAACAACAACTAGTTTTACCTCCACAACAACAACAACAACAACTTGTGCA
ACAGCAAATC
91 V L P Q Q S P F S Q Q Q Q L V L P P Q Q Q Q Q Q L V Q Q Q I
361
CCTATTGTTCAGCCATCCGTTTTGCAGCAGCTAAACCCATGCAAAGTATTCCTCCAGCAGCAGTGCAGCCCTGTAGCAAT
GCCACAACGT
121 P I V Q P S V L Q Q L N P C K V F L Q Q Q C S P V A M P Q R
451
CTTGCTAGGTCACAGATGTGGCAGCAGAGCAGTTGCCATGTGATGCAACAACAATGTTGCCAGCAATTGCAGCAAATCCC
CGAACAATCC
151 L A R S Q M W Q Q S S C H V M Q Q Q C C Q Q L Q Q I P E Q S
541
CGCTATGAAGCAATCCGTGCCATCATCTACTCCATCATCCTGCAAGAACAACAACAGGGCTTTGTCCAACCTCAGCAGCA
ACAACCCCAA
181 R Y E A I R A I I Y S I I L Q E Q Q Q G F V Q P Q Q Q Q P Q
631
CAGTCAGGTCAAGGTGTCTCCCAATCCCAACAGCAGTCGCAGCAGCAGCTCGGACAATGTTCTTTCCAACAACCTCAAC
AGCAACTGGGT
211 Q S G Q G V S Q S Q Q Q S Q Q Q L G Q C S F Q Q P Q Q Q L G
721
CAACAGCCTCAACAACAACAACAACAGGTACTACAGGGTACCTTTTTGCAGCCACACCAGATAGCTCACCTTGAGGCGG
TGACTTCCATT
241 Q Q P Q Q Q Q Q Q V L Q G T F L Q P H Q I A H L E A V T S I
811
GCACTCCGTACCTTGCCAACGATGTGCAGTGTCAATGTGCCGTTGTACAGCGCCACCACCAGTGTGCCATTCGGCGTTGG
CACCGGAGTT
271 A L R T L P T M C S V N V P L Y S A T T S V P F G V G T G V
901 AGTGCCTAC*TGATAA*
301 S A Y * *

Fig. 4. The ORF and deduced amino acid sequence of *177-21*. The start (ATG) and double stop (TGATAA) codons were in *italics* and *underlined*. The signal peptide was *underlined*. The Cys residues were *boxed*. Specific PCR primer set (P3 and P4) was *double-underlined*.

Discussion

Wheat LMM-GS plays an important role in determining dough quality. In recent years, lots of attention has been paid to the isolation of LMM-GS genes from cultivated wheat and its wild relatives. More than one hundred LMM-GS genes have been cloned and sequenced from wheat and its relative grasses, including *177-21* (according to the data in NCBI). However, the contribution or loci of many individual LMM glutenin subunit genes were not fully studied.

With the application of different one or two-dimensional separation systems to nullisomic-tetrasomic, ditelocentric and inter-cultivar chromosome substitution lines of cv. Chinese Spring in early researches, it is widely proved that the majority of LMM glutenin subunits are controlled by genes on *Glu-A_3*, *Glu-B_3*, and *Glu-D_3* loci on the short arms of chromosome 1A, 1B,

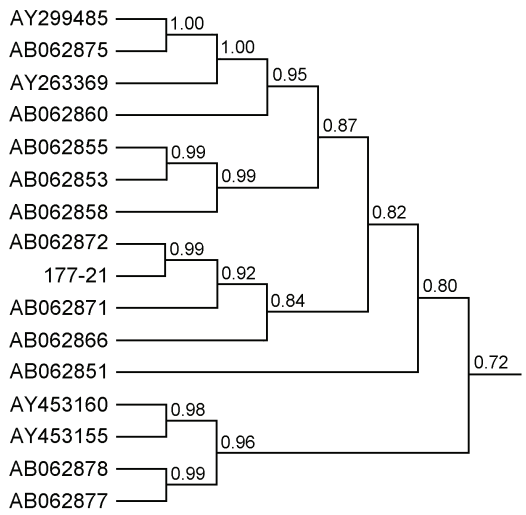

Fig. 6. Phylogenetic tree based on the deduced amino acid sequences of *177-21* and other 15 genes located in *Glu-3* loci according to data of NCBI. AY453160, AY453155, AB062878, AB062877, and AB062871 belong to *Glu-A3* loci. AY299485, AY299485, AB062858, AB062855, and AB062853 belong to *Glu-B3* loci. AY263369, AB062875, AB062872, AB062866, AB062851 belongs to *Glu-D3* loci.

and 1D respectively (Gupta *et al.* 1989; Payne 1987; Singh and Shepherd 1988). Further, Van *et al.* (1995) designed several primer sets specific for each of the *Glu-3* loci, and successfully determined the chromosomal locations of five *LMM-GS* genes. Ikeda *et al.* (2006) localized a few more *LMM-GS* genes on their deduced N-terminal sequence and through PCR and tetrosomic-nullisomic analysis. However, these works were limited by the shortage of sequence information as well as the difficulty in designing PCR primers and in distinguishing different amplified DNA of the same or similar nucleotide number. Other most commonly applied methods in gene location for wheat include FISH (fluorescence *in situ* hybridization) and cDNA crossing. However, the first of which are negatively effected by the polyploidy nature of wheat and the large number of genes present in the *LMM-GS* family, that the existing probes are far from covering the whole genome; while the use of cDNA library are tampered with the low frequency of some desired cDNA in the library and the incompleteness of the individual cDNA molecules. So till to now, only 47 *LMM-GS* genes have been located (according to NCBI), within the 144 *LMM-GS* genes. In this study, we carried out PCR of *177-21* with nullisomic-tetrasomic lines of Chinese Spring lacking group-1 chromosomes, which suggested that *177-21* is located on 1D chromosome.

Taking into consideration the primary structure of the gene *177-21* (AY994364), it seems very likely to act positive influence on quality of wheat flour. Firstly, out of its eight Cys residues, the first and seventh should form inter-molecular disulfide bond, which could be a potential extender in "glutenin polymer" (Masci *et al.* 1998, 2002, Luo *et al.* 2005). Secondly, it has a repetitive domain of more than 20 repeats, while repetitive domains often have beta spiral structure, which consists of consecutive beta-pleated sheets, and elasticized the *LMM-GS* like a spring. Furthermore, in the 299 amino acids that make up the corresponding protein of 177-21, there are 97 glutamines and 31 prolines. According to Tanaka *et al.* (2005), proline gives the LMM-GS extensibility by making the amino-acid chain form a zigzag shape, whereas glutamine is linked by hydrogen bonds between the chains that form a zigzag shape, and strengthens the gluten network. Therefore, number of glutamine, especially in the repetitive domain, may also affect the dough strength of wheat flour. It's possible to further test the contribution of the gene to wheat quality by express it in prokaryote system to use the obtained protein to study its structure and function through micro-mixographic analysis, or evaluate the strength and the stability of the dough by incorporating small amounts of purified protein in a base flour, and re-oxidation experiments to study their different behaviour in polymer constitution. In this study, The SDS-PAGE analysis clearly presented the expression of expected LMM glutenin subunit, then large amounts of protein 177-21 could be overproduced and purified to be used for further studies.

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Suárez, M.F., Bozhkov, P.V. (ed.): **Plant Embryogenesis** - Humana Press, Totowa 2008. 184 pp. USD 99.50. ISBN 978-1-58829-931-4, e-ISBN 978-1-59745-273-1

I wish to recommend a recent addition to the series "Methods in Molecular Biology" named "Plant Embryogenesis" edited by M.F. Suárez and P.V. Bozhkov. The study of plant embryology is fundamental for the understanding of the whole process of plant development. The methods in plant embryogenesis have undergone a rapid evolution recently. A comprehensive handbook has been missing for a long time. Now we can whole-heartedly express our thanks to all authors - leading experts in the field of plant embryology for very useful book. They focus on the common developmental process and compile the tools and methods important for studying of plant embryology.

The text is divided into 2 parts. Part I is devoted to the description of three plant embryonic models: *Arabidopsis*, maize and spruce. The authors describe the morphology of embryos in developmental stages during zygotic and somatic embryogenesis. Both molecular and genetic aspects are explained. The methods of study are recommended for these plant models. Part I is accompanied by a rich list of references.

Part II – "Cellular, Genetic and Molecular Mechanisms of Plant Embryogenesis" is composed of detail protocols used in different laboratories for studying plant embryos. The topics of chapters are: "*In Vitro* Fertilization with Isolated Higher Plant Gametes"; "*In Vitro* Culture of *Arabidopsis* Embryos"; "Culture and Time-Lapse Tracking of Barley Microspore-Derived Embryos"; "Isolation of Embryo-Specific Mutants in *Arabidopsis*: Plant Transformation"; "Isolation of Embryo-Specific Mutants in *Arabidopsis*: Genetic and Phenotypic Analysis"; "Laser-Capture Microdissection to Study Global Transcriptional Changes During Plant Embryogenesis"; "Promoter Trapping System to Study Embryogenesis"; "Visualization of Auxin Gradients in Embryogenesis"; "Intercellular Trafficking of Macro-molecules During Embryogenesis"; "Immunolocalization of Proteins in Somatic Embryos: Applications for Studies on the Cytoskeleton"; "Detection of Programmed Cell Death in Plant Embryos".

The authors aim this book primarily at reasearchers who are relatively new to the field of plant embryology. Nevertheless I am certain that this volume will be very useful text in all laboratories dealing with plant development and embryology. It can inspire students as well as full-fledged plant embryologists.

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