

Expression and Cellular Localization of *dab5-1*,
a Delayed Abscission Mutant,
in *Arabidopsis thaliana*

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Signature Page

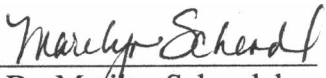
This thesis for honors recognition has been approved for the
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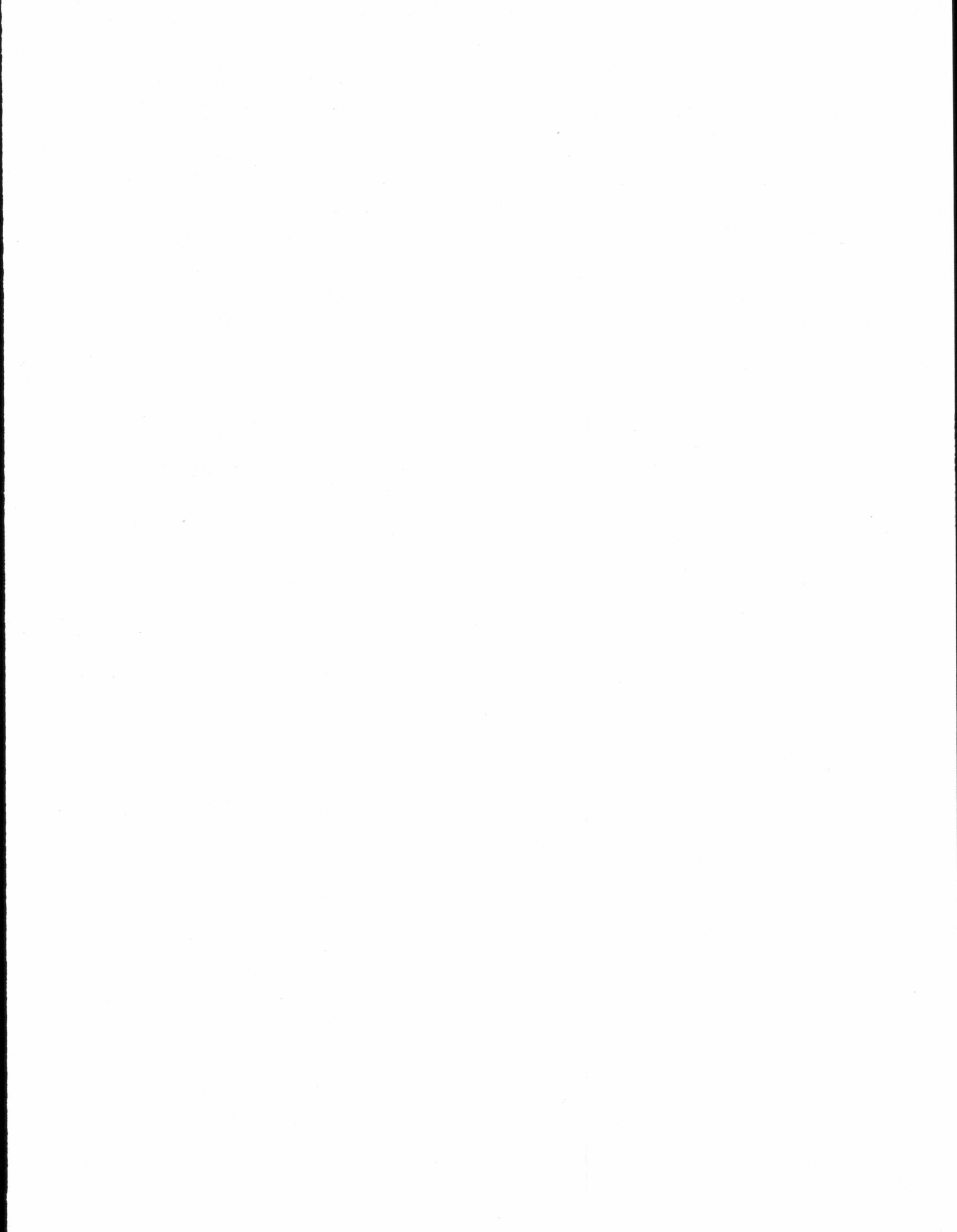


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Abstract

Abscission, an active process resulting in the removal of an organ from the main body of a plant, occurs naturally in response to pathogens, disease, or when the plant part is no longer needed. Several delayed abscission mutants have been identified from the University of Wisconsin T-DNA tagged mutant populations in *Arabidopsis thaliana*. One of the identified mutants, *dab5-1*, is characterized by a delay in abscission causing the floral organs to remain attached past position ten; however, all other plant functions are normal. *DAB5* has been thought to be involved in the secretory pathway. The present study was conducted to further characterize *DAB5* expression at the cellular, tissue, and organelle levels using reporter gene constructs, light microscopy, scanning electron microscopy, and RT PCR. *DAB5* expression was found in the roots, root tips, cotyledons, meristem, abscission zone, and anthers. However, no *DAB5* expression was present in the hypocotyl.

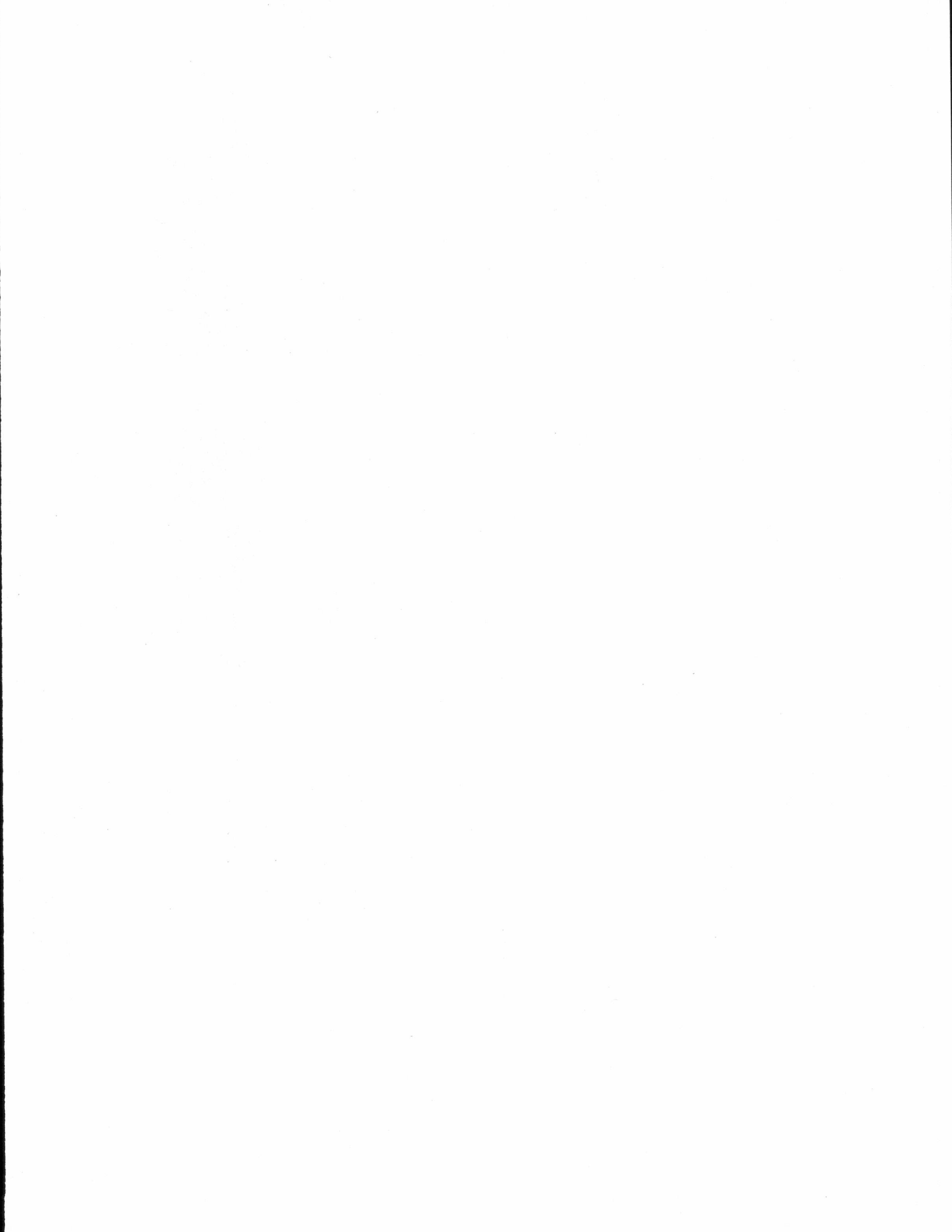
Introduction

Arabidopsis thaliana, a common weed of Eurasia, has become the plant of choice among research geneticists particularly because its genome has been mapped. Although the *Arabidopsis* genome is quite small with only 120 Mb, compared to maize with 2,500 Mb and wheat with 16,000 Mb, it contains all the necessary genes for development, metabolism, environmental responses, and disease resistance (Meyerowitz and Somerville, 1994). *Arabidopsis* plants have a short life cycle and are easily grown inside in controlled conditions. They can be both cross- and self-pollinated and each generation will produce hundreds of thousands of offspring. *Arabidopsis* is a member of the mustard

family (*Brassicaceae* or *Cruciferae*) and its developmental and physiological processes may be used as a model for many crop plants.

Abscission is an active process resulting in detachment of organs from the main body of a plant (Esau, 1977). Organs are abscised in response to pathogens or disease; as well as when they no longer contribute to the plant. Cell separation and cell wall degradation are common to the abscission process. Some processes involving cell separation include: the emergence of radicles from germinating seeds, primary root penetration, expansion of leaves and cotyledons, pollen release from anthers, fruit softening, pod dehiscence, lateral root emergence, dust tube formation, and the shedding of organs (Roberts, J. et al., 2002). Abscisic acid (ABA) was initially thought to be responsible for abscission. However, over 100 years ago, Dimitry Neljubov noticed the effects of ethylene gas from illuminating gas produced from coal that was used for lighting. He observed that the plants around leaky gas lines showed early senescence and abscission. By 1930, scientists had discovered that plants produced ethylene; thus supporting Neljubov's claim that ethylene is an endogenous regulator of plant growth and development (Schaller and Kieber, 2002).

The abscission zone consists of a small, dense area of cells generally four to six layers deep (Bleecker and Patterson, 1997). Cells in this zone are predetermined early in development and are characterized by increased rough endoplasmic reticulum associated with the plasma membrane and the Golgi (Patterson, 2001). During the process of abscission we observe changes in hormone levels, modification of elemental compounds, and an increased expression of cell wall hydrolytic enzymes (Patterson, 2001). The process of abscission involves the establishment of the abscission zone, the dissolution of



the middle lamella, the loosening of the cell wall, and finally cell separation leading to detachment of the organ (Patterson, 2001). Many genes associated with abscission have been identified in the *Arabidopsis thaliana* genome and further studies have led to the identification of several delayed abscission mutants including *dab5*. The mutant *dab5-1* was chosen prior to this study for its delayed abscission phenotype (Fig. 1). Abscission of floral organs in wild-type plants occurs around position six to eight while *dab5-1* retains the floral organs past position ten. Position one, or anthesis, is defined as the first floral bud in which the white petals can be seen (Fig.2). *dab5-1* shows normal development in all other aspects.



Figure 1. Wild type (WS) versus *dab5-1* mutant. Floral organs are retained past position 10 in *dab5-1* and serve as the phenotypic marker for this mutant.

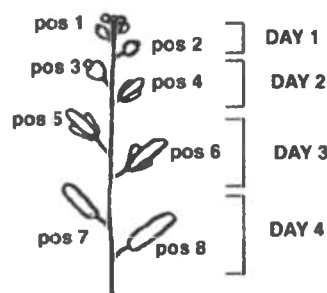
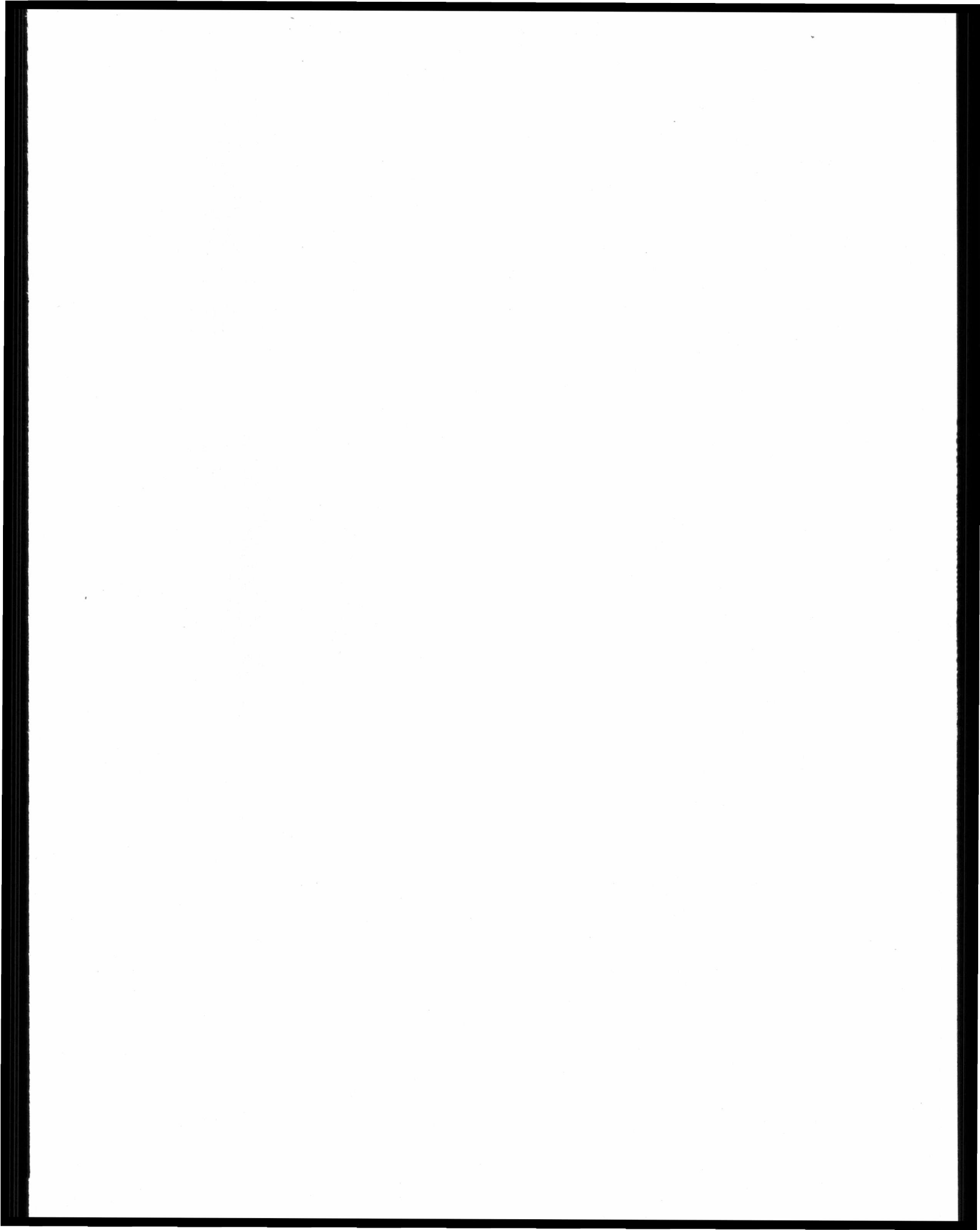


Figure 2. Positions in *Arabidopsis thaliana*. Position one is defined as the first flower showing its petals.

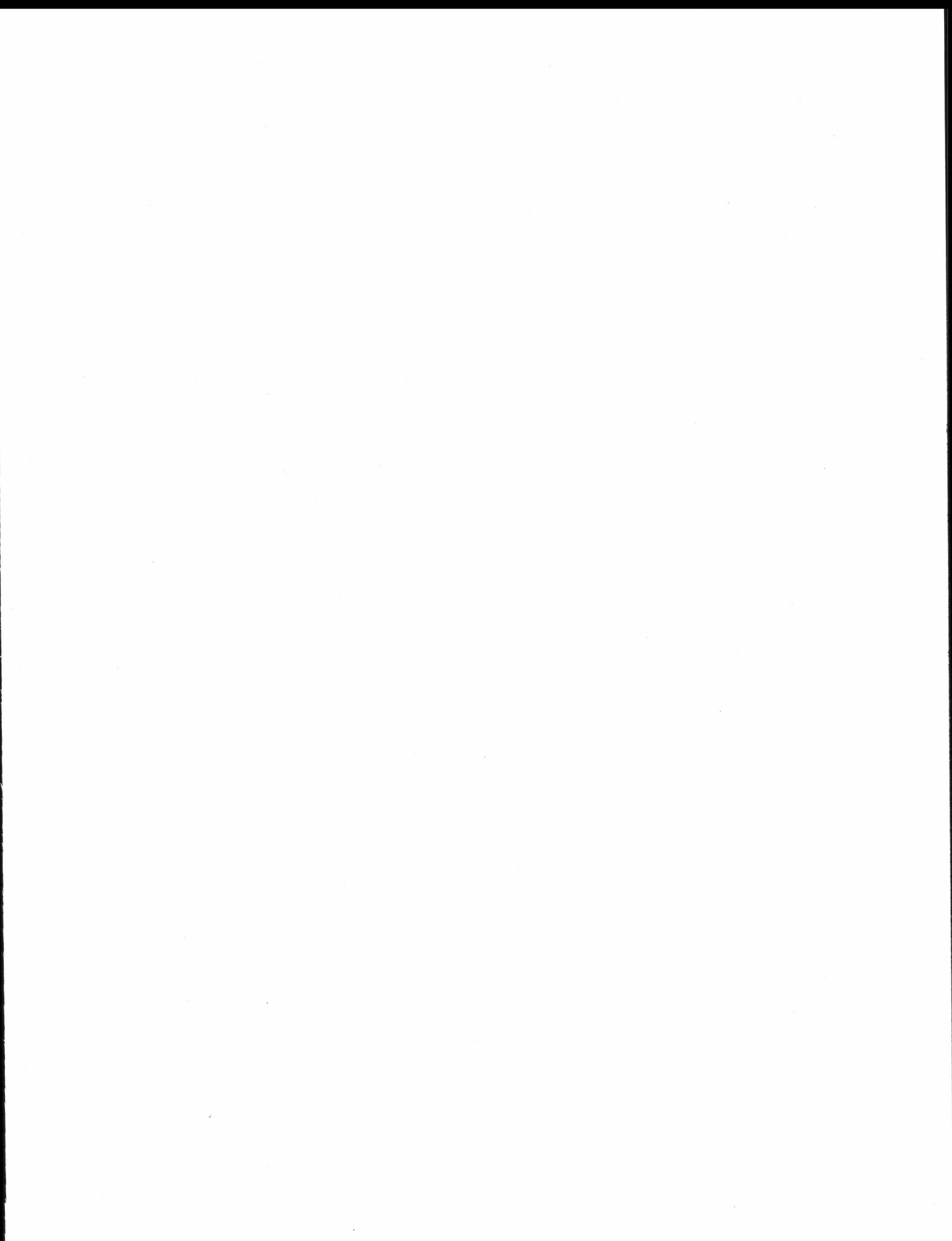
The *DAB5* gene is thought to be involved in the secretory pathway due to the similarity to mammalian secretory genes (NCBI geneseq BLAST). The secretory pathway is made up of a series of cisternae, small vesicles, vacuoles, endosomes, and other membrane bound compartments. It is along this pathway that proteins are shuttled after synthesis in the endoplasmic reticulum to various locations including the plasma membrane and the vacuole. Traffic flows in both directions along the pathway with the major biosynthetic route flowing from the endoplasmic reticulum (ER) to the plasma membrane (PM) (Raikhel, and Chrispeels, 2000). For a protein in the biosynthetic



pathway, the journey begins in the rough ER as the protein is synthesized and folded after which it continues through the Golgi where it is sorted and sent to the vacuole or plasma membrane.

Further knowledge of the mechanism of abscission may be used in agriculture and horticulture to extend the longevity of the crop or plant. Delayed abscission was one of the first traits selected for by humans. For example, the collection of some fruits and seeds in crops such as wheat (*Triticum monococcum*), rice (*Oryza sativa*), and many legumes was only possible when fruit abscission or pod dehiscence were delayed therefore retaining the seeds on the stalk (Patterson, 2001). Farmers were able to increase the yield from their crops by extending the harvesting time. Floriculture may also benefit from delayed abscission of floral organs in ornamental plants as consumers may pay more for example, for a rose that retains its petals for weeks rather than days.

The objective of this study was to better understand the timing of structural and compositional changes within the cell wall through the analysis of gene expression in tissues, cells, and organelles as well as the localization of the *DAB5* gene in *Arabidopsis*. I hypothesize that I will see expression of reporter gene constructs associated with *DAB5* in cells involved in cell to cell adhesion as well as changes in the endoplasmic reticulum, Golgi, and plasma membrane as *DAB5* is expressed. Based on preliminary microarray and RT PCR data (Kusner, 2002), I also hypothesize that *DAB5* will be expressed at many stages of development although; the levels of expression are unknown.



Methods and Materials

Plant Material and Growth Conditions

Wild type *Arabidopsis thaliana* plants were of the Wassilewskija (WS) ecotype unless otherwise noted. The *dab5-1* mutant plants were developed from the University of Wisconsin T-DNA AP3:GUS insertion lines (collection # 1807, ecotype WS) (Krysan *et al.*, 1996; www.biotech.wisc.edu/Arabidopsis). Seeds were sterilized (10% bleach and one drop 0.1% Tween 20) and then plated on agarose plates with MS media (Murashige and Skoog, 1962). The seeds underwent a cold treatment for two days at 4°C prior to being transferred to continuous light at 25°C for germination. Seedlings were transplanted in a 2:1 (v/v) mixture of Jiffy Mix soil (Jiffy Products of America, Batavia, IL) and Perlite (Midwest Perlite, Appleton, WI) after one week. The plants were allowed to fully develop at 24°C under a 16-h photoperiod.

β-glucuronidase (GUS) Assay

To obtain a visualization of *DAB5* expression at the tissue level, seedlings containing a

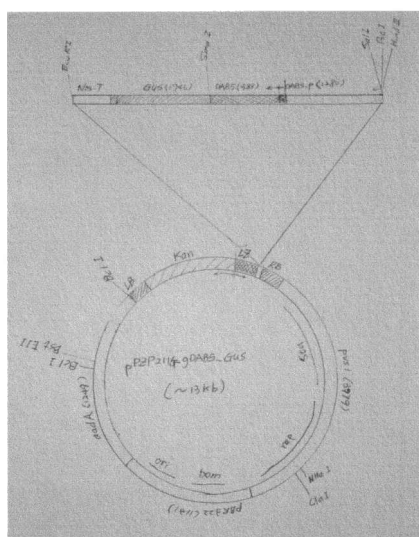
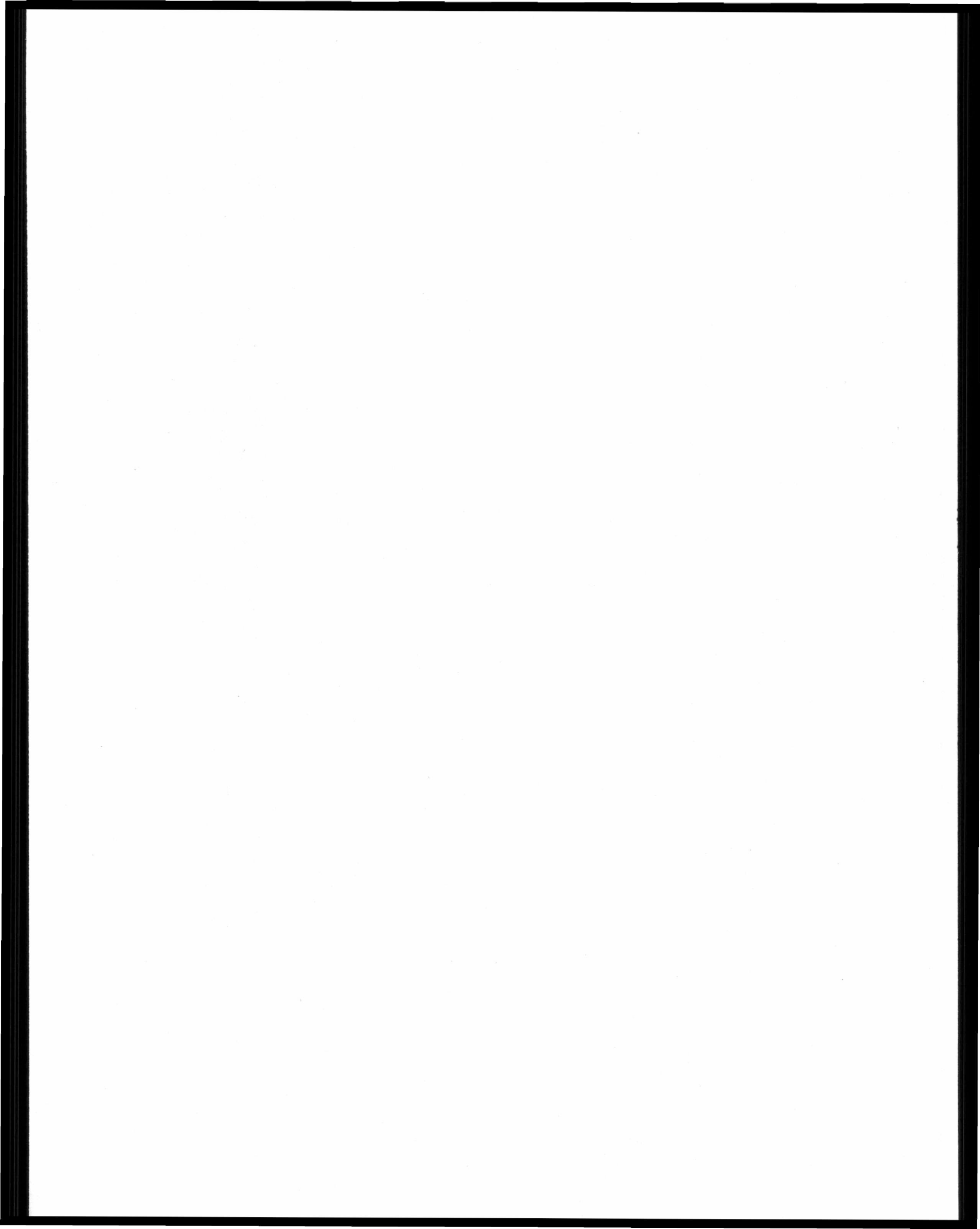


Figure 3. Drawing of *DAB5* promoter-GUS construct. See appendix 1 for larger image.

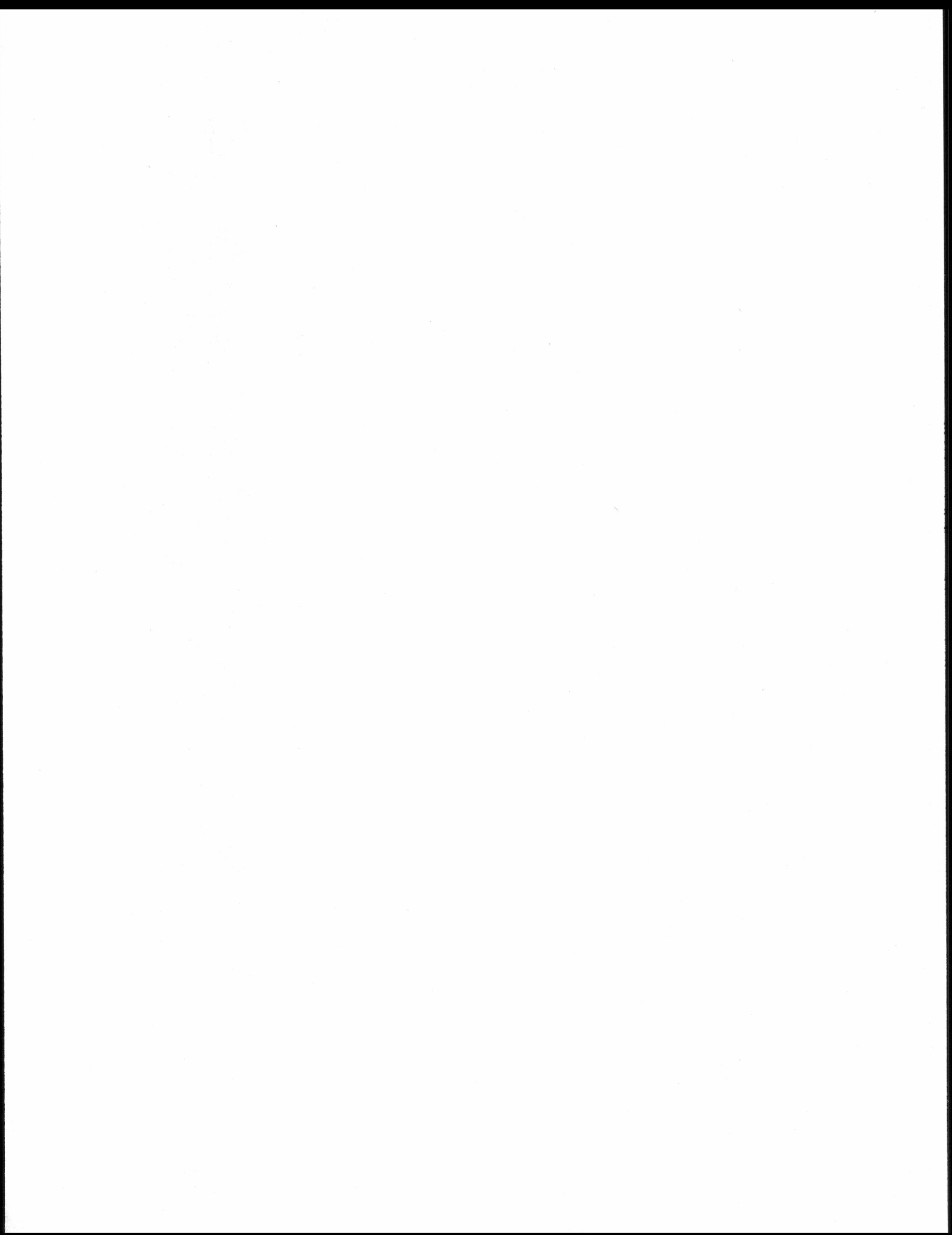
DAB5 promoter-GUS construct (Fig. 3) were harvested every seven days for five weeks for use in the histochemical β-glucuronidase (GUS) assay. The seedlings were then incubated with 0.5 mg/ml X-gluc (Research Organics, Cleveland, Ohio), 0.825 mg/ml ferricyanide, 1.0 mg/ml ferrocyanide, and 0.05% betamercaptoethanol (Jefferson, 1987) for 4-12 hours at 37°C until there were visible signs of blue staining.



The insoluble blue precipitate occurs only in the areas where *DAB5* is expressed. Staining was followed by three rinses with sterile water to remove the X-Gluc buffer. A second set of rinses with 70% ethanol was performed to bleed out the chlorophyll thereby facilitating the GUS visualization (bluestaining). The tissues were then kept in 70% ethanol at 4°C. For comparison the whole mount tissues were examined using an Olympus SZX12 dissecting microscope and photographed using an Olympus DP12 Microscope Digital Camera (Olympus Optical Co., Tokyo, Japan) both prior to and following the ethanol rinses.

Light Microscopy

Flowers at positions 1/2, 4/5, 7/8, 10/11, 13/14, and 15/16 (where position 1 = anthesis) were collected from the primary inflorescences of approximately twelve *DAB5* GUS plants to determine the cellular expression of *DAB 5*. The flowers were placed in 0.4% (v/v) gluteraldehyde (Sigma, St. Louis) in 0.05 M potassium phosphate for collection. After rinsing in potassium phosphate the flowers were placed in X-gluc and were allowed to stain as stated above. Following GUS staining the tissues were fixed in 4% (v/v) gluteraldehyde, rinsed in potassium phosphate buffer, and dehydrated with a graded ethanol series to 100%. The tissues were then embedded in London Resin White resin (Medium grade; Ted Pella, Inc. Redding, CA). Using glass knives on a Sorvall MT-2 ultramicrotome (Ivan Sorvall, Inc., Morwald, CT); 2 μ m-sections were cut and heat fixed to glass slides. Slides were covered with #1.5 coverslips (Corning, Corning, NY), annealed with Cytoseal 60 (Thomas Scientific, Riverdale, NJ), viewed unstained using dark-field microscopy with the Olympus BX60 microscope, and photographed using the



Olympus DP12 Microscope Digital Camera. Images were assembled for figures using Adobe Photoshop 4.0 Software (Adobe, Inc., Mountain View, CA).

Green Fluorescent Protein (GFP)

To determine *DAB5* expression at the cellular level, *DAB5* was fused with a green fluorescent protein (GFP) construct using a 35S CAM~promoter (Fig. 4) (Cutler and

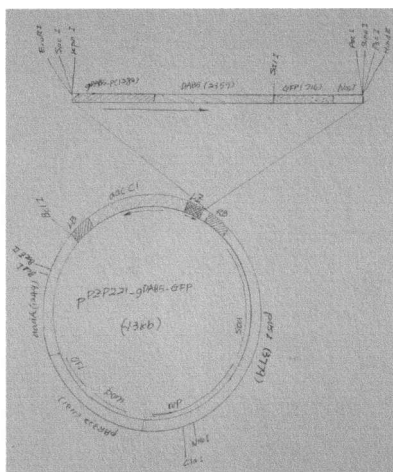
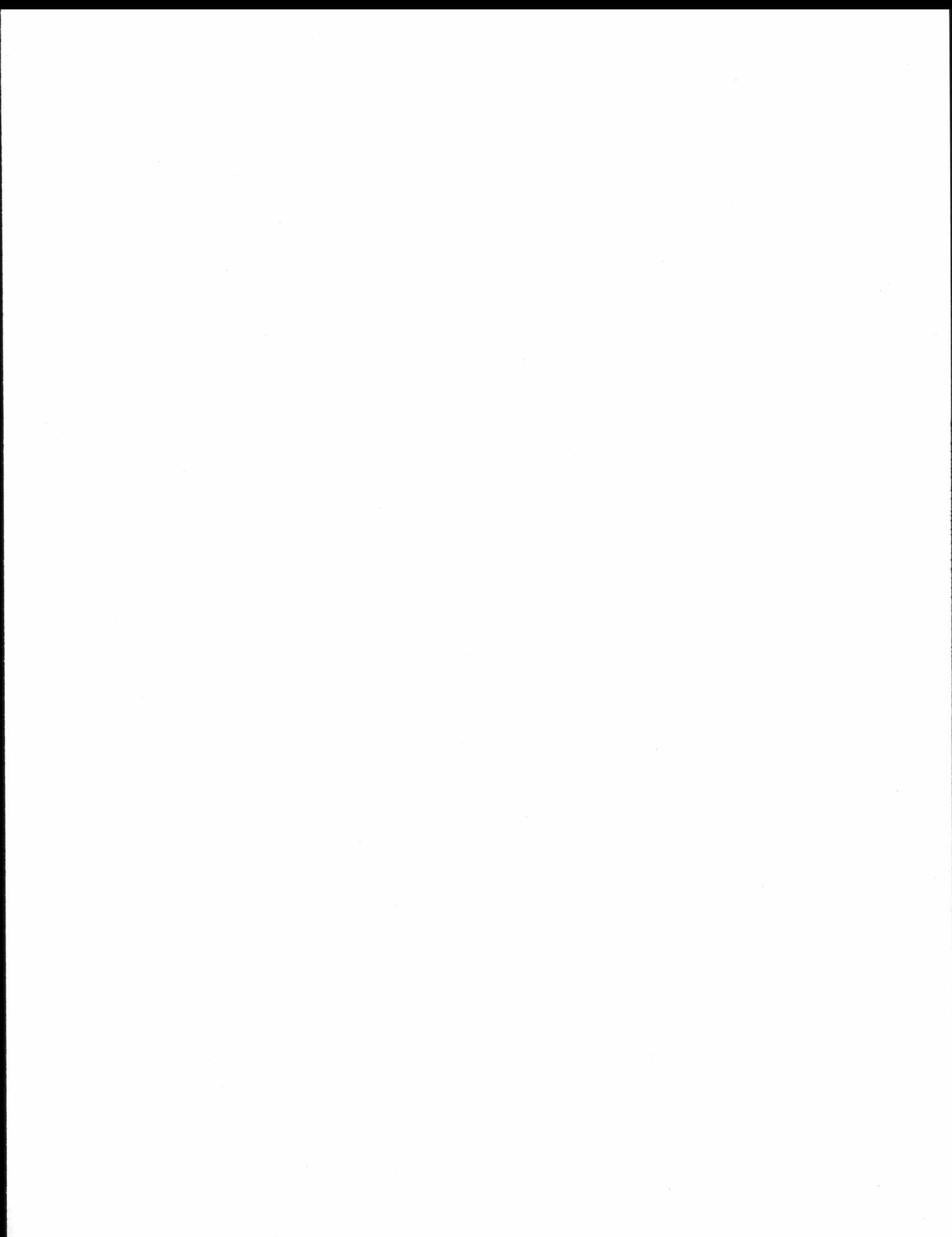


Figure 4. Drawing of GFP *DAB5*-GFP construct. See appendix 1 for larger image.

Ehrhardt, 2000). *DAB5*-GFP whole mount seedlings were first examined under fluorescence with an Olympus BX60 microscope and photographed using the Olympus DP12 Microscope Digital Camera to confirm that the GFP construct was present. The whole mounts were then examined using confocal microscopy (Olympus) at 4, 7, and 14 days. To decrease photobleaching an antifade solution, 1:9 Tris:glycerol, 1 mg/ml p-phenylenediamine (Ruzin, 1999) was added to the slides.

Scanning Electron Microscopy (SEM)

Wild-type and *dab5-1* seedlings were collected at two and seven days into 4% glutaraldehyde in 50mM potassium phosphate buffer, pH 7.4, dehydrated in an ethanol series, and critical point dried in liquid carbon dioxide. The samples were then mounted on aluminum SEM mounts (cat #16222, Ted Pella Inc.), sputter coated with gold palladium and viewed on a scanning electron microscope (model S-570; Hitachi) at a voltage of 10kV.

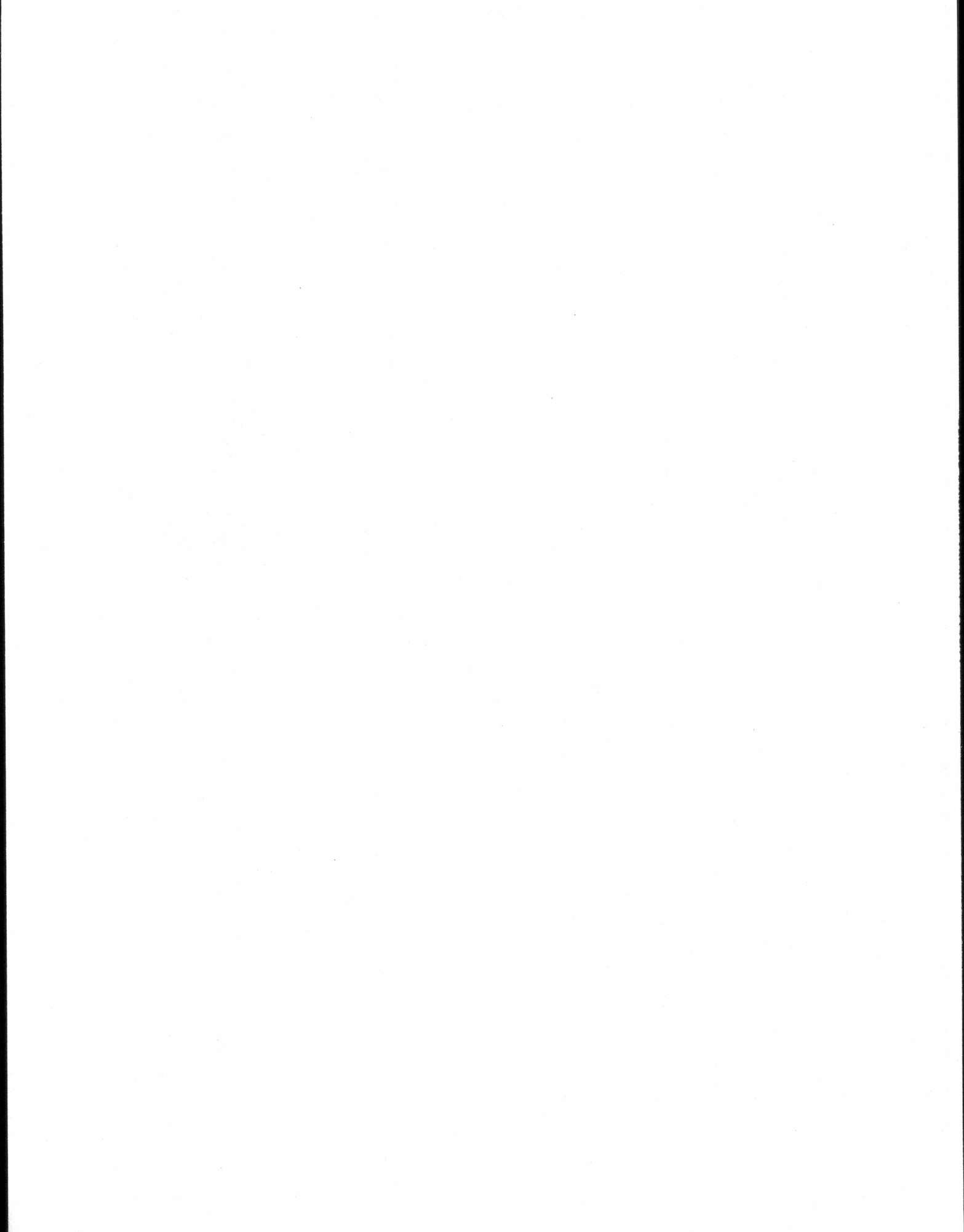


Nucleic Acid Isolation and Quantification

Nucleic acids from *dab5-1* and wild-type (WS ecotype) plant tissue were isolated from abscission zones at positions 1/2, 5/6, 7/8, 9/10, and 13/14 using a modified method of Maniatis *et al* (1982). Tissues were harvested and immediately frozen in liquid nitrogen. The frozen tissues were ground with a mortar and pestle and lysed by vortexing in TES-Lysis (50 mM Tris pH 8, 5 mM EDTA, 50 mM NaCl, 1% (w/v) SDS, 1% (w/v) sarkosyl). Extraction with a phenol:chloroform:isoamyl alcohol mix (25:24:1) followed. Nucleic acids were precipitated at 4°C with isopropanol and 10 M ammonium acetate and ethanol. Samples were resuspended in TE (10 mM Tris, 10 mM EDTA, pH 8.0) and the RNA was separated from the DNA using lithium chloride precipitation. The RNA contained in the LiCl-precipitated pellet was washed with ethanol and resuspended in DEPC-treated H₂O (1 µg/µl). The DNA was precipitated from the supernatant using 10 M NH₄OAc (1/3 volume) and ethanol followed by resuspension in TE. Yields of DNA and RNA were quantified using a Biorad Spectrophotometer and nucleic acid integrity was verified by gel electrophoresis.

RT-PCR

Using the RNA prepared above, first strand cDNA was constructed using 1 µg of RNA treated with DNase. The RNA was incubated with 1 µl Oligo dT (18)(500 µg/ml) and 1 µl 10 mM dNTP mix at 70°C for 10 minutes. Four µl of 5x first-strand buffer (250 mM Tris-HCl, 375 mM KCl and 15 mM MgCl₂), 2 µl of 0.1 M DTT, 1 µl of SUPERSCRIPT II Reverse Transcriptase (200 units/µl) (Invitrogen, Carlsbad, Ca.) were then added and the reaction was incubated at 42°C for 35 minutes and inactivated for 10 minutes at 70°C. One µl of the cDNA was used in 20 µl PCR reactions. Specific primers,



determined by BLAST searches, used for RT-PCR of the *DAB 5* gene were designed (F) RTPCR (5'-ACCACGATCCCAATCCTTTTCGATGA-3') and (R) RTPCR (5'-AACCCATAGACTCAGCAGTGACTCCAA-3').

Results

Expression as Determined by GUS

To determine expression of *DAB5* at the tissue level GUS assays were carried out. Figure 5 (a-c) shows GUS staining at 2, 7, and 14 days. GUS expression was detected in the anthers, roots, and floral organ receptacle; however, there was no detectable *DAB5* expression in the hypocotyl. In mature plants *DAB5*-GUS expression was further analyzed using thin section microscopy. The thin sections showed GUS staining in the abscission zones (Figure 5, d and e) and in the dehiscent zone (data not shown).

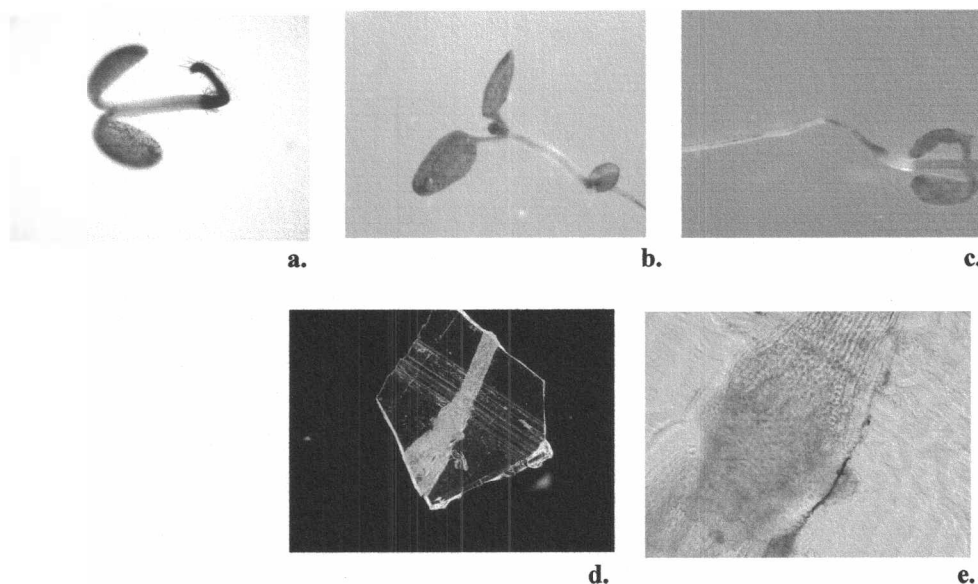
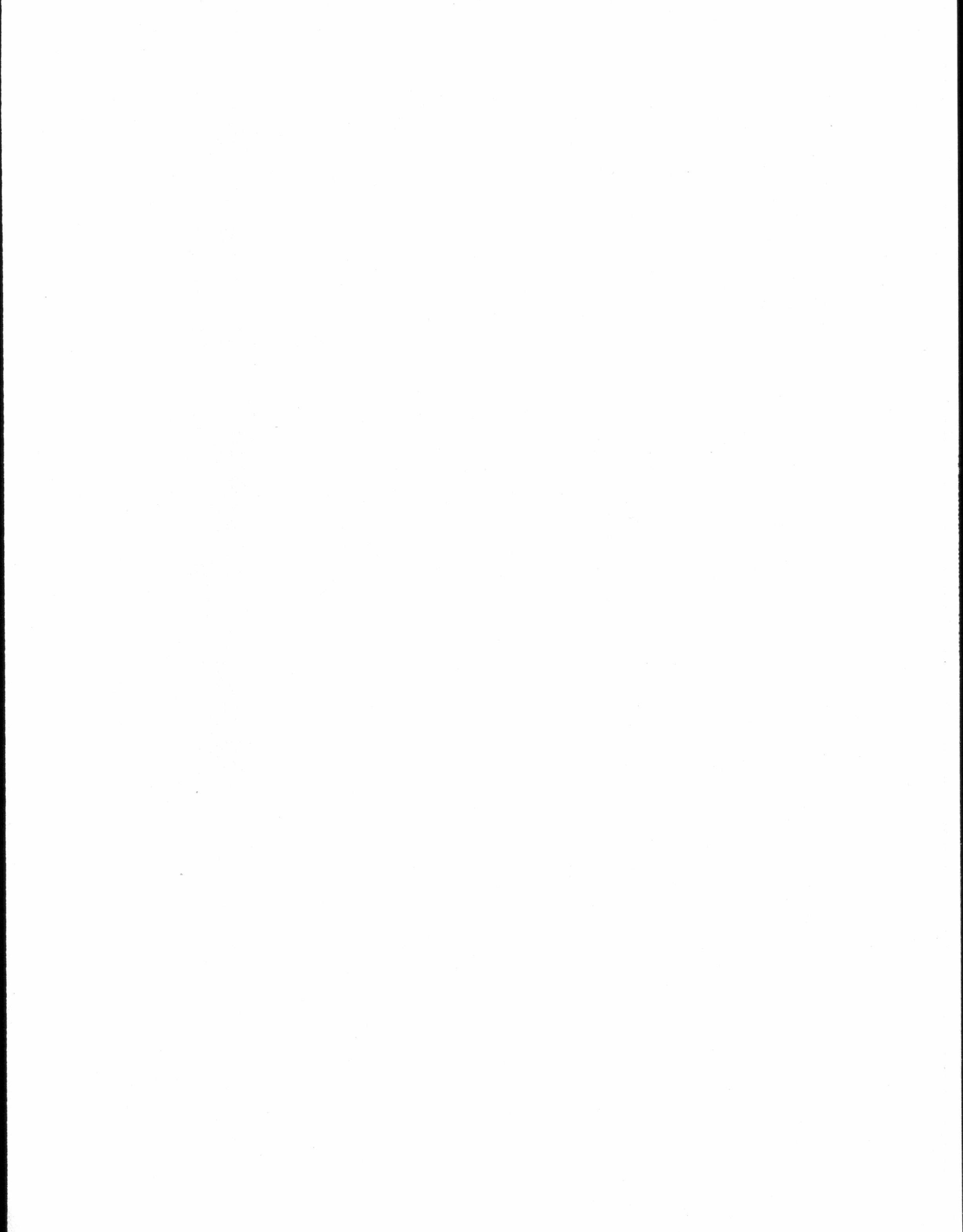
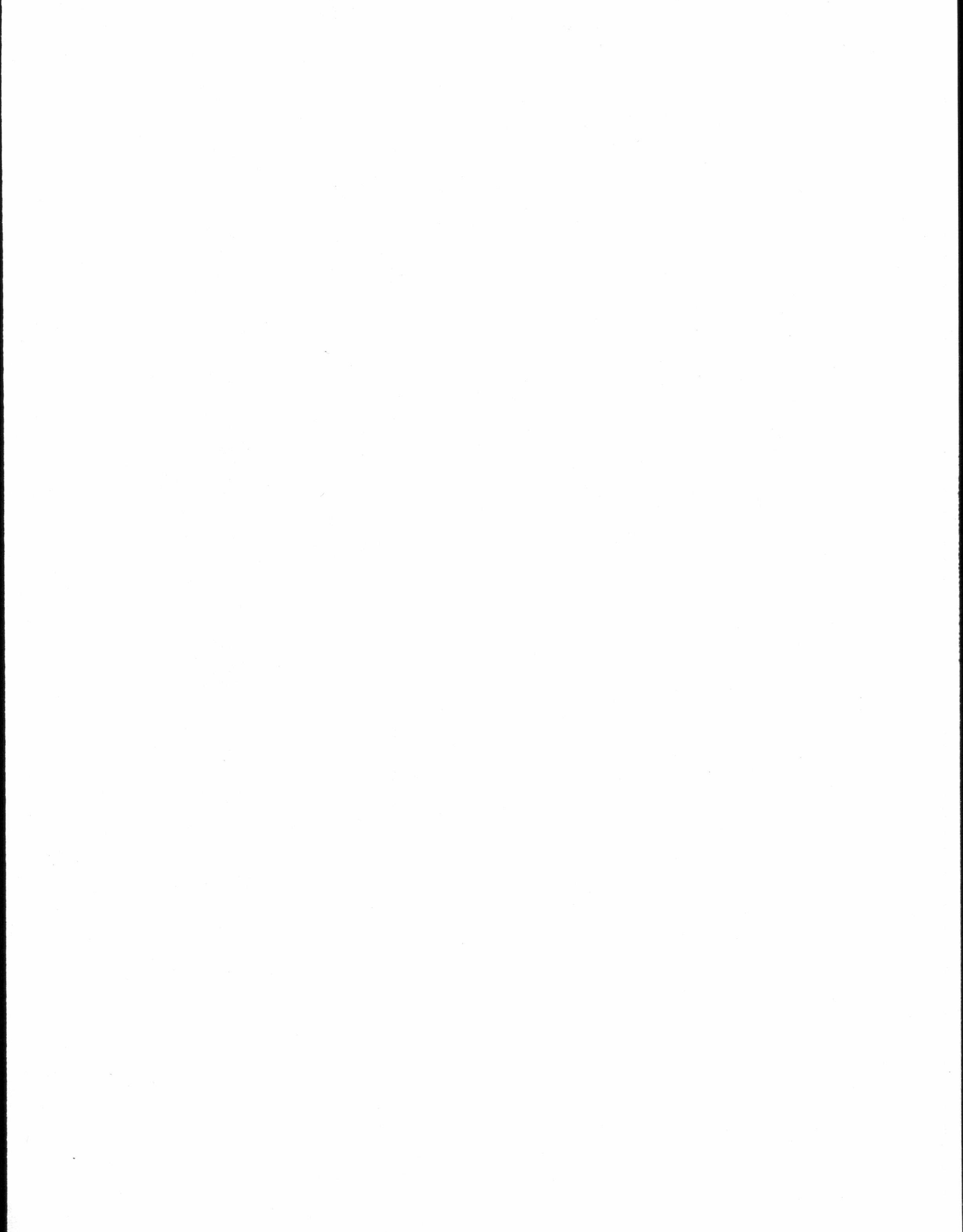


Figure 5. GUS staining of 2 (a), 7 (b), and 14 (c) day seedlings showing GUS expression throughout the tissues with the exception of the hypocotyl. D and e show GUS staining in the abscission zone as seen in thin sections.





Expression as Determined by GFP

DAB5-GFP fusion plants showed *DAB5* expression at the hypocotyl-root junction and in the anthers, floral organ abscission zones and cotyledons (Figure 6). However, the resolutions of the microscopes were insufficient to determine the location of *DAB5* within the cell. GFP seedlings were also analyzed using confocal microscopy as seen in Figure 7. Expression was apparent in the root and at the root-hypocotyl junction. Thus far the abscission zones have not been analyzed using confocal microscopy.

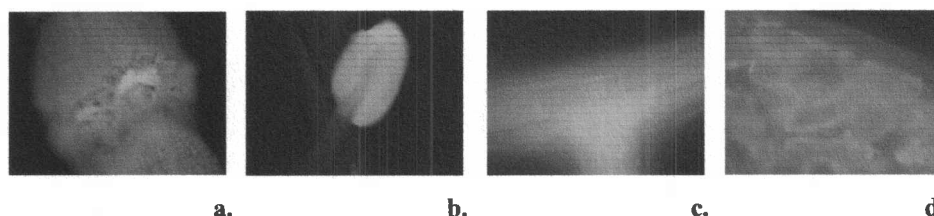


Figure 6. Images taken with epifluorescence on a compound microscope illustrating *DAB5*-GFP expression in the (a) abscission zone, (b) anther, (c) cotyledon, and (d) root/ root tip junction.

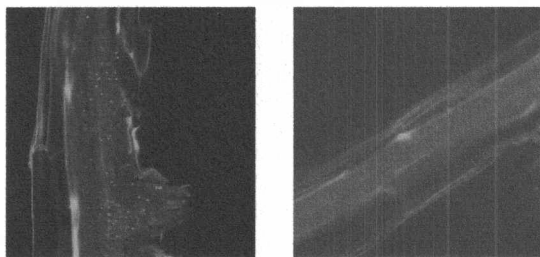
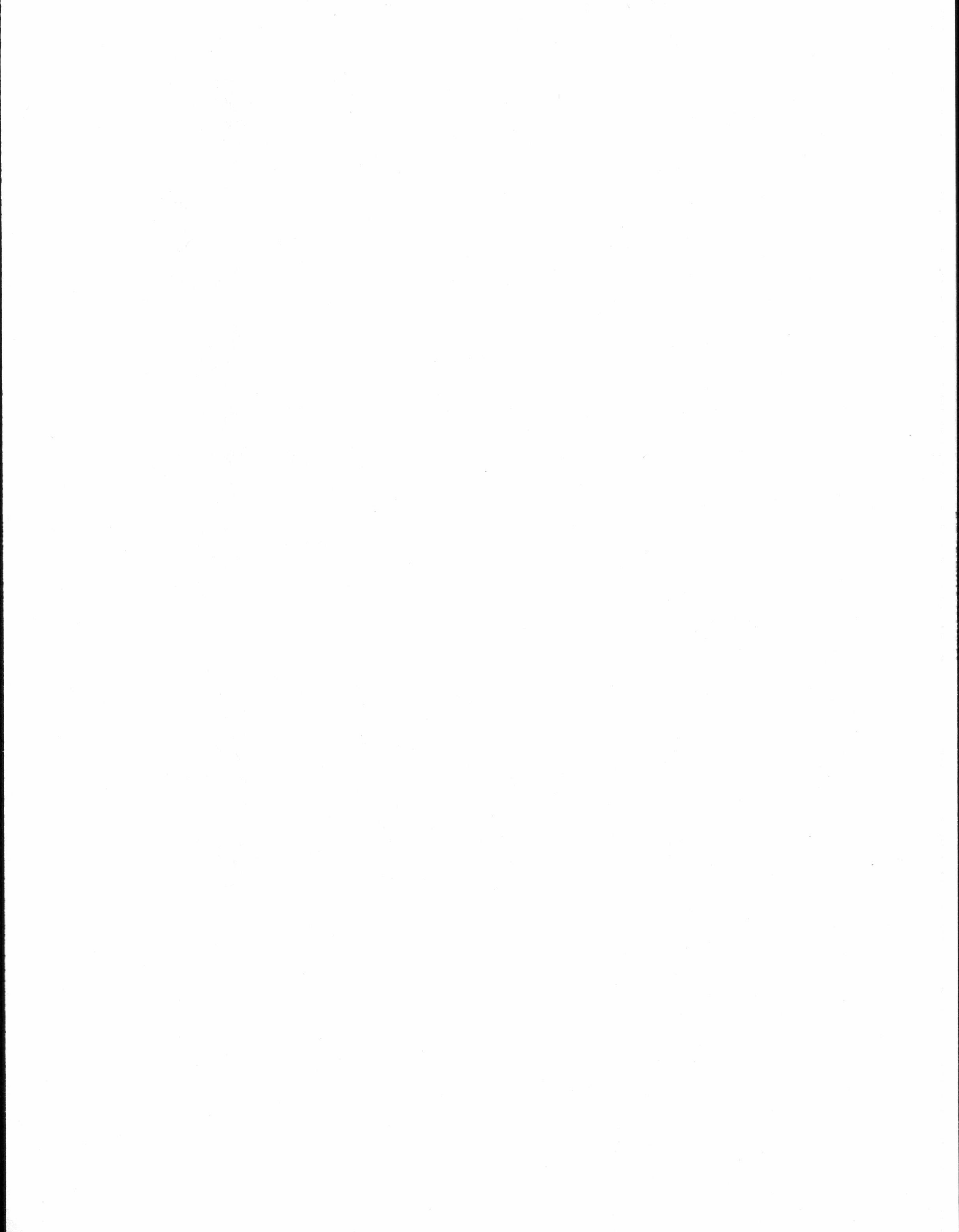
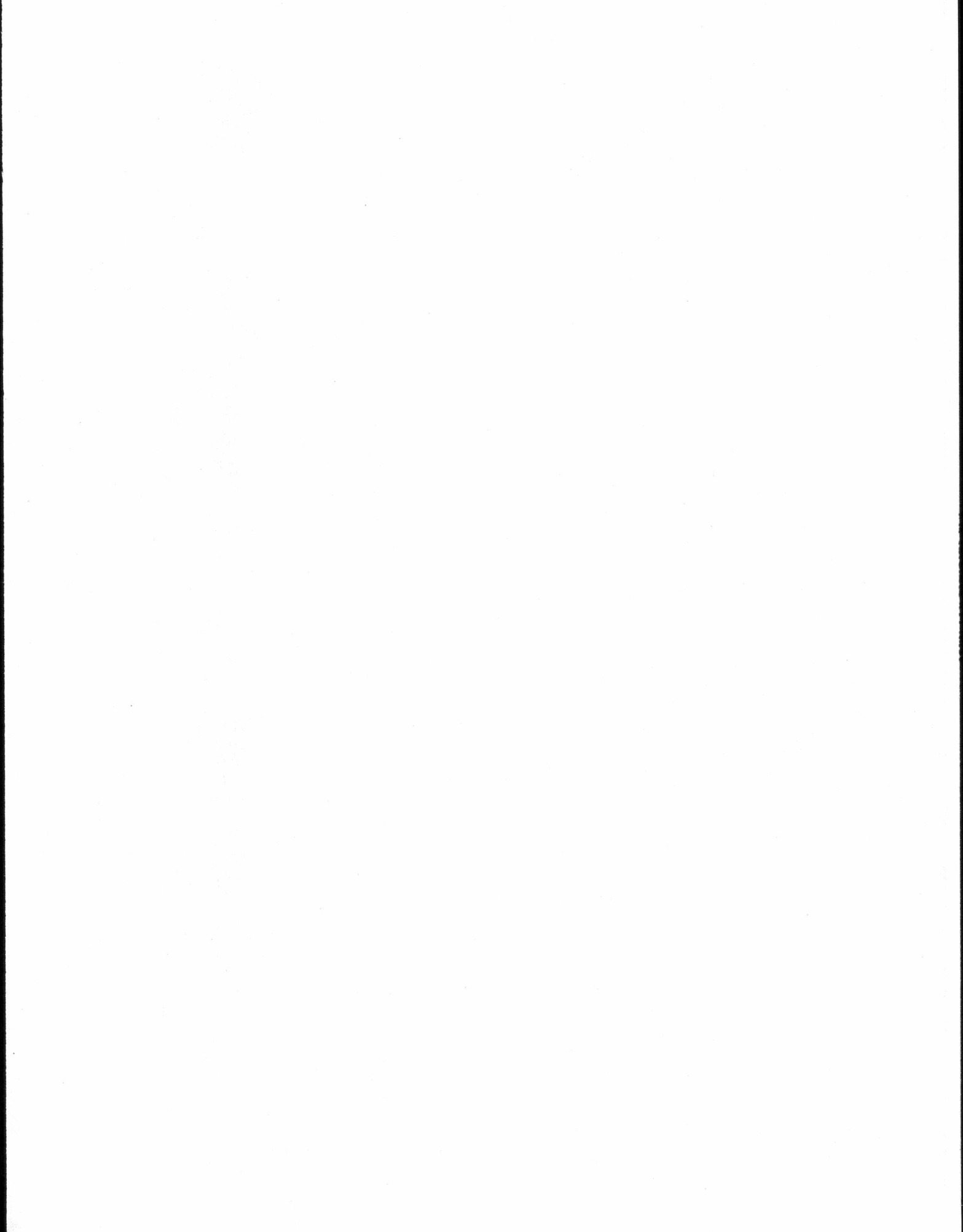


Figure 7. Images taken on the confocal microscope showing *DAB5*:GFP expression in the roots localized to the plasma membrane or cell wall.

Phenotypic Characterization

Scanning electron micrograph images (Figure 8) show no significant difference between *dab5-1* and WS seedlings at two and seven days. I observed root meristem, lateral root, hypocotyl-root junction, and apical meristem tissues (data not shown). Hypocotyl and cotyledon SEM results are shown in Figure 8.





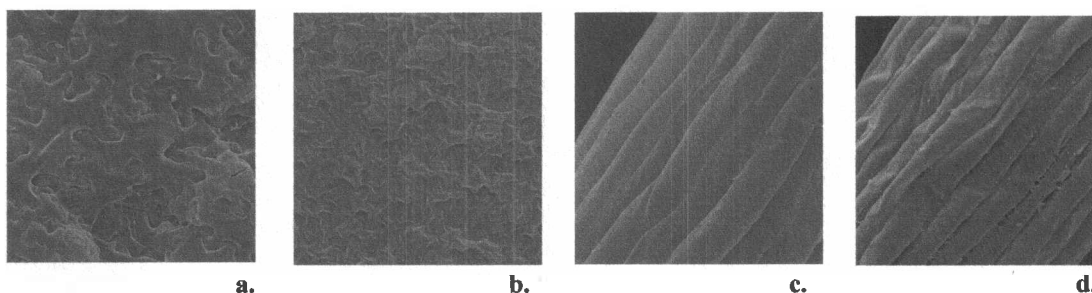


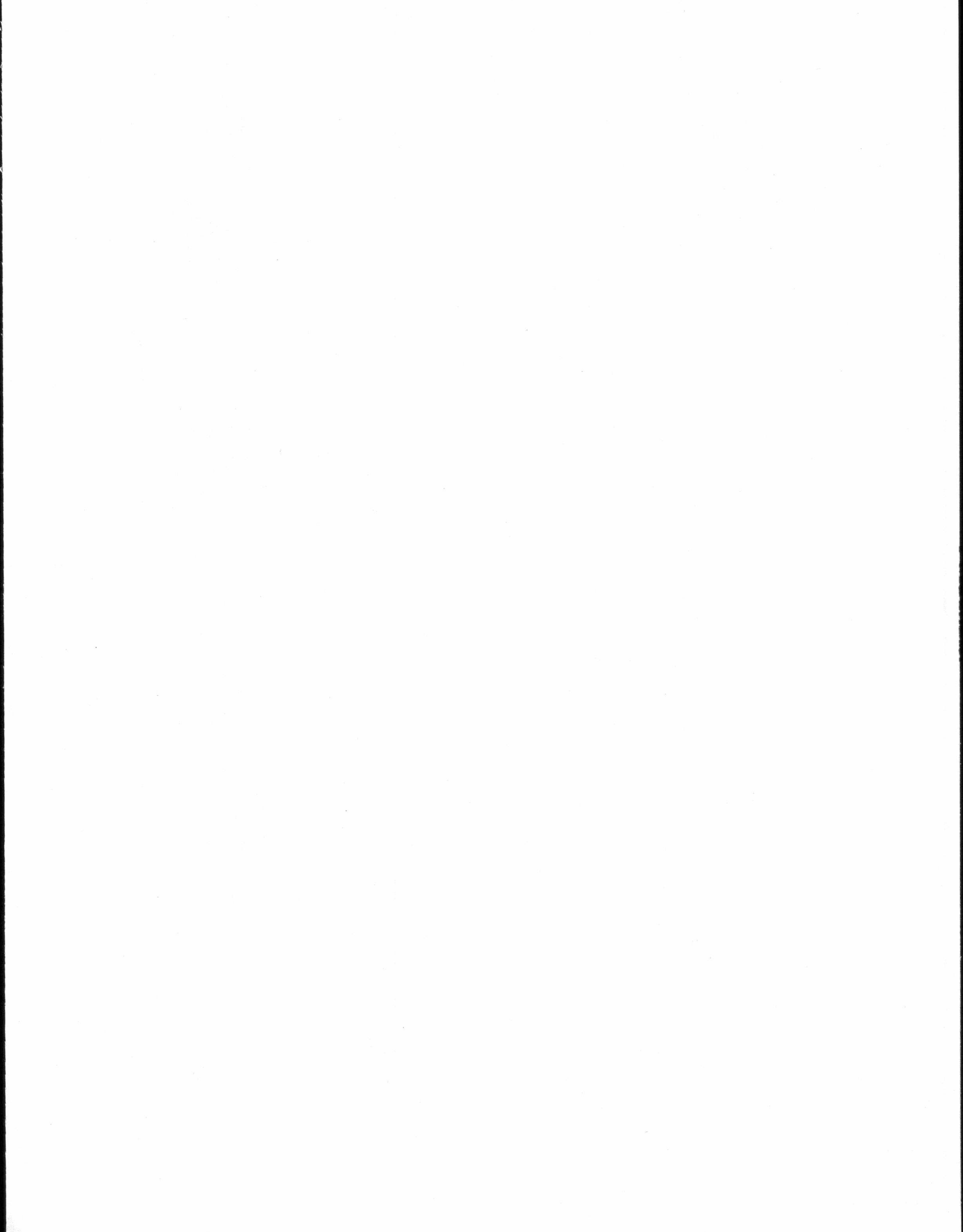
Figure 8. SEM images showing 7 day cotyledons (a,b) and 7 day hypocotyls (c, d). a and c are wildtype while b and d are *dab5-1* images.

RT PCR Quantification

The RT PCR data (not shown) for *DAB5-1* expression in the abscission zones indicate that *DAB5-1* is expressed at positions 5/6, 7/8, 9/10, and 13/14. *DAB5-1* was not expressed in the earlier positions: 1/2, and 3/4. However in the mutant *dab5-1*, *DAB5* is only expressed at position 3/4.

Discussion

The secretory pathway is of great importance to all biological systems as cell-to-cell communication is fundamental to life. In plants many processes of cell separation, including abscission, fruit dehiscence, fruit ripening, and seed germination, are dependent on this pathway. The *DAB5* gene has been identified as a putative secretory carrier membrane protein (SCAMP) primarily involved in vesicle trafficking between the Golgi and plasma membrane (Fernandez-Chacon and Sudhof, 2000). One possible function of *DAB5* is delivering hydrolytic enzymes necessary for cell separation. There are four additional homologous genes to *DAB5* in the *Arabidopsis* genome and all show great sequence similarity as well as a high level of domain conservation (Fernandez-Chacon and Sudhof, 2000). However, RT-PCR and microarray indicate that there is variance in



the amount of expression of each gene in various tissues suggesting that regulatory factors may play a large role in the function of the individual genes (Kusner, 2002).

The goal of this research was to look more specifically at *DAB5* expression during different stages of development to better understand its function in vesicle trafficking in the secretory pathway. Microarray data have shown expression of *DAB5* at many developmental stages; and as vesicle trafficking is essential to plant function, I anticipated *DAB5* to be expressed throughout the life of the plant. However, during specific developmental stages of growth, such as abscission, one might hypothesize unique regulation of the secretory pathway and subsequent increases in expression of *DAB5*. It is interesting to note that *DAB5* is the only one of these five closely related genes that shows expression in abscission. Experiments were designed to look at the tissue, cellular, and organelle levels of *DAB5* expression.

A *DAB5*-GUS construct was transformed into wildtype and *dab5-1* plants to provide a means of visual analysis of *DAB 5* expression at the tissue and cellular levels. In wildtype high levels of *DAB5* expression were found in the cotyledons, meristem, roots, root tip, abscission zone and anthers; however, there was no *DAB5* expression in the hypocotyl (Figure 5). The hypocotyl is laid down during embryogenesis; thus other genes in the secretory pathway may be regulating cell-to-cell adhesion and cell separation. It is also possible that one of the other homologous SCAMPs are involved in the regulation of abscission processes in the hypocotyl, thus providing compensation in the mutant *DAB5* plants. This hypothesis also coincides with the lack of GUS expression in seeds. Seedlings screened at 2, 7, and 14 days showed similar patterns of expression as seen in Figure 5. Originally 7 and 14 day seedlings were tested for expression and they

showed no detectable GUS expression in the hypocotyl. Experiments using two day seedlings were then performed to determine whether GUS expression was decreasing in the hypocotyl as the seedling aged; however this hypothesis was not supported since the same pattern of expression was seen at 2, 7, and 14 days. Additional testing using *dab5-1* mutant plants and double and triple SCAMP mutants may elucidate further understanding of the lack of expression in the hypocotyl.

Green fluorescent protein (GFP) was fused to *DAB5* to allow for an alternative analysis of *DAB5* expression. Seedlings showed similar expression patterns to GUS seedlings with a high GFP signal in the roots and cotyledons and a low signal in the hypocotyl (Figure 6, c and d). Examination of mature plants showed GFP expression in the abscission zones as well as in the anthers (Figure 6, a and b). Although the GFP experiments confirmed *DAB5* expression at the tissue level, an additional goal of using GFP was to examine expression at the cellular and organelle levels. The confocal microscopy data show that *DAB5* is localized to either the cell wall or plasma membrane (Figure 7). Since I was not able to obtain a great enough resolution on the microscope to differentiate the location of expression it would be useful to increase the cellular specificity of GFP markers for specific organelles, such as the vacuole or plasma membrane, as well as the cell wall by using antibodies to indicate function.

In response to my findings of the lack of *DAB5* in the hypocotyl, I examined two and seven day seedlings using scanning electron microscopy to look for differences in cell structure. Preliminary analysis of cotyledon, hypocotyl, and root tissues showed no discernable differences between WT and *dab5-1* plants (Figure 8) as the wrinkles seen in the *dab5-1* hypocotyl (d) were most likely due to the fixation process. This is not

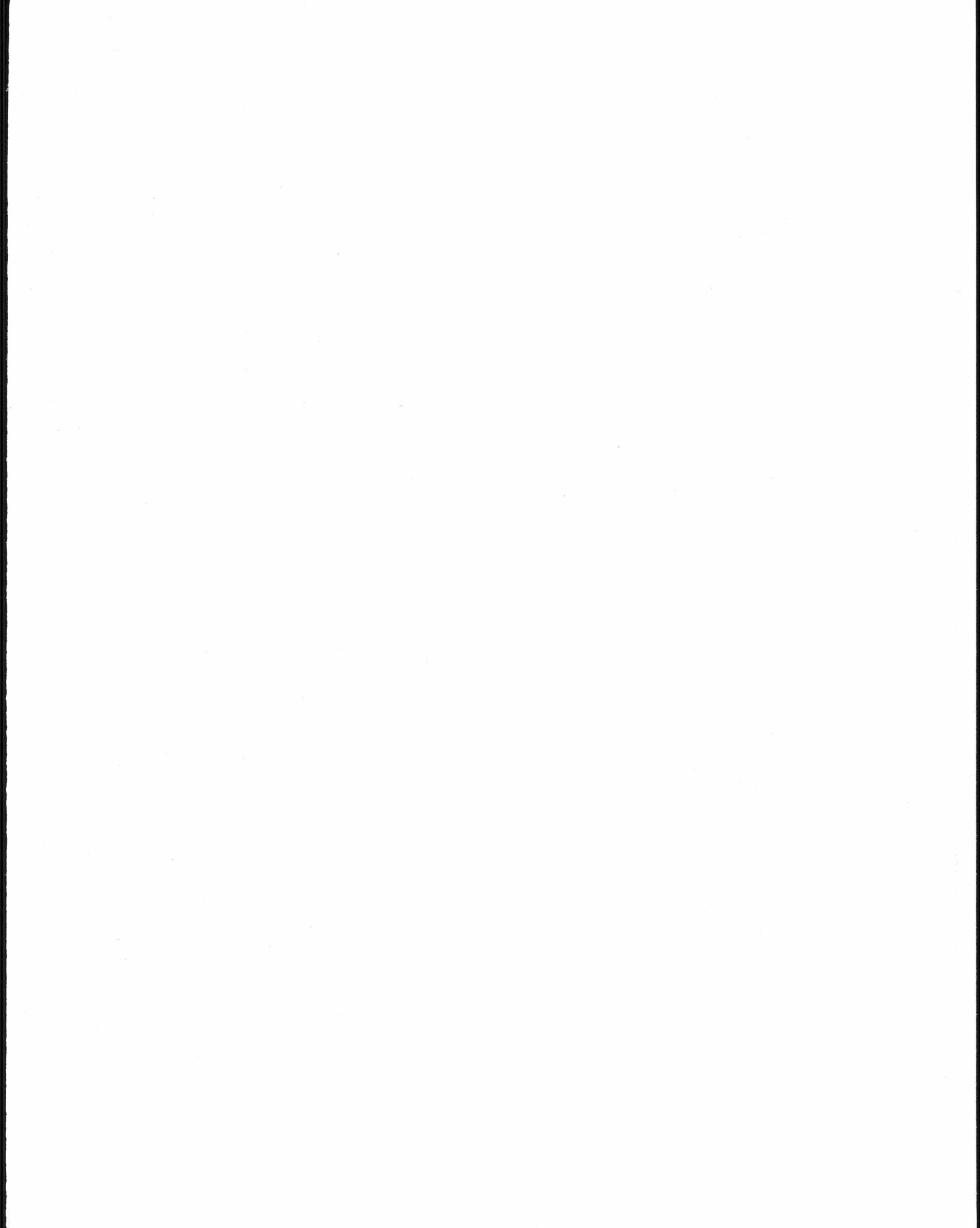
surprising as *dab5-1* plants are phenotypically normal in every way except for the delayed abscission phenotype. Although *dab5-1* delays abscission, abscission still occurs. I hypothesize that in tissues with expression but no phenotypic difference from wildtype, the products transported by *DAB5* may be compensated for by the other homologous genes or *dab5-1* may be working at a reduced efficiency. I suggest that this is due to a mutation that may have changed gene regulation, as it sits in the promoter of the gene. Overall, the integrity of the cell wall and the cell structure is not phenotypically altered in the *dab5-1* mutant.

The RT PCR abscission zone data indicate that *dab5-1* is expressed in the later stages of development, namely positions four through fourteen. It is possible that the lack of *DAB5* expression at the early positions is indicative of compensation by one of the other SCAMPs thereby giving reason for the lack of *DAB5* expression in the hypocotyl. Further testing of expression by the other four SCAMPs in the abscission zones has yet to be completed.

In summary, *DAB5* expression is detectable in all tissues except the hypocotyl during the early stages of development. Further studies are being conducted to determine expression in mature plants. The GUS stained tissues prepared for thin sectioning will be utilized for transmission electron microscopy and to determine if specific changes in the vesicles or plasma membrane can be observed. The lack of expression of *DAB5* in the hypocotyl may be caused by compensation by other genes of the secretory pathway or by the lack of function of *DAB 5* in the hypocotyl. Knockouts of homologous genes are being isolated along with the generation of double and triple mutants to test overlapping function between the five closely related genes.

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APPENDIX 1

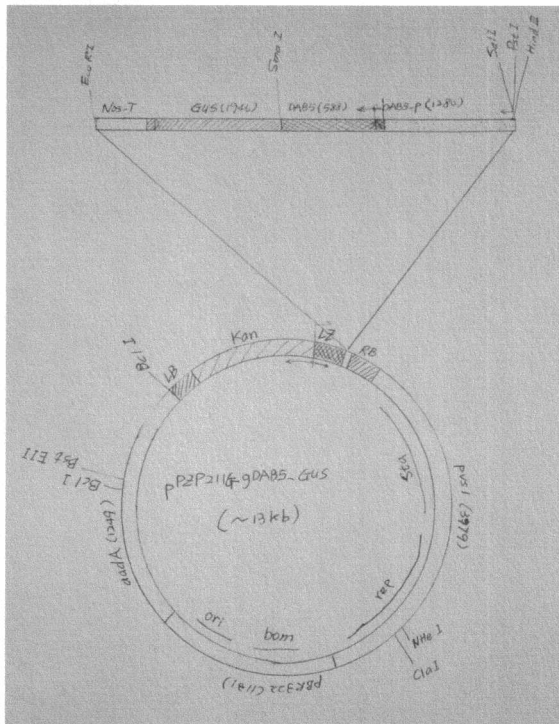


Figure 3. Drawing of *DAB5* promoter-GUS construct.

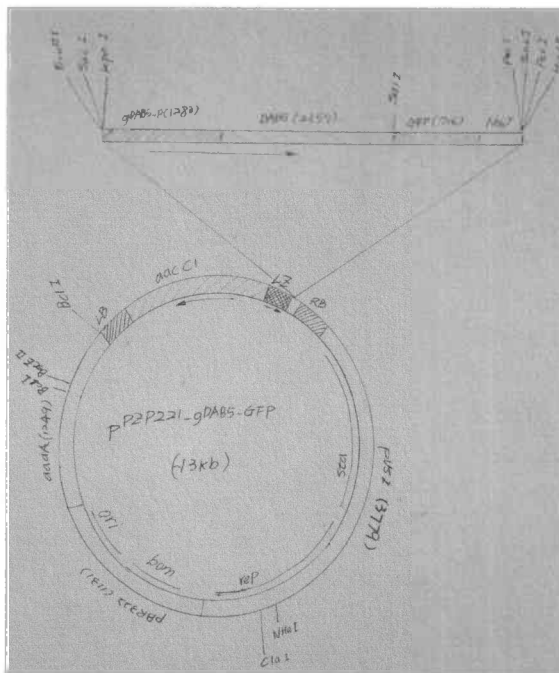
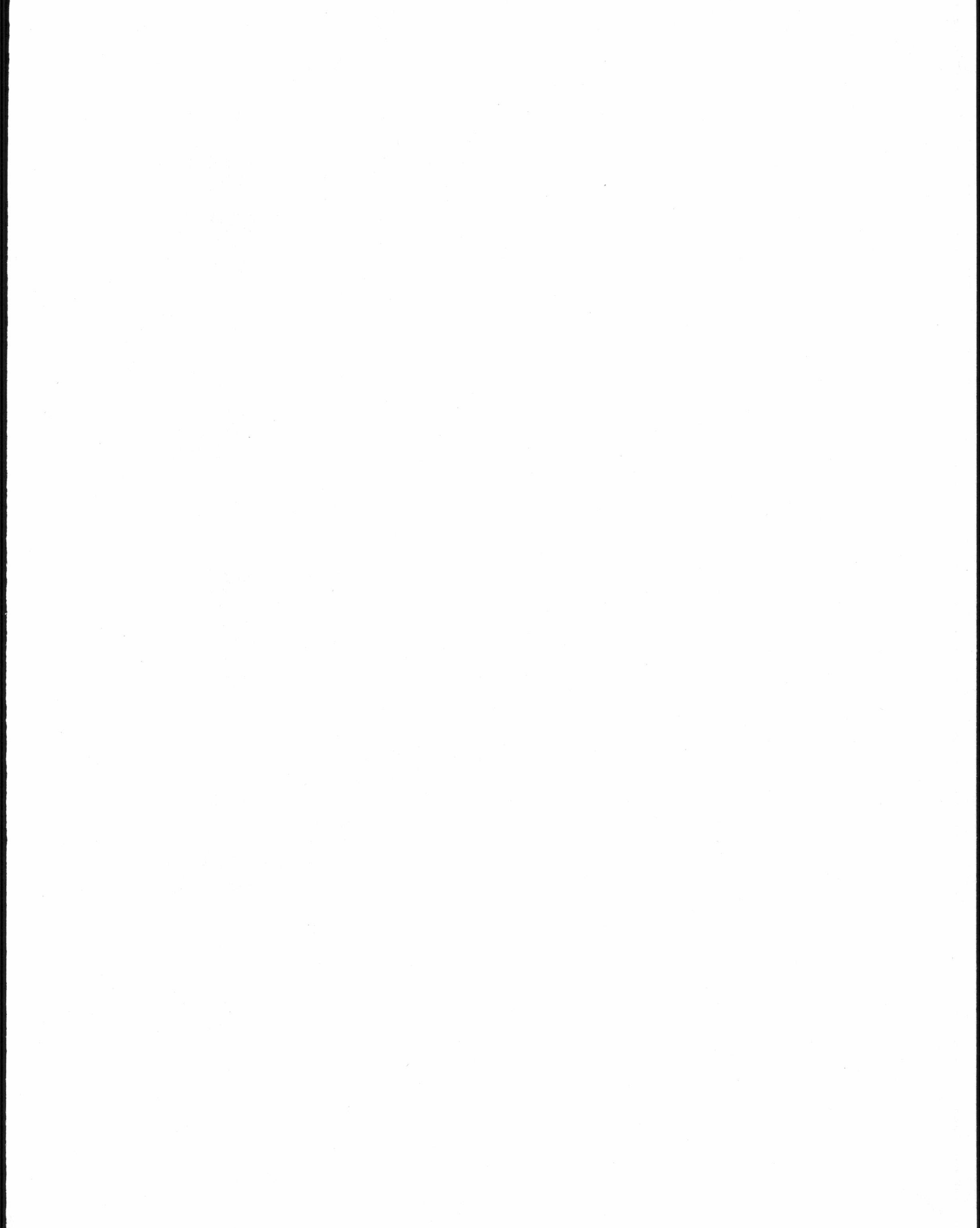


Figure 4. Drawing of GFP *DAB5*-GFP construct.



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