AN ABSTRACT OF THE THESIS OF

Alvaro R. Monteros for the degree of Master of Science in Crop Science presented on December 6, 2002.

Title: Dormancy and Germination of True Potato (*Solanum tuberosum* L.) Seeds: Characterization of Endo-β-Mannanase Genes.

Abstract approved

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True potato (*Solanum tuberosum)* seed (TPS) is used for preservation of variable genetic lines of wild and cultivated potatoes (Hawkes et al., 2000) and for propagation of food crops in some developing countries. TPS has advantages over seed potato tubers in storage and transportation and favors lower virus infection levels in fields. However, TPS has thermodormancy and will not readily germinate at 25°C and above (D'Antonio and McHale, 1988; Pallais, 1995a, b; Alvarado et al., 2000). TPS can be extremely unreliable when planted directly in fields due to poor emergence related to diseases and soil crusting.

Germination tests were conducted with two lots of TPS derived from cvs. *EB-8109* and *All Blue*, respectively, to study dormancy mechanisms. Seeds were germinated under four temperature regimes (10°C, 15°C, 20°C and 25°C). The two lots showed distinctly different germination characteristics. *EB-8109* seeds showed only thermodormancy whereas *All Blue* seeds showed very deep dormancy.

A carotenoid synthesis inhibitor, fluridone, which blocks abscisic acid (ABA) synthesis, effectively broke thermodormancy in *EB-8109* TPS but did not break primary dormancy in *All Blue* seeds. Additional treatments, including pre-chilling and hormonal regimes, also failed to break *All Blue* deep dormancy. When the micropylar region of the endosperm (endosperm cap) was removed from seeds of both seed lots, radicle elongation was observed, suggesting that mechanical resistance from the endosperm cap restrains radicle protrusion, and that weakening of the endosperm cap is requisite for TPS germination.

Endo-β-mannanase expression was measured to help characterize mechanism underlying the weakening of endosperm cap tissues. This enzyme is thought to permit radicle protrusion by degrading cell walls thereby weakening the tissues of the endosperm cap (Groot et al., 1988). The coding region of germination-specific mannanase was isolated from the potato genome by use of polymerase chain reaction (PCR) with primers specifically designed for the tomato germination-specific mannanase gene (*LeMAN2*, Nonogaki et al., 2000). The cDNA of the TPS mannanase was identical to that of *LeMAN2*. The expression of mannanase mRNA was detected in the endosperm cap of germinating TPS after 72

h of imbibition at 15°C, while no expression was detected at 25°C (thermodormant condition). Fluridone induced mannanase expression in the micropylar region of the endospem at 25°C. Thus, there was a correlation between induction of mannanase and dormancy breakage.

A major increase in TPS post-germinative endo-β-mannanase activity was detected by use of gel diffusion assay. Two isoforms of mannanases were detected in the protein extracts of germinated TPS by activity staining of native polyacrylamide gel electrophoresis. The post-germinative mannanase was detected in the whole endosperm of germinated TPS by using tissue printing with the *LeMAN1* (Bewley et al., 1997) RNA probe. These results suggest that, as with tomato, TPS also expresses post-germinative mannanase activity.

The promoter region of a new tomato mannanase was isolated during this research. This promoter was shown to be involved in anther-specific expression of mannanase.

Dormancy and Germination of True Potato (*Solanum tuberosum* L.) Seeds: Characterization of Endo-β-Mannanase Genes

by
Alvaro R. Monteros

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CONTRIBUTION OF AUTHORS

Dr. Sergei Filichkin, Department of Crop and Soil Science and Dr. Jeffrey Leonard, Department of Environmental and Molecular Toxicology at Oregon State University were involved in the experiments described in Chapter 5.

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DEDICATION

To my loved wife Emilia and my little daughter Domenica for encouraging me to complete this work. Even though we were physically distant part of this time, we were really close in our hearts.

To my parents Manuel and Alicia and my sisters Aracely, Ivanova and Priscila for their moral support from the distance.

Dormancy and Germination of True Potato (*Solanum tuberosum* L.) Seeds: Characterization of Endo-β-Mannanase Genes

Chapter 1

General Introduction

Alvaro R. Monteros

Potato (*Solanum tuberosum* L.) is widely cultivated in many countries and ranks fourth in food production after, wheat, maize and rice (Hawkes and Hjerting, 1989; Hawkes, 1990). Potato grows under very diverse agro-ecological conditions (Struik and Wiersema, 1999). Domestication first occurred in the Andes of Peru and Bolivia, where the largest diversity of cultivated potato is observed; a minor center also occurs in southern Chile (Hawkes and Hjerting, 1989; Ortiz, 2001). Wild species of potatoes are widely distributed from the southern United States southward through Mexico, Central America and the Andes; however, the main centers of diversity are situated in south-central Mexico and the Andes (Hawkes and Hjerting, 1989; Struik and Wiersema, 1999; Hawkes et al., 2000). The International Potato Center (Lima, Peru) has assembled the World Potato Collection, which includes 5,000 classified cultivated accessions and 1,500 wild accessions (CIP, 1984). Different cultivars of potatoes produce diverse tuber characteristics.

In conventional seed potato production systems, potatoes are vegetatively propagated by means of "seed tubers". True potato seeds (TPS) are the genuine botanical seeds of potato. TPS are extracted from tomato-shaped berries of approximately 2-3 cm in diameter that grow in inflorescences (Fig. 1). Individual true seeds measure 1.2-1.8 mm and weigh on average 50-100 mg per 100 seeds (Fig. 2) (FAO, 1991; Struik and Wiersema, 1999; Upadhya and Cabello, 2000).

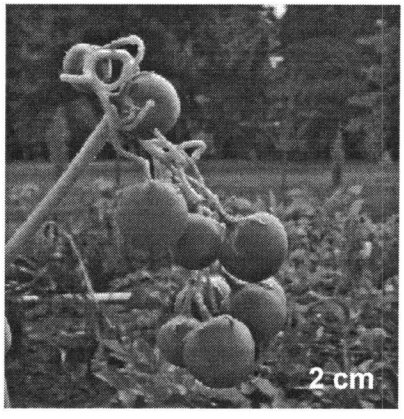

Figure 1. Potato fruits. Plant were field-grown in Corvallis Oregon.

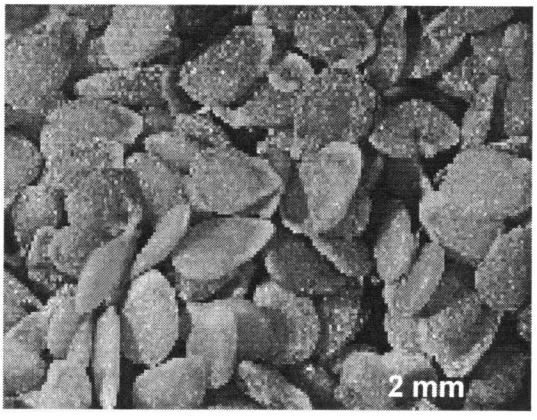

Figure 2. True potato seeds (TPS) Sexual reproductive botanical or "true potato seeds.

The use of TPS reduces planting and storage costs, and also minimizes tuber-transmitted diseases such as viruses (CIP, 1984; Struik and Wiersema, 1999). As little as 100 grams of TPS will plant as much land as two tons of tubers (Pallais et al., 1989). TPS can be stored for many years with very little loss in germination whereas seed tubers last one season at best.

Research on potato production from TPS was first initiated by the International Potato Center (CIP) in Lima, Peru in 1977 (CIP, 1984; Pallais, 1995a). TPS use has been increasing in tropical and subtropical regions (Pallais, 1995b). Studies on commercial acceptability and profitability of TPS have also been conducted (Upadhya, 1994; Chilver et al., 1994). Some relatively stable and uniform hybrids are available and currently grown from TPS in many parts of the world (CIP, 2000). Numerous cultivated and wild species of potatoes are being

conserved long-term as TPS in national gene banks, including ARS/USDA collection at Sturgeon Bay, Wisconsin, the National Autonomous Institute for Agricultural Research, National Department of Plant Genetic Resources and Biotechnology in Ecuador (INIAP, DENAREF) and many others (FAO, 1998).

TPS germination is very important for regeneration of potato genetic resources in many gene banks as well as for commercial potato production. TPS is known to germinate poorly at relatively high temperature (25°C and above), due to a condition called "thermodormancy" (Alvarado et al., 2000). Thermodormancy is problematic in field production and regeneration of potatoes. Characteristics of TPS thermodormancy were investigated in this research and results are presented in Chapter 2.

Previous studies on tomato (*Lycopersicon esculentum* Mill.) seeds have shown that endo-β-mannanase enzymes are involved in hydrolysis of mannan-rich cell walls of the endosperm during and following germination (Nonogaki et al., 1995; Bewley et al., 1997; Nonogaki et al., 2000). In tomato, since the micropylar region of the endosperm (endosperm cap) provides mechanical resistance to the radicle, weakening of the endosperm cap is required for radicle emergence (Nonogaki et al., 2000). Tomato and potato are closely related genetically (Bonierbale et al., 1988, Tanksley et al., 1992). TPS morphology is very similar to that of tomato seeds (see Chapter 3). Therefore, it is likely that similar mechanisms of endosperm weakening occur in potato seed germination. Although tomato seeds have become a model system for germination research, they do not have deep

dormancy. Therefore, TPS may be a very good model system for seed dormancy research. Research on TPS has significant merit in basic seed science as well as in economically important agricultural applications.

It was shown by RNA gel blotting that a tomato mannanase homologue is expressed in imbibed TPS, although the cDNA of potato mannanase has not yet been isolated (Alvarado et al., 2000). Isolation and characterization of the cDNA of germination-specific mannanase from potato (*StMAN2*) are described in Chapter 3. The expression of this gene in the endosperm and dormancy breakage of TPS are discussed in this chapter.

It is also known that endo-β-mannanases are expressed in germinated seeds of tomato (Nonogaki et al., 1995) and lettuce (Nonogaki and Morohashi, 1999). The expression of mannanases in the endosperm after germination is thought to be significant for reserve mobilization of cell wall mannans to support seedling establishment. Since seedling establishment is an important stage for crop production, post-germinative mannanases in TPS were also characterized in this study. Biochemical characterization of post-germinative mannanases is described in Chapter 4.

In the course of this study on seed-specific endo-β-mannanase genes, new mannanase genes were found in the potato and the tomato genome. These related mannanase genes are characterized in Chapter 5. The promoter regions of these genes are analyzed by using reporter gene in transgenic *Arabidopsis*. Possible

physiological roles of these mannanases and potential applications of the gene promoter in basic and applied seed biology are discussed.

General conclusions from the entire study are described in Chapter 6.

Chapter 2

Germination and Dormancy of True Potato (*Solanum tuberosum* L.) Seeds

Alvaro R. Monteros, Alvin R. Mosley and Hiroyuki Nonogaki

Unpublished

INTRODUCTION

Visible radicle penetration of the structures surrounding the embryo is used as a criterion for germination (Bewley, 1997). However, in a physiological and biochemical sense, seed germination *sensu stricto* encompasses only the events occurring in imbibed seeds prior to radicle emergence (Bewley and Black, 1994). That is, one can recognize the occurrence of germination only by observing radicle emergence, which is the result of germination. On the other hand, dormancy is the inability of the embryo to germinate because of some inherent inadequacy (Bewley and Black, 1994). Although significant progress has been made in understanding mechanisms of seed germination in the last decade, the mechanism of seed dormancy is not yet fully understood. Intensive research on mechanisms of seed dormancy is needed. For this purpose, a good model system for seed dormancy is necessary. TPS may present such model.

Mature TPS typically undergo a period of primary-dormancy lasting from 6 to 18 months after extraction from the berries (D'Antonio and McHale, 1988; Pallais et al., 1991; Pallais, 1995b; Struik and Wiersema, 1999). The duration of TPS dormancy depends on stages of seed development, seed moisture content, storage temperature and genotype (Simmonds, 1964; D'Antonio and McHale, 1988; Pallais et al., 1989; Pallais et al., 1991; Pallais, 1995b; Struik and Wiersema, 1999). After the first deep dormancy is broken, TPS begin to germinate at relatively low temperature but still will not germinate at high temperatures. This condition is

called "thermodormancy". Thermodormancy is very obvious and can be used as a good model system for biochemical and molecular biological research to elucidate the mechanisms of seed dormancy. Basic phenomena of TPS thermodormancy are investigated in this chapter.

MATERIALS AND METHODS

Plant Materials

Seeds of two potato (*Solanum tuberosum* L.) clones, *EB-8109* and *All Blue* were used in this study.

Seeds of *EB-8109* were extracted from berries by maceration in a blender and yeast-fermentation in water for two days. Seeds were then removed and dried to 4.8% moisture content (MC). *EB-8109* seeds were stored at room temperature for approximately 1 year and then stored in a 15 ml centrifuge tube at -20°C before testing.

All Blue berries were harvested from randomly selected plants in the field (Lewis-Brown Farm, Oregon State University, Corvallis) in August, 2001. Seeds were manually extracted from berries and dried at room temperature (~ 18°C) for 2 days and at 25°C for 4 days until they reached 4.9% MC. The seeds were then stored as described above.

Seeds of two accessions of *Solanum phureja,* PI 195191 and PI 283123, collected in Ecuador were obtained from the Potato Introduction Station, Sturgeon Bay, WI. and stored at 4°C.

Seed Germination

For germination tests, seeds were imbibed on two layers of filter paper moistened with either 20 mL distilled water or test solutions in plastic containers (12 cm x 12 cm) and incubated at four temperatures (10°C, 15°C, 20°C and 25°C) in the dark. Three replicates of 50 seeds each were tested. The stock solution of fluridone (1-methyl-3-phenyl-5-[3-trifluoromethyl-(phenyl)]-4-(1H)-pyridinone) (100 μM) was prepared according to the method of Ober and Sharp (1994).

De-tipping

Seeds were imbibed at 15°C (*All blue*) or 25°C (*EB-8109*) for 24 h. After imbibition, the micropylar region of the endosperm adjacent to the radicle tip (endosperm cap) was carefully removed with a razor blade and forceps under a dissection microscope (MZ6, Leica, Bellevue, WA) so that the surface of the intact radicle tip was exposed (Fig. 3). These seeds were termed "de-tipped seeds". The de-tipped seeds were incubated for 6 more days. Radicle elongation (germination) was examined after incubation. Two replicates of 10 de-tipped seeds each were prepared for each treatment.

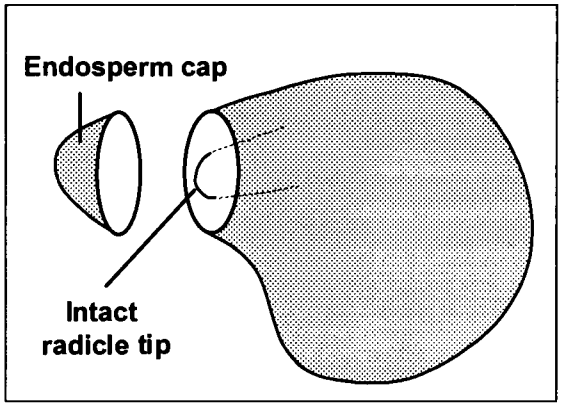

Figure 3. Schematic presentation of "de-tipping".

Seed Storage

All blue seeds were stored long-term (8 months) at room temperature (~18°C), 4°C and -20°C to examine changes in the status of TPS dormancy. After 5 months at -20°C, an aliquot of seeds were held at 42°C for 3 months. Three replicates of 25 seeds from each storage temperature were subjected to germination tests at 15°C.

RESULTS AND DISCUSSION

TPS Dormancy

Germination tests were conducted at 10°C, 15°C, 20°C and 25°C to determine temperature dependency for TPS germination. *All Blue* seeds did not germinate at any temperature (data not shown), indicating very deep dormancy (discussed later). *EB-8109* seeds germinated at all four temperatures (Fig. 4). *EB-8109* TPS germinated well at 10°C, 15°C and 20°C and best (89%) at 15°C. Final

germination percentage declined markedly at 25°C (Fig. 4). This reduction is consistent with previous reports (Pallais, 1995a; Alvarado et al., 2000). Thus, *EB-8109* showed typical thermodormancy.

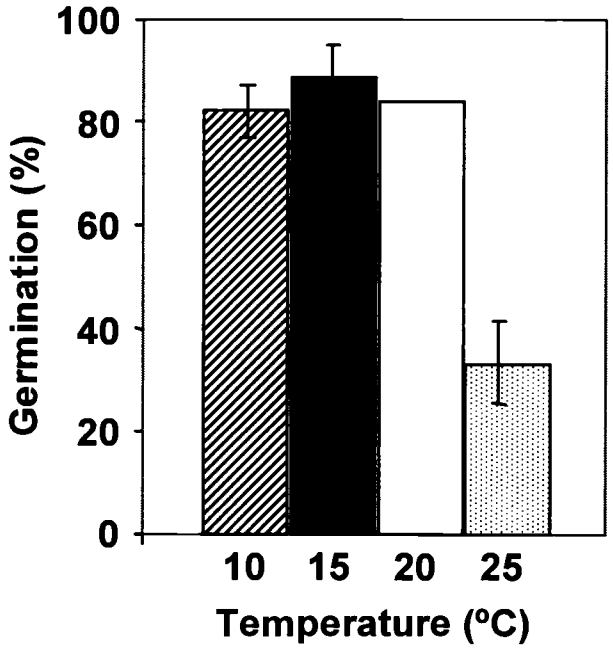

Figure 4. Final germination percentages of TPS (*EB-8109*) after 14 d at four temperatures. Values are means of 3 replicates ± SD

Although final germination percentages of *EB-8109* TPS (14 d) at 10°C, 15°C and 20°C did not significantly differ, the germination rate (7 d) at 10°C was significantly lower than at 15°C and 20°C (Fig. 5). The results showed that 15°C and 20°C were the optimal temperature for *EB-8109* seeds. We decided to use

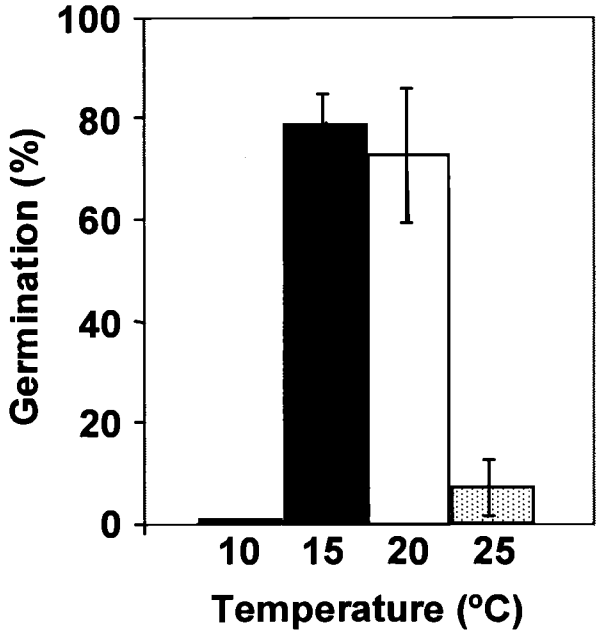

Figure 5. Germination rate of TPS *(EB-8109)* after 7 d at four temperatures. Values are means of 3 replicates ± SD.

15°C and 25°C as "non-dormant" and "dormant" conditions, respectively, for biochemical and molecular biological experiments (see later chapters).

We also characterized seed germination in a diploid potato species, *Solanum phureja* (Accessions PI 195191 and PI 283123). Accession PI 195191 seeds showed 18%, 74%, 88% and 80% germination at 10°C, 15°C, 20°C and 25°C, respectively (Fig. 6). Accession PI 283123 seeds showed germination percentages

of 0%, 38%, 84% and 72% at 10°C, 15°C, 20°C and 25°C, respectively (Fig. 7). The temperature dependency of *S. phureja* TPS differed somewhat from that of the tetraploid species (*S. tuberosum*). *S. phureja* did not show significant thermodormancy at 25°C (Fig. 6, 7). TPS of diploid species is known to be less dormant than that of cultivated tetraploid species (Simmonds, 1964; D'Antonio and McHale, 1988). These results are consistent with previous reports on thermodormancy. On the other hand, germination of *S. phureja* at low temperatures (10°C and 15°C) was relatively lower than that of *S. tuberosum* (Fig. 4, 6, 7). Although *EB-8109* seeds germinated slowly at 10°C, the final germination percentage at 10°C was not significantly reduced compared to percentages at 15°C and 20°C (Fig. 5). Germination of *S. phureja* TPS may be genetically less tolerant to cold conditions than that of broadly cultivated species. Alternatively, it is possible that *S. phureja* seeds used in these studies were partially deteriorated and had low vigor after prolonged storage in the gene bank. According to The Genetic Resources Information Network (GRIN, http:/www.ars-grin.gov.), these two accessions, PI 195191 and PI 283123, were logged into the Wisconsin Inter-Regional Potato Introduction Station in 1951 and 1962, respectively. Regeneration dates, if any, were unavailable. Thus, low germinability of *S. phureja* TPS at low temperature may be due to aging during long-term storage.

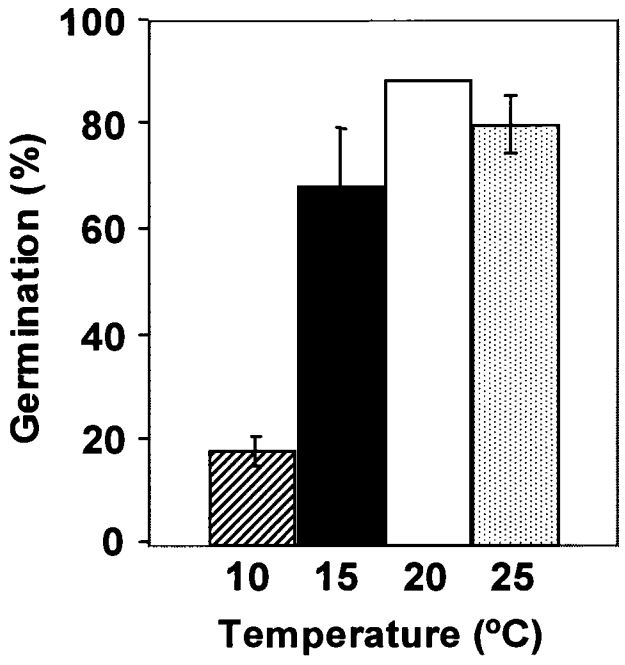

Figure 6. Final germination percentages of *S. phureja* (PI 195191) seeds after 14 d at four temperatures. Values are means of 3 replicates ± SD.

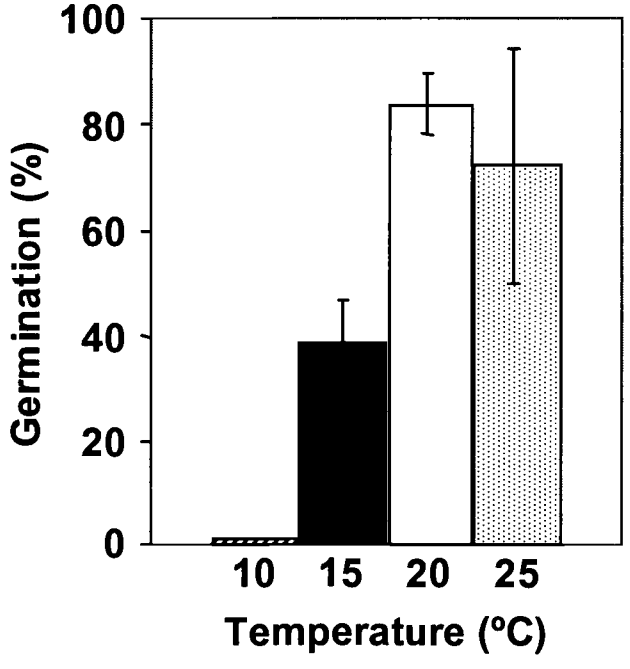

Figure 7. Final germination percentages of *S. phureja* (PI 283123) seeds after 14 d at four temperatures. Values are means of 3 replicates ± SD.

Dormancy Breakage Treatments

It is known that fluridone, an inhibitor of the carotenoid synthesis pathway, breaks thermodormancy of TPS (Alvarado et al., 2000). The project examined effects of fluridone (50 μM) on thermodormancy of *EB-8109* seeds. Fluridone dramatically stimulated germination of *EB-8109* at 25°C. Germination of fluridone-treated seeds reached 89% after 13 d at 25°C (Fig. 8).

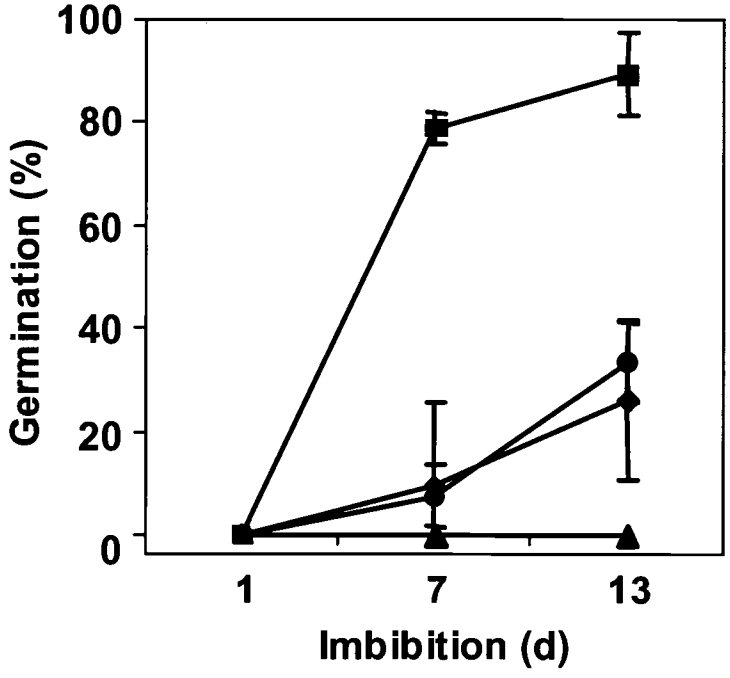

Figure 8. Breakage of thermodormancy of *EB-8109* TPS at 25°C by Fluridone. TPS were germinated in water (●), 50 μM fluridone (■), 100 μM ABA (▲) and 50 μM fluridone plus 100 μM ABA (◆) at 25°C for 13 d.

Fluridone blocks the carotenoid synthesis pathway, which is the upstream event of ABA synthesis. Therefore, fluridone is thought to break seed dormancy by blocking ABA synthesis (Yoshioka et al., 1998). Application of 100 μM ABA with 50 μM fluridone cancelled thermodormancy-breaking effects of fluridone and reduced the germination percentage to 26%, similar to the germination percentage (33%) of water-imbibed seeds at 25°C (Fig. 8). These results support the hypothesis that the breakage of TPS thermodormancy by fluridone results from blockage of the ABA synthesis pathway. It appears that ABA synthesis maintains TPS thermodormancy. A study using ABA-defficient *sit*^{w} mutant tomato also demonstrated that ABA is essential for the induction of dormancy in tomato seeds (Groot and Karssen, 1992). Toyomasu et al., (1994) demonstrated a decrease in ABA content in lettuce seeds when the seeds were subjected to dormancy breakage treatments such as gibberellic acid (GA) and red light. This also suggests that ABA synthesis maintains seed dormancy. It has been suggested that GA and red light treatments accelerate the degradation of ABA rather than block ABA synthesis (Grappin et al., 2000). Further research on the expression of genes regulating ABA synthesis enzymes is necessary to clarify this question.

Although fluridone effectively broke thermodormancy of *EB-8109* at 25°C, it did not affect the deep dormancy of *All Blue* (data not shown).

Table 1. Dormancy breakage treatments used for deeply dormant TPS.

Medium	Concentration	Temperature and Duration
Water	-	4°C, 6 d → 15°C incubation
Gibberellic Acid	1.5 mM	15°C incubation
	1.5 mM	4°C, 6 d → 15°C incubation
	1.0 mM	~18°C, 1 d → dried → 15°C incubation
Fluridone	50 μM	15°C incubation
	50 μM	4 °C, 6 d → 15°C incubation
KNO_3	2 mM	15°C incubation
	2 mM	4 °C, 6 d → 15°C incubation
	20 mM	15°C incubation

Many other treatments were examined for deep dormancy breakage (Table 1). However, none induced germination of *All Blue* even at 15°C (data not shown). Previous studies have shown that the deep dormancy of TPS can last for 8 to 18 months after fruit harvest (Simmonds, 1963; D'Antonio and McHale, 1988; Pallais, 1989, 1991; Pallais et al., 1989). *All Blue* seeds used for these experiments, were tested only 2 months after harvest, whereas *EB-8109* seeds had been stored one year before testing.

Mechanical Resistance of the Endosperm

In tomato seeds, the micropylar region of the endosperm (endosperm cap) provides mechanical resistance to the radicle (Groot and Karssen, 1987). TPS has very similar morphology to tomato seeds but TPS are smaller (Fig. 9, 10).

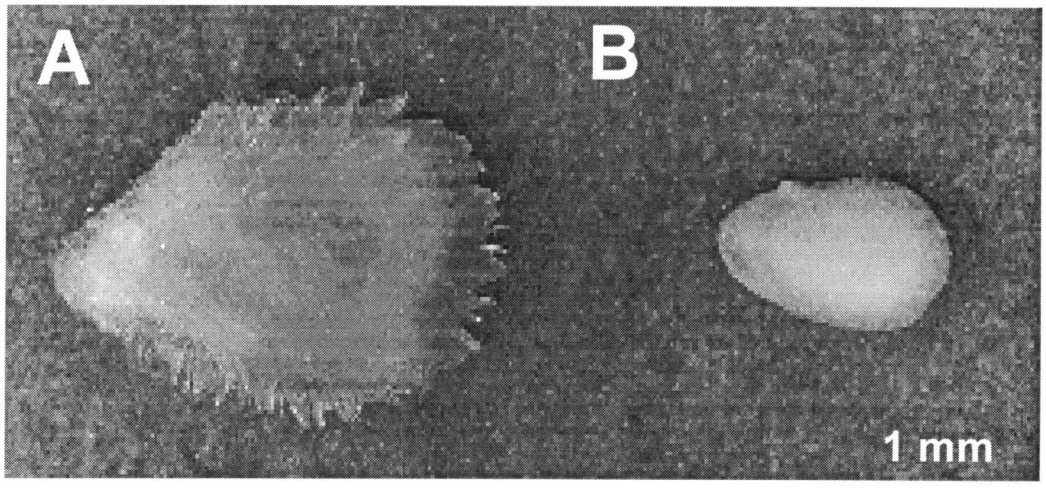

Figure 9. Tomato seed (A) and TPS (B).

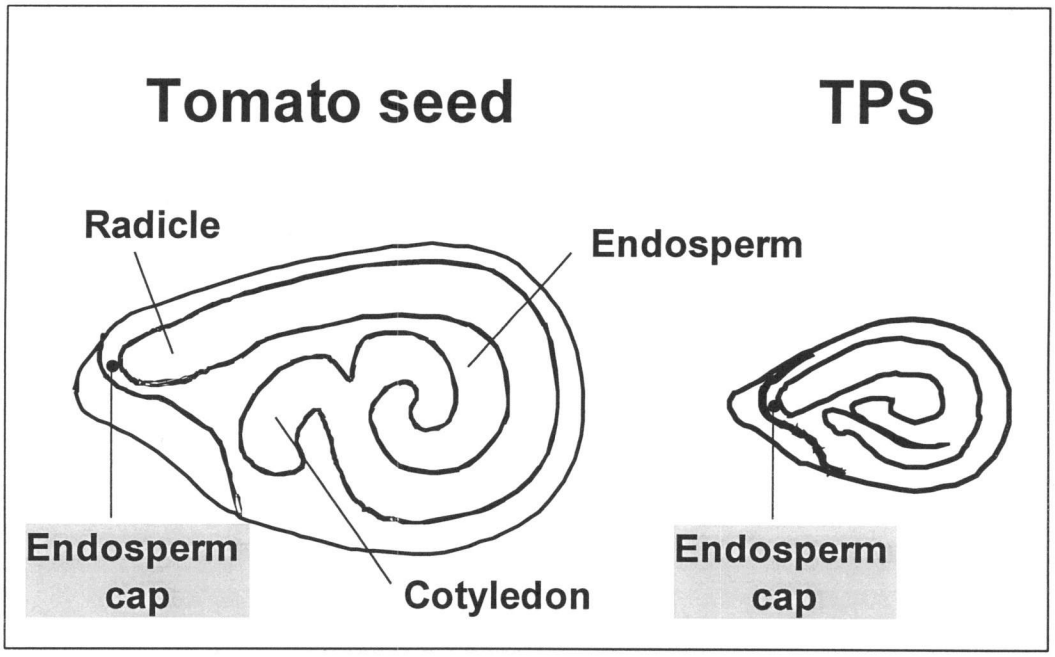

Figure 10. Schematic presentation of tomato and potato seed morphology.

It is likely that the endosperm cap inhibits radicle emergence in TPS as in tomato. To examine this possibility, the endosperm cap was removed from TPS "de-tipped seeds" (Fig. 3) and conducted germination tests. Radicle elongation was observed from the de-tipped seeds of thermodormant *EB-8109* seeds under thermodormant conditions (25°C) (Fig. 11C). This suggests that the embryos have enough growth potential to elongate the radicle and that the endosperm cap provides mechanical resistance to radicle emergence under thermodormant conditions. The germination of *EB-8109* de-tipped seeds reached 95% after 6 d at 25°C (Fig. 12). It is likely that the thermodormancy of *EB-8109* seeds is mainly due to the mechanical resistance of the endosperm cap. Interestingly, *All Blue* seeds which had deep dormancy at 15°C also showed radicle elongation after de-tipping, indicating that the endosperm cap has the same effect on the deep dormancy of *All Blue* seeds (Fig. 11D). However, the germination percentage of de-tipped *All Blue* seeds at 15°C reached only 15% (Fig. 12) suggesting that the embryos of *All Blue* seeds still have some deficiency in terms of growth potential. There must be another important factor causing *All Blue* seeds to remain deeply dormant at 15°C.

Dormancy and Seed Storage

There was not efficient chemical treatment to break the deep dormancy of *All Blue* seeds. Then, seeds were stored under several storage regimes.

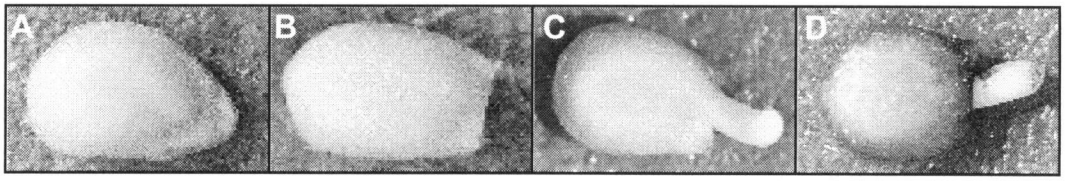

Figure 11. Radicle elongation from de-tipped seeds. A, intact seed (*EB-8109*); B, ungerminated de-tipped seed (*EB-8109*); C, germinated de-tipped seed (*EB-8109*, 25°C); D, germinated de-tipped seed (*All Blue*, 15°C).

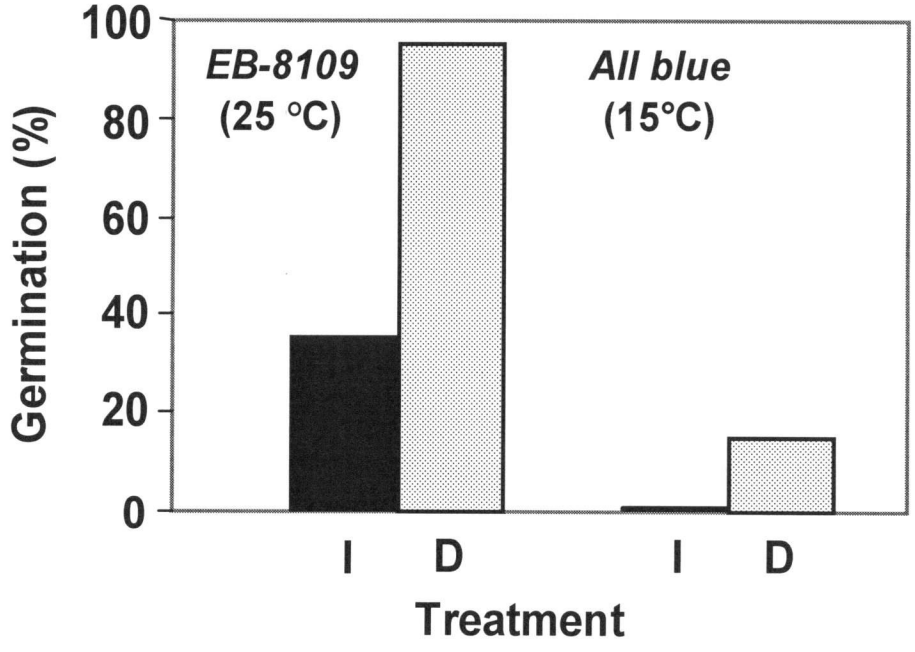

Figure 12. Germination of de-tipped seeds. De-tipped seeds of *EB-8109* and *All Blue* were tested at 25°C and 15°C, respectively. I and D, Intact and De-tipped, respectively.

First, aliquots of seeds were stored at room temperature (~18°C), 4°C and -20°C for 5 months; none germinated. An aliquot of the seeds stored at -20°C for 5 months was transferred to 42°C and held another 3 months; we observed 37% germination from this treatment compared to only 6.7%, 1% and 1% from seeds stored at room temperature, 4°C and -20°C, respectively. D'Antonio and McHale (1988) showed that the deep dormancy of hybrid TPS was partly broken after 10 months at 4°C and 7 months at room temperature but residual dormancy persisted for 2 years at 4°C and 1 year at room temperature. Data from this study also showed that TPS dormancy is long lasting. The relatively high temperature of 42°C broke dormancy more effectively as suggested by Pallais (1995a). However, the effect was still incomplete. Although it is known that storage at -20°C is optimal for conserving TPS materials long-term in seed banks (Hong et al., 1998), the optimal conditions to break dormancy (temperature, humidity and so on) remain to be determined.

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Chapter 3

Expression of the Endo-β-Mannanase Gene in Germinating True Potato (*Solanum tuberosum* L.) Seeds

Alvaro R. Monteros, Alvin R. Mosley and Hiroyuki Nonogaki

Unpublished

INTRODUCTION

Mechanisms underlying dormancy breakage and induction of seed germination are not fully understood. To elucidate these mechanisms it is necessary to focus on germination *sensu stricto.* Germination *sensu stricto* comprises the physiological events occurring before radicle protrusion. This stage is also called the germinative stage. This chapter focuses on biochemical and molecular mechanisms of germinative events.

In endospermic seeds, radicle protrusion is controlled by two opposing forces - embryo growth potential and mechanical resistance of the endosperm. An increase in embryo growth potential or a decrease in mechanical resistance of the endosperm, or both must occur to induce radicle protrusion in endospermic seeds. In tomato (*Lycopersicon esculentum* Mill.) seeds, the major barrier to germination resides in the endosperm enclosing the radicle tip (endosperm cap) (Ni and Bradford, 1993). The endosperm cap of tomato is weakened before radicle emergence (Groot and Karssen, 1987). The previous chapter described the thermo-dormancy of *EB-8109* seeds at 25°C and the deep dormancy of *All Blue* at 15°C. In both conditions, the endosperm cap enclosing the radicle tip presents a mechanical barrier to germination of TPS. Therefore, it is likely that weakening of the endosperm cap is prerequisite for induction of germination in TPS as well as in tomato.

The mechanical resistance of the tomato endosperm cap is due to the thick cell walls of this tissue. The cell walls consist mainly of mannan polymers, including galactomannans (Groot et al., 1988). The concerted action of three hydrolases, endo-β-mannanase, β-mannosidase and α-galactosidase is required for the complete degradation of galactomannans (Fig. 13). Endo-β-mannanase plays a significant role in weakening of the endosperm cap of tomato seeds (Nonogaki and Morohashi, 1996; Nonogaki et al., 2000). It is also known that imbibed TPS expressed a homologue of the tomato mannanase cDNA during the germinative stages (Alvarado et al., 2000).

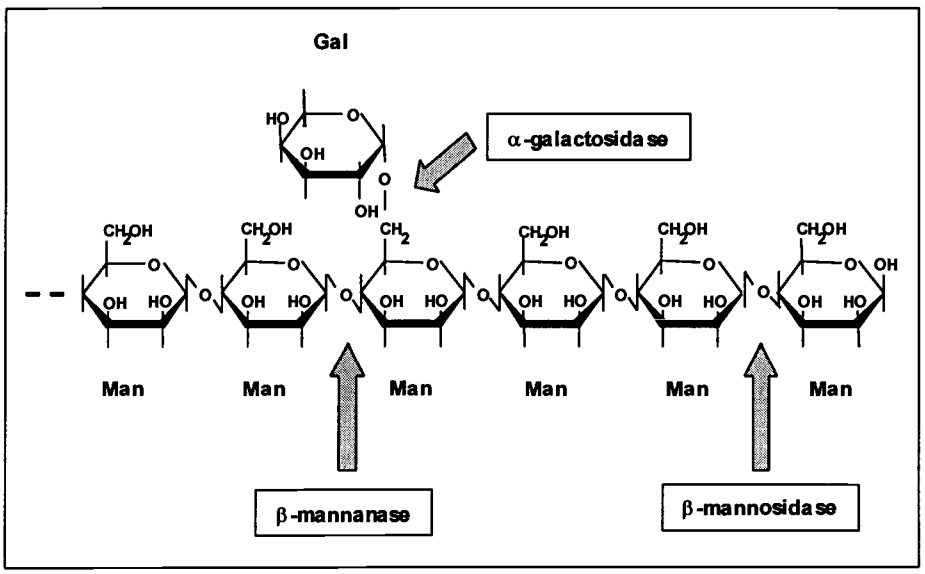

Figure 13. Structure of galactomannan and three hydrolases involved in degradation of galactomannan. Gal, galactose; Man, mannose.

It is likely that endo-β-mannanase is involved in dormancy breakage of TPS. We isolated potato mannanase cDNA and characterized the expression of the mannanase gene in TPS by using tissue printing. The correlation between mannanase induction and breakage of dormancy is discussed in this chapter.

MATERIALS AND METHODS

Genomic DNA extraction

The genomic DNA was extracted from potato leaves and seedlings of *EB-8109* using Dneasy Plant Mini Kit (QIAGEN, Valencia, California) or the protocol from the Wisconsin Knockout Facility, University of Wisconsin, Madison (http://www.biotech.wisc.edu/Arabidopsis/).

Polymerase Chain Reaction (PCR)

To amplify the coding region of potato seed germination-specific mannanase, the genomic DNA of *EB-8190* was used as a template for PCR. Fifty μL PCR reactions were prepared by using the genomic DNA (400 ng), Taq polymerase (ExTaq, Takara Shiga, Japan), 10X ExTaqBuffer (20 mM) (Takara, Shiga, Japan), dNTP mixture (10 mM) (Takara, Shiga, Japan) and specific primers. The specific primers were designed to the cDNA of tomato germination-specific mannanase (*LeMAN2*, Nonogaki et al., 2000) (SP-1 forward, 5-

GGCACGAGAAATAATGGC-3'; SP-2 reverse, 5'-CTTATTTTCTTCCATGT CCTC-3') (Table 2, Fig. 14). The following program in the PCR thermalcycler (Eppendorf, Hamburg, Germany) was used for PCR: the initial denaturation at 94°C (4 min), 7 cycles 94°C (15 sec), 62°C ~ 55°C (touchdown PCR) and 72°C (30 sec) and, then 25 cycles at 94°C (15 sec), 55°C (15 sec) and 72°C (30 sec) followed by extension at 72°C (7 min). PCR products were examined on 1% agarose gel. DNA-bands on the gel were visualized in a Fluor-S Multimager (Bio-Rad, Hercules, CA).

Table 2. Primers used for amplifying and sequencing the potato mannanase gene

Primer Number	Primer Sequence
SP1	5' – GGC ACG AGA AAT AAT AAT GGC – 3'
SP2	5' – CTT ATT TTC TTC CAT GTC CTC – 3'
SEQ1	5' – CAC GTT CAT CAT AAA CAC CAG – 3'
SEQ2	5' – CTT GGG CTT TTA GTG ATG GAG – 3'
SEQ3	5' – CTG GTG TTT ATG ATG AAC GTG – 3'
SEQ4	5' – CTG GAA ATA GGA ATG GAA GGA – 3'
SEQ5	5' – GAC CAA TGG TTA TCT GGT CAA – 3'

DNA Sequencing

DNA sequencing was done in the Central Sequencing Laboratory in the Center for Gene Research and Biotechnology at Oregon State University. The sequencing primers (Table 2, Fig. 14) were used for sequencing the coding region of the potato mannanase gene. *DNASTAR* software (DNASTAR Inc., Madison, WI) was used to analyze sequence data.

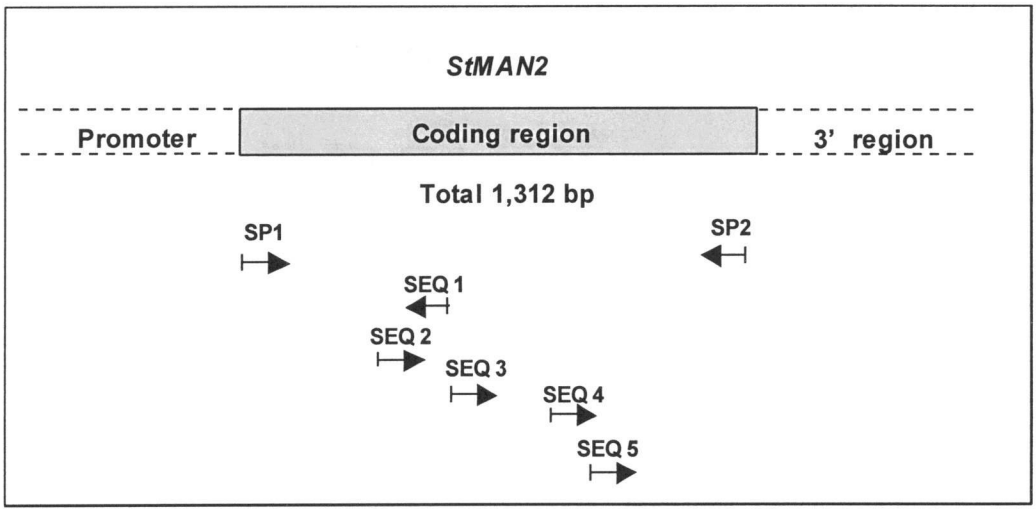

Figure 14. Schematic presentation of the positions of the primers used for amplifying and sequencing potato mannanase.

DNA Cloning

The PCR product (approximately 1.3 kb) was cloned into pCR4-TOPO vector (Invitrogen Corporation, Carlsbad, CA.) according to the manufacturer's instructions in the TOPO TA Cloning Kit for sequencing (Invitrogen Corporation, Carlsbad, CA.). The reaction was placed on ice and proceeded with One Shot Chemical transformation: Two µL of the previous TOPO cloning reaction was incubated on ice for 30 minutes, heat shocked for 30 seconds at 42°C and tubes were then transferred back to ice. LB broth (10 g NaCl, 10 g Tryptone, 5 g of yeast extract in 1 liter water, pH 7.0, autoclaved) was added. Samples were incubated with shaking for 1 h at 37°C then centrifuged at 10,000*g* to collect the bacterial pellets. The pellet was dissolved in 50 µL LB broth and spread on the LB agar plate containing either Kanamycin (25 µg mL^{-1}) or Ampicillin (50 µg mL^{-1}) and

incubated overnight at 37°C. The individual colonies grown on the plate were separately picked up and used to inoculate 3 mL LB media containing the antibiotics to prepare overnight cultures. The plasmid was isolated from the overnight cultures using Wizard Plus Minipreps DNA Purification Systems (Promega, Madison, WI.) and used for PCR to verify the presence of the cloned DNA. The bacterial cultures (500 μL) were mixed with the same volume (500 μL) of 30% (v/v) glycerol. The glycerol stocks were stored at -80°C as laboratory stocks.

Tissue Printing

TPS were bisected with a razor blade. The cut surfaces were pressed for approximately 15 sec onto a positively charged membrane (Hybond-N+, Amersham Pharmacia Biotech, Inc., Piscataway, New Jersey, USA) supported on many layers of paper towel. After tissues were removed, the membranes were UV-cross-linked and hybridized with tomato cDNA probe *LeMAN2* (GenBank accession no. AF184238, Nonogaki et al., 2000). Hybridization was routinely done overnight at 50°C with a hybridization buffer containing 50% (v/v) deionized formamide, 2% (w/v) blocking reagent (Boehringer Mannheim, Germany), 2% (w/v) SDS, 25% (w/v) 20X SSC, and approximately 100 ng mL^{-1} RNA probe. Membranes were prehybridized for 15 minutes at the same temperature. The next day membranes were washed for 20 min with 2X SSC, 0.1% (w/v) SDS at 50°C and twice more for 20 min with 0.2X SSC, 0.1% (w/v) SDS at the same

temperature. The membranes were blocked for 1 h with 5% (w/v) non-fat milk in 0.1 M Maleic acid buffer, pH 7.5, containing 0.15 M NaCl and 0.3% (v/v) Tween 20 (buffer A) and then incubated with alkaline phosphatase-conjugated anti-DIG antibody (1:15,000 dilution) for 2 h at 25°C. After washing with buffer A, the membranes were subjected to colorimetric detection by using: 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in Dimethylformamide (DMF) (10 mg mL^{-1}); Tris pH 8.8 (1.5 M), Nitroblue tetrazolium (NBT) 1 mg mL^{-1} and MgCl (1 M), for 24 h. Observations and photographs were done using a light microscope (Leica, Bellevue, Washington).

RESULTS AND DISCUSSION

Isolation of the Coding Region of Germination-Specific Mannanase from Potato Genome

To isolate a potato mannanase gene, genomic DNA was extracted from *EB-8109* TPS and used for PCR with specific primers designed for tomato germination-specific mannanase *LeMAN2* (Primers SP1 and SP2, Table 2). A 1,307-bp product was obtained from PCR. The PCR product showed a strong similarity to tomato *LeMAN2*. The putative potato mannanase was cloned into pCR4-TOPO vector and further sequenced. To obtain accurate sequencing data, multiple sequencing primers were designed and used for sequencing the potato mannanase (Table 2, Fig. 14).

The DNA sequence of the potato mannanase gene turned out to be exactly the same as that of *LeMAN2* cDNA (data not shown). The genomic DNA of the seed-specific *LeMAN2* does not have introns, while other fruit- and anther-specific mannanase genes in the tomato genome do (S. A. Filichkin, Jeff Leonard, H. Nonogaki, unpublished results) The potato gene isolated (termed as *StMAN2*) did not have introns, suggesting that this is also a seed-specific mannanase in potato. It is surprising that the seed-specific mannanase in potato had exactly the same sequence as that of the tomato seed-specific mannanase. *StMAN2* contained an open reading frame encoding protein of 415 amino acids (Fig. 14). A putative signal peptide sequence of 22 amino acids was identified at the amino terminus of the protein. The mature protein of 393 amino acids was encoded from the Cys residue at nucleotide 83 to Ile at nucleotide 1,259, with a predicted molecular M_r of 44,379 and pI of pH 5.7. The predicted amino acid sequence of *StMAN2* was compared with that of other seed mannanases from different species (Fig. 15).

Expression of the Germinative Endo-β-Mannanase Gene in TPS

To characterize the expression of *StMAN2* mRNA in TPS, tissue printing and hybridization were done by using *LeMAN2* antisense RNA probe. The TPS of *EB-8109* and *All Blue* were imbibed for 72 h and subjected to tissue printing. When the expression of mannanase mRNA in *All Blue* seeds incubated at 15°C for 72 h was

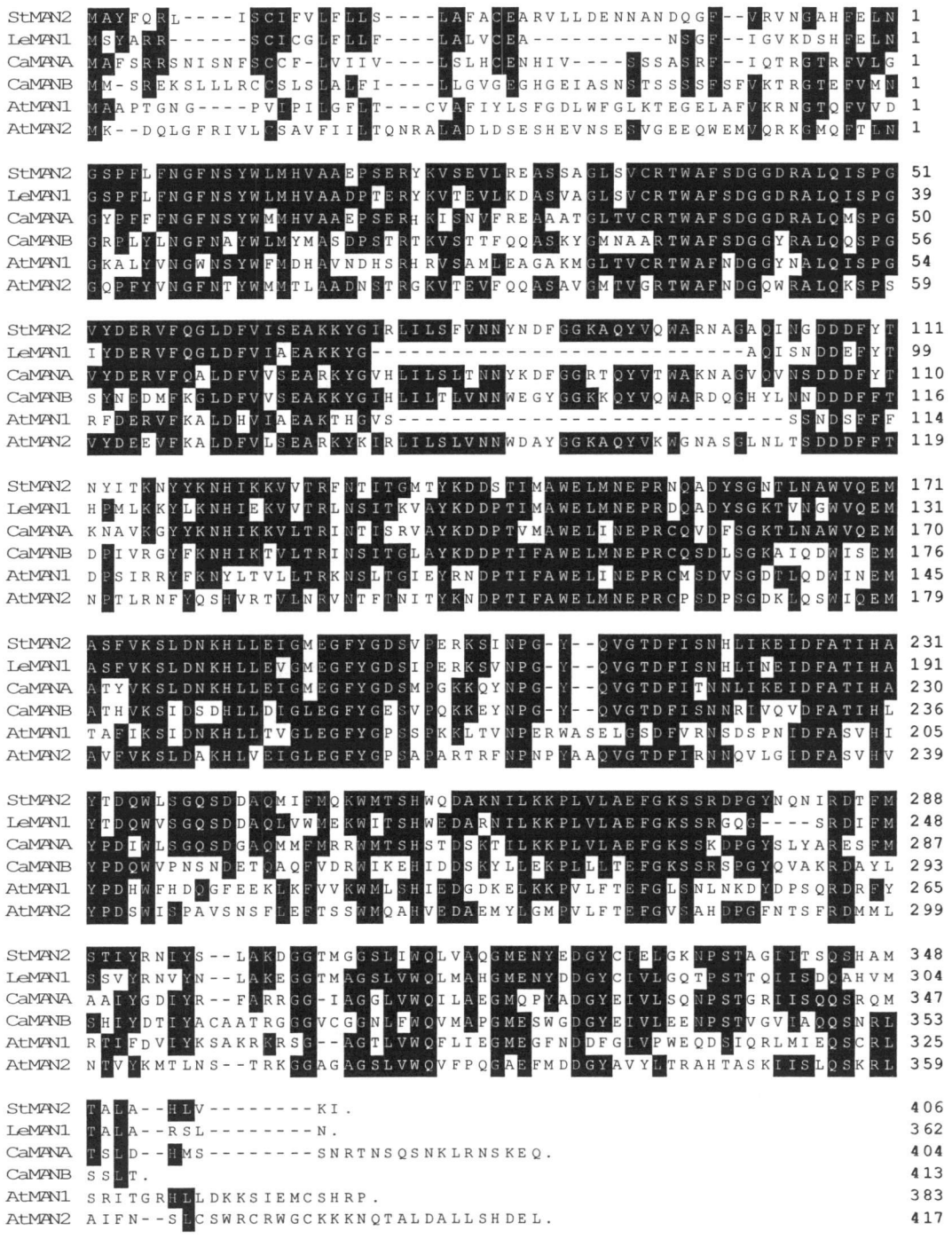

Figure 15. Alignment of amino acid sequences of *StMAN2* and other seed mannanases. The potato StMAN2, tomato LeMAN1 [AAB87859], coffee (*Coffea arabica*) CaMANA [CAC08208], CaMANB [CAC08442]), and *Arabidopsis* AtMAN1 (NP179660), AtMAN2 (NP195813) are aligned. Amino acids identical in three or more of the sequences are highlighted by reverse shading.

Examined, signal of mRNA expression in the tissue print was detected (Fig. 16A). However, a clear signal in the tissue print of *EB-8109* seeds was detected. The expression of the mannanase mRNA was detected exclusively in the endosperm cap of the seeds (Fig. 16B). Therefore, the localization of the expression of potato mannanase gene was exactly the same as that in tomato seeds (Nonogaki et al., 2000). This suggests that the endosperm cap of TPS is also weakened by the action of endo-β-mannanase before radicle emergence. As discussed in Chapter 2, *All Blue* seeds did not germinate at 15°C because of deep dormancy, while 89% of *EB-8109* seeds germinated at this temperature (Fig. 4). The patterns of expression of mannanase mRNA at 15°C were consistent with the results of germination: germinable seeds (*EB-8109*) expressed mannanase mRNA while ungerminable seeds (*All Blue*) did not. Seeds of *EB-8109* did not express mannanase mRNA in the thermodormant condition (25°C) when incubated in water (Fig. 16C). Application of fluridone (50 μM) induced the expression of mannanase mRNA in the endosperm cap of *EB-8109* seeds incubated at 25°C (Fig. 16D). As described in Chapter 2, fluridone breaks thermodormancy of *EB-8109* TPS and induces germination at 25°C (Fig. 8). The expression of mannanase mRNA was consistent with germination results at 25°C. Thus, there was a correlation between mannanase expression and dormancy breakage (germination).

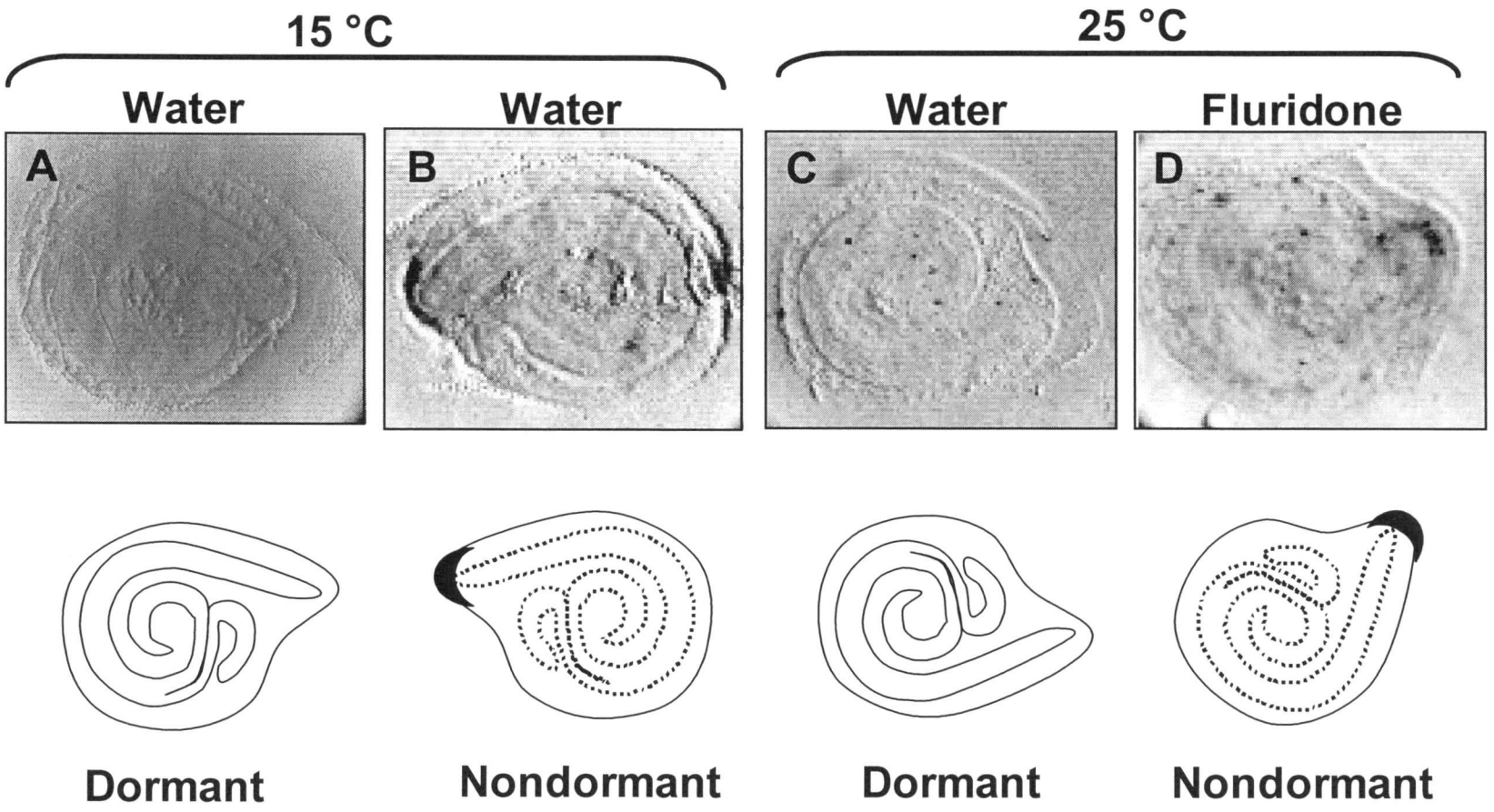

Figure 16. Tissue printing and hybridization of TPS for endo-ß-mannanase. TPS were imbibed for 3d and bisected into halves A, *All Blue*; B, C and D, *EB-8109*. The half seeds were used for tissue printing. The DIG-labeled antisense RNA probe of *LeMAN2* was used for hybridization. The dark areas indicate the expression of mRNA.

Alvarado et al. (2000) showed that the expression of fluridone-induced mannanase mRNA in thermodormant TPS was not inhibited by ABA, although ABA cancelled fluridone-induced germination. This suggests that mannanase expression may not be the sole factor involved in induction of TPS germination. However, mannanase was never induced in dormant conditions in these experiments. Conversely, mannanase was induced whenever germination occurred. It is likely that mannanase expression is indispensable for radicle protrusion. Since mannanase was always induced when dormancy was broken, mannanase expression can be a good marker for dormancy breakage. If the promoter region of this gene could be isolated and construct a reporter cassette constructed by using reporter genes such as green fluorescent protein genes, one should be able to visualize the timing of dormancy breakage on a single seed basis. This will enhance basic research on the mechanism of seed dormancy and may also be useful in seed processing.

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Chapter 4

Expression of the Endo-β-Mannanase Gene in Germinated True Potato (*Solanum tuberosum* L.) Seeds

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Unpublished

INTRODUCTION

After germination, seeds start to degrade storage reserves for seedling establishment. The mobilization of seed reserves is primarily a post-germination event (Bewley and Black, 1994). Seedlings depend on the mobilization of seed storage reserves before they become autotrophic through photosynthesis. Seeds accumulate storage oils, proteins and carbohydrates during developmental stages. Tomato and potato seeds also accumulate oils and proteins in oil bodies (OB) and protein storage vacuoles (PSV), respectively. The carbohydrates are stored in the cell walls in the case of tomato seeds, and possibly TPS as well. The major carbohydrate reserve in tomato seeds is the cell wall mannans (Groot et al., 1988).

In tomato, germinated seeds also express mannanase activity (Nonogaki et al., 1995; Nonogaki and Morohashi, 1996; Bewley et al., 1997). The activity of the post-germinative mannanase in germinated tomato is not restricted to the endosperm cap but spread to the rest of the endosperm (lateral endosperm) (Nonogaki et al., 1998, 2000). The physiological role of the post-germinative mannanase is to mobilize the stored mannans in the cell walls of the lateral endosperm for seedling establishment.

We found that TPS also express the post-germinative mannanase activity after germination. This chapter, characterizes the different isoforms of post-germinative mannanase and their expression patterns. Tissue printing was also used to localize the expression of the post-germinative mannanase gene in TPS.

MATERIALS AND METHODS

Gel Diffusion Assay

The proteins were extracted from 20 germinated seeds (*EB-8109*) after 4, 6 and 8 d of imbibition at 15°C, with 50 mM potassium phosphate buffer, pH 6.8. The extract was centrifuged at 10,000*g* for 2 min. The supernatant was used for gel diffusion assay of endo-β-mannanase activity (Downie et al., 1994). Ten μL of the enzyme solution was applied to the wells of the gel plate. The gel plate was incubated at 25°C for 24 h, stained with 0.5% (w/v) Congo red for 30 min and washed with 1 M NaCl for 30 min. The clearing zone produced by the enzyme activity was visualized by adding 0.1 M citrate-phosphate buffer, pH 7.0 and photographed. The enzyme activity was quantified using a commercial fungal (*Asperguillus niger*) mannanase (Megazyme, Wicklow, Ireland) as a standard.

Native Polyacrylamide Gel Electrophoresis (PAGE)

Native PAGE was done using 7.5% gels [2.7% (w/w) N, N'-methylene-bis(acrylamide)] according to Davis (1964) except that ammonium persulfate was used in place of riboflavin in the stacking gel.

Activity Staining

Activity staining was done as previously described (Nonogaki et al., 1995). After native PAGE, the gel was placed on the blue-colored gel containing 0.5% (w/v) dyed galactomannan. These two gels were placed between two layers of filter paper moistened with 80 mM Na-citrate buffer, pH 4.0 and incubated at 25°C for 24 h. During the incubation, low molecular mass products produced by the action of endo-β-mannanase were blotted to filter paper (Whatman No 2, Maidstone, England) in contact with the substrate-containing blue gel, and stained the filter paper blue at the positions corresponding to the enzyme bands in the native gel.

Tissue Printing

Tissue printing and hybridization were done as described in Chapter 3, except that the cDNA of the post-germinative mannanase *LeMAN1* (GeneBank accession no. AF017144, Bewley et al., 1997) was used for probe synthesis.

RESULTS AND DISCUSSION

Post-germinative Endo-β-Mannanase Activity

To analyze post-germinative mannanase activity, proteins were extracted from *EB-8109* seedlings after 4, 6, and 8 d of imbibition at 15°C and subjected to activity assay. Endo-β-mannanase activity was detected in the seedlings after 4 d (Fig. 17). However, the activity level in 4-d seedlings was the same as that of 3-d

ungerminated seeds (380 pmol min^{-1} $seedling^{-1}$), indicating that there was no increase in the post-germinative mannanase activity in 4-d seedlings. Post-germinative mannnanase activity increased dramatically after 6 d and reached 1,745 pmol min^{-1} $seedling^{-1}$ (Fig. 17) after 8 d.

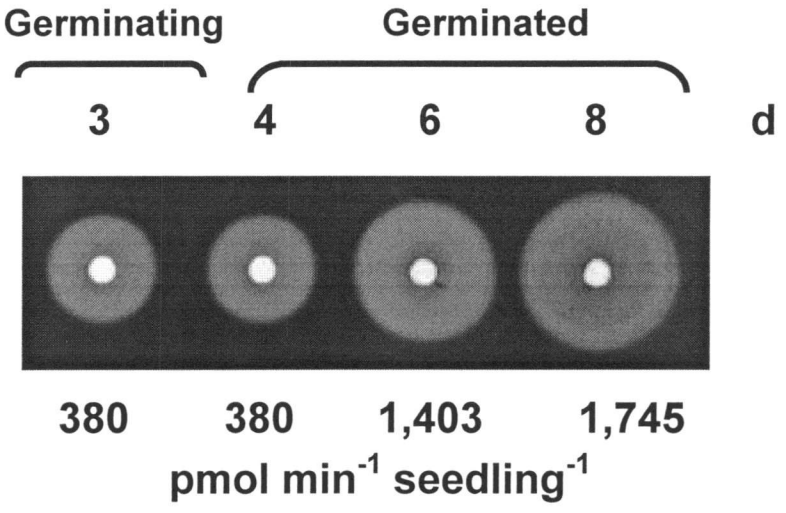

Figure 17. Gel diffusion assay of endo-β-mannanase activity in germinating and germinated TPS. Proteins were extracted from ungerminated (3 d) and germinated (4, 6 and 8 d) seeds. Ten μL of the extracts were applied in each well. Activity was measured by using commercial mannanase as a standard (see MATERIALS AND METHODS).

These results indicate that TPS express post-germinative mannanase, consistent for results with tomato (Nonogaki et al., 1995) and carrot seeds (T. Homrichhausen, J. Hewitt, H. Nonogaki, unpublished results). Mobilization of stored mannans is probably an important source of nutrition for potato seedlings.

It is known that three different isoforms of endo-β-mannanases, M1, M2 and M3 are expressed in germinated tomato seeds with M1 and M3 being the major ones (Nonogaki et al., 1995). Multiple isoforms of endo-β-mannanases are also known to be present in germinated lettuce seeds (Nonogaki and Morohashi, 1999). To examine whether there are multiple forms of endo-β-mannanases in potato seedlings, native PAGE and activity staining were conducted. Two distinct bands of endo-β-mannanases were detected in activity staining (Fig. 18).

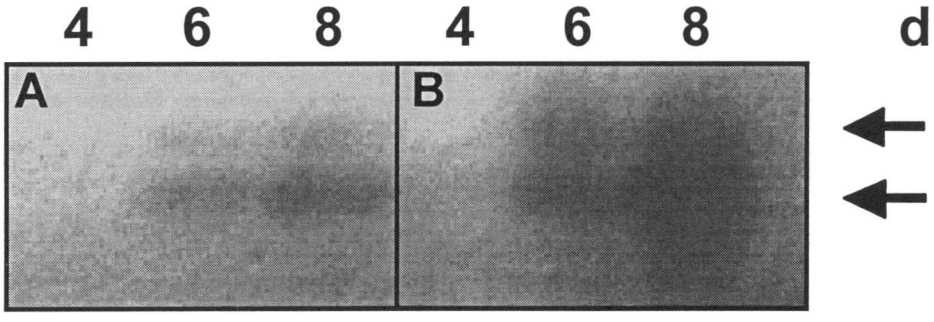

Figure 18. Native PAGE and activity staining of extracts from germinated TPS. The proteins were extracted from 4, 6 and 8 d seedlings. Ten μL and 20 μL of the same sets of the extracts were applied in panels A and B, respectively.

The relative mobilities of these two activity bands were similar to those of the major mannanases, M1 and M3 in tomato seeds (data not shown). The activities of both isoforms increased after 6 d of imbibition (Fig. 18). The pattern of the increase in total mannanase activity detected in activity staining of native gel was the same as that detected in gel diffusion assay (Fig. 17, 18). It seems that TPS and tomato seeds have very similar post-germinative mannanases expression patterns.

Expression of a Post-germinative Mannanase Gene in TPS Endosperm

Bewley et al. (1997) isolated the cDNA (*LeMAN1*) of the post-germinative mannanase from tomato. This gene is expressed in the lateral endosperm of germinated tomato seeds. This study also examined the localization of the post-germinative mannanase in TPS by using *LeMAN1* RNA as a probe. The mRNA of the post-germinative mannanase was expressed in whole lateral endosperm of TPS (Fig. 19). The signal was not detected from the embryo, indicating that the post-germinative mannanase was endosperm-specific. This result suggests that the post-germinative mannanase in TPS is involved in endosperm reserve mobilization. This is consistent with results for tomato seeds which have morphology similar to that of TPS.

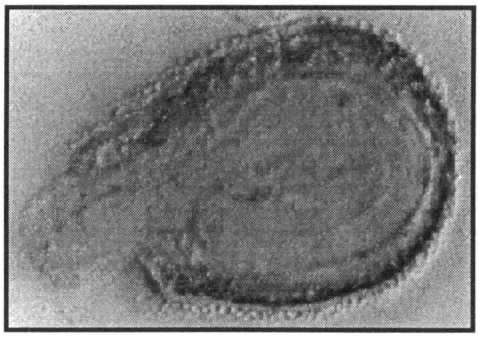

Figure 19. Tissue printing of mannanase mRNA in germinated TPS. Mannanase is expressed in the whole endosperm.

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Chapter 5

Characterization of Other Related Endo-β-Mannanases in Potato *(Solanum tuberosum* L.*)* and Tomato (*Lycopersicon esculentum* Mill.)

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INTRODUCTION

Previous studies have shown the existence of multiple endo-β-mannanase genes in the tomato genome (Bewley et al., 1997; Nonogaki et al., 2000). Some are expressed in germinating and germinated seeds and play significant roles in seed germination and reserve mobilization during post-germinative stages (Bewley et al., 1997; Nonogaki et al., 2000). Other tomato mannanases are known to be expressed in fruits, although the physiological roles of tomato fruit mannanases are not known (Bewley et al., 2000; Banik et al., 2001; Bourgault et al., 2001). Eight putative mannanase genes have been reported for *Arabidopsis* (ecotype *Columbia*). The expression sequence tag (EST) data show that some of *Arabidopsis* mannanases are expressed in the pods and developing seeds (The TIGR *Arabidopsis thaliana* Genome Annotation Database, http://www.tigr.org/tdb/e2k1/ath1/ath1.shtml). However, the expression of mannanase genes in other plant tissues has not been well characterized.

During this research, we found a new mannanase gene in tomato and isolated the promoter region. A similar promoter was also isolated from the potato genome. The tomato promoter was characterized by using the reporter gene system in transgenic *Arabidopsis* as well as RNA gel blot analysis and activity assay in tomato. The potential physiological roles of the new mannanase in tomato and potato flowers are discussed in this chapter.

MATERIALS AND METHODS

Genomic DNA Extraction

Genomic DNA was extracted from tomato (*Lycopersicon esculentum* cv. *Moneymaker*) and potato (*Solanum tuberosum, EB-8109*) as described in Chapter 3.

Isolation of the Promoter Region of the New Tomato Mannanase Gene (*LeMAN5*)

The 5' untranslated region of a new mannanase gene from tomato (*LeMAN5,* described later) was isolated using a "Genome Walker" kit (Clontech Laboratories, Ink., Palo Alto, CA) according to the manufacturer's instructions. The gene-specific reverse primers GSP1 (5'-TGCATTTCGCGCCCATTGAACATATTGAGC-3') and GSP2 (5'-TTCATCAAGTAAAACCCTAGCTTCACATGC-3') were designed to the cDNA sequence of tomato germination-specific mannanase *LeMAN2*, and used for the first and second PCR, respectively.

Sequencing of *LeMAN5* Genomic Clone

The BAC library (LA483) of wild species of tomato, *Lycopersicon cheesmannii* (courtesy of NSF Tomato Genome Project), was screened using tomato germination-specific mannanase *LeMAN2* as a probe (Nonogaki et al., 2000). The clone 241 P6 that hybridized with the probe was used for further analysis. The BAC clone was purified using a Qiagen plasmid purification kit

(QIAGEN, Valencia, California) and used for sequencing. Based on the sequence data obtained from the BAC clone of the wild species, two specific primers (5'-TCGATCCCATAGACTAGAGA-3' and 5'-GTCTATAGCGAATCTAGTGAC-3' were designed and used to ampify the genomic DNA of *LeMAN5* from *Lycopersicon esculentum*, cv *Moneymaker*. A 3.7 kb DNA fragment was cloned into TOPO pCR4.0 vector (Invitrogen Corporation, Carlsbad, CA.) and used for further sequencing.

Vector Construction for the Reporter Gene Cassette

The 572-bp *LeMAN5* promoter region was amplified using the forward primer (5'-CTGAGCTCAAATGTCTACAAAGCTTA-3') and the reverse primer (5'-CAGTCGACTGATAGACAAATGCATGT-3') flanking with *Sac*I and *Sal*I restriction sites, respectively. The PCR product was digested with *Sac*I and *Sal*I and cloned into *Sac*I and *Sal*I restriction sites of pRJG23 shuttle vector (Grebenok et al., 1997). The resulting plasmid pRJG 572 was digested with *Sac*I and *Spe*I restriction enzymes to cleave the GFP-GUS reporter genes driven with *LeMAN5* promoter out. The 3.9-kb reporter cassette was cloned into the *Sac*I and *Xba*I restriction sites of pGPTV-KAN binary vector (Becker *et al.*, 1992). The binary vector was used for plant transformation.

Plant Transformation

Arabidopsis thaliana (ecotype *Columbia*) plants were grown at 23°C under a 14 h photoperiod. For transformation, a 5 mL overnight culture of EHA 105 strain of *Agrobacterium tumefaciens* harboring the binary vector was grown in YEP medium supplied with 50 μg/mL of kanamycin, 50 μg/mL of streptomycin and 50 mM of MOPS buffer, pH 6.0. The medium (200 mL) was inoculated with 5 mL of overnight culture and incubated for an additional 16 h at 28°C with vigorous shaking. The bacterial cells were harvested by centrifugation at 5,000*g* and resuspended in 400 mL of a 5% (w/v) sucrose solution containing 0.02% (w/v) Silwet L-77 detergent (Lehle Seeds, Round Rock, TX). Transformation was done by using a floral dip method as described previously (Clough and Bent, 1998).

Isolation and Identification of Transgenic *Arabidopsis*

Matured *Arabidopsis* seeds were collected and incubated at 4°C for 2 days. Seeds were sterilized in 96% (v/v) ethanol for 1 min and in 50% (v/v) bleach plus 0.1 % (v/v) Tween 20 for 10 min followed by several extensive washes with sterile water. Kanamycin-resistant plants were selected on 1% (w/v) Phytagar (Gibco BRL, Rockville, MD) plates supplied with 4.3 g/L MS salts, 3% (w/v) sucrose and 25 μg/mL of kanamycin. To verify the presence of *LeMAN5* sequence in the genomic DNA of the transformants, the genomic DNA was isolated from kanamycin-resistant plants and subjected to PCR using primers 5' -

CTGAGCTCAAATGTCTACAAAGCTTA - 3' and 5' – CAGTCGACTGATAGA CAAATGCATGT-3'.

Glucuronidase (GUS) Staining

The substrate, 5-Bromo-4-chloro-3-indolyl-ß-D glucuronide (Research Products International Corp., Mt. Prospect, IL), was dissolved in N,N-dimethylformamide (DMF). Then, 10 µL of the substrate was mixed with 3 mL 100 µM sodium phosphate buffer pH 7.0, containing 0.1% (v/v) triton X-100 and used for GUS staining. Sample tissues from all parts of the *Arabidopsis* plants (leaves, stems, pods, roots, seeds) were collected separately in 1.5 mL microfuges, infiltrated in the substrate solution under vacuum for 10 min and incubated for 24 h at 37°C. The staining buffer was removed after 24 h and 70% ethanol was added. Samples were kept at 4°C until the tissues were examined under a dissection microscope.

Gel Diffusion Assay

The gel diffusion assay for endo-β-mannanase activity was performed as described in Chapter 4.

RESULTS AND DISCUSSION

Isolation of the Promoter Region of a New Tomato Mannanase Gene

We conducted the genome walking experiment to isolate the promoter region of the seed-specific *LeMAN2* gene from tomato (*Lycopersicon esculentum* cv. *Moneymaker*). In these experiments, we used the specific reverse primers (GSP1 and GSP2, see MATERIALS AND METHODS) designed to the 5' region of *LeMAN2* cDNA. We happened to isolate the 5' upstream, untranslated region of a new mannanase gene. We found the down stream, coding region of this gene in the BAC clone 241 P6 in the BAC library (LA483) of the wild tomato species, *Lycopersicon cheesmannii* (NSF Tomato Genome Project). We sequenced the genomic DNA of the clone of wild tomato and designed the primers to amplify the gene from *Lycopersicon esculentum* cv. *Moneymaker*. We obtained a 3.7-kb fragment of this gene and termed as *LeMAN5*. The coding region of *LeMAN5* was almost the same as that of the seed-specific *LeMAN2* except that *LeMAN5* lacked the first 8 bases of *LeMAN2* (data not shown). There were 4 introns in *LeMAN5* (Fig. 20), whereas *LeMAN2* had no introns (data not shown).

The promoter region of the potato homologue (termed *StMAN5*) was also amplified by PCR using the primers (5'- TCG TGT TCG TGT TGG TAC ATG -3', 5'- TTC ATC AAG TAA AAC CCT AGC -3'). The promoter sequences of *LeMAN5* and *StMAN5* show a high similarity (Fig. 21).

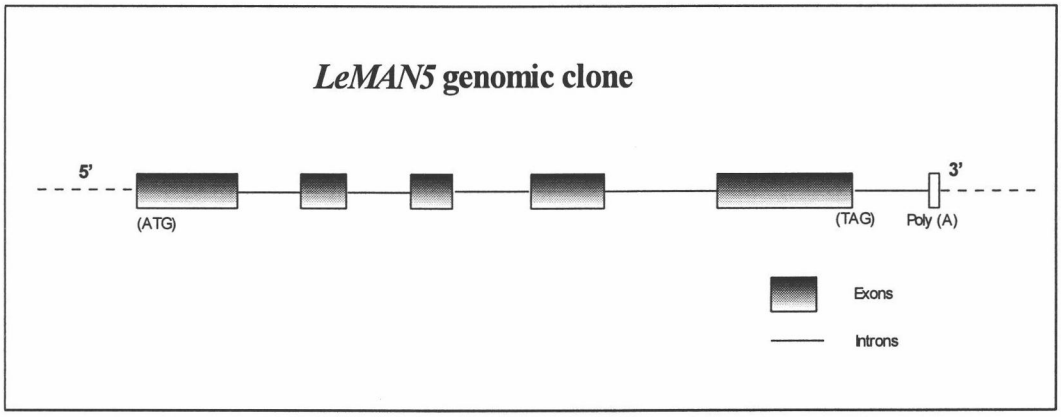

Figure 20. Structure of a new endo-β-mannanase gene from tomato.

Characterization of the *LeMAN5* promoter

To characterize the tomato promoter, a glucuronidase (GUS)-reporter gene fused to the *LeMAN5* promoter was constructed and used to transform *Arabidopsis*. Multiple transformants were obtained from two independently transformed plants. The T_1 plants were self-pollinated to produce seeds. The resultant T_2 generations were examined for kanamycin resistance. Three lines showed 3:1 Mendelian segregation in the T2 generation (data not shown).

The homozygous tomato lines were obtained from the T2 progeny by seeking 100% kanamycin resistance in the T_3 seed population. The kanamycin-resistant plants were tested by PCR for the presence of the promoter sequence in the genomic DNA. The transgenic plants that had the *LeMAN5* promoter were used for further experiments.

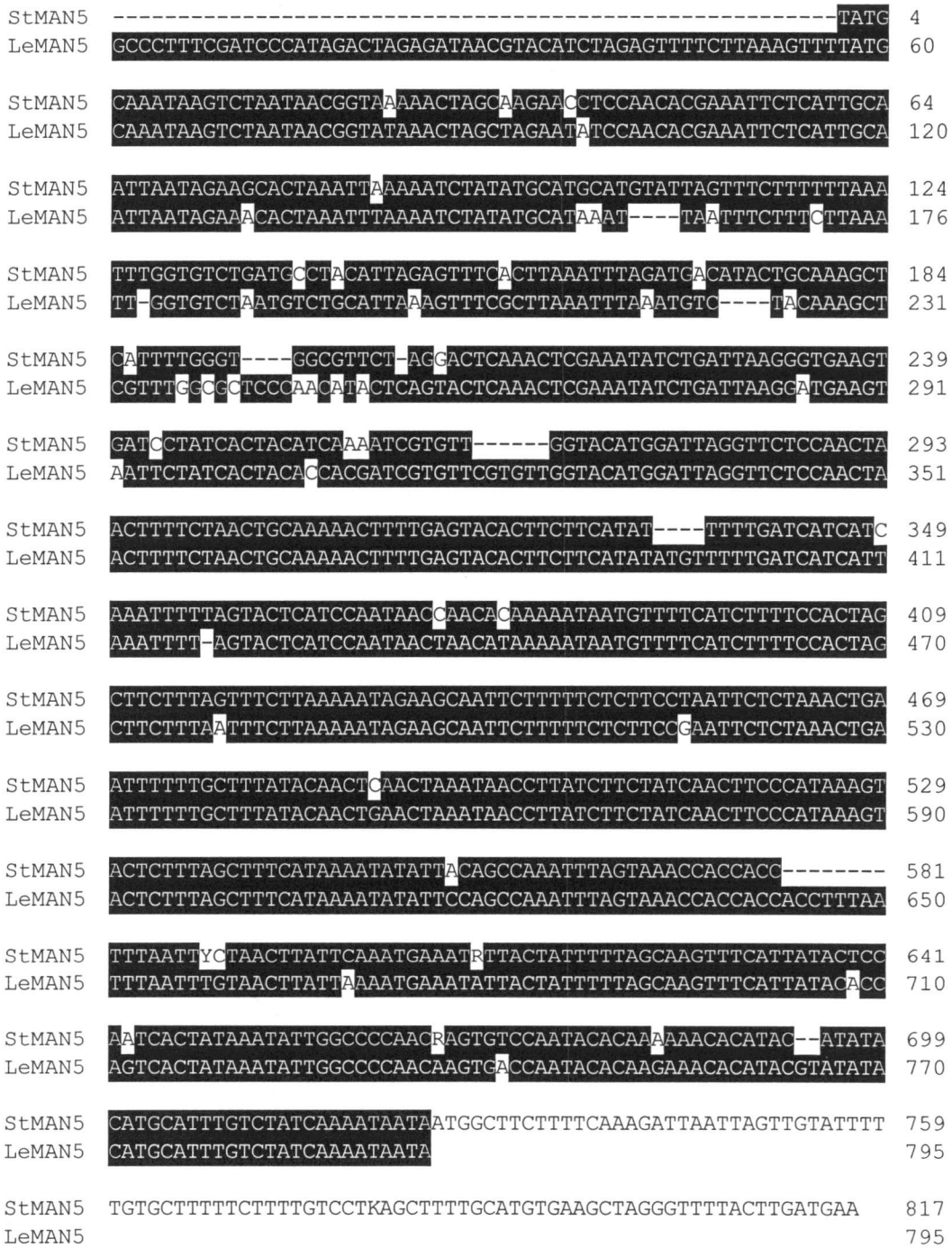

Figure 21. Alignment of DNA sequences of the promoter regions of tomato (*LeMAN5*) and potato (*StMAN5*) genes.

GUS activity was not observed in *Arabidopsis* roots, stems, leaves or seeds (data not shown). However, there was a strong signal of GUS activity in *Arabidopsis* flowers (Fig. 22A). The signal was detected mainly in the anthers. The signal was barely detectable during early stages of flower development but became very clear during relatively later stages (Fig. 22A). Both anthers and released pollen grains showed GUS activity (Fig. 22B). Thus, the *LeMAN5* promoter was activated in *Arabidopsis* flowers in tissue- and stage-specific manners. These results suggest that *LeMAN5* is an anther-specific endo-β-mannanase in tomato

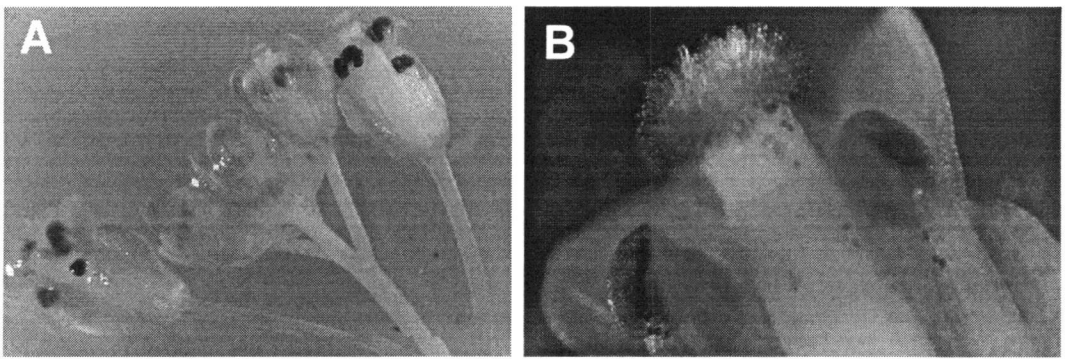

Figure 22. GUS reporter gene assay of the tomato mannanase (*LeMAN5*) promoter. A. Flowers-Anthers. B. Pollen.

To examine this possibility, endo-β-mannanase activity in tomato anthers at different stages was monitored by using a gel diffusion assay (Downie et al., 1994). Endo-β-mannanase activity was detected in tomato anthers at all stages examined

(Fig. 23). Peak activity was detected at relatively late stages, consistent with the timing of promoter activation in *Arabidopsis* flowers (Fig. 22, 23). These results suggest that *LeMAN5* is an anther-specific mannanase in tomato. To our knowledge, this is the first report of an anther-specific expression of mannanase. Since the expression of anther–specific mananase in tomato flowers occurs at the relatively late stages of flower development, this enzyme might play an important role in pollen maturation or anther dehiscence to release pollen.

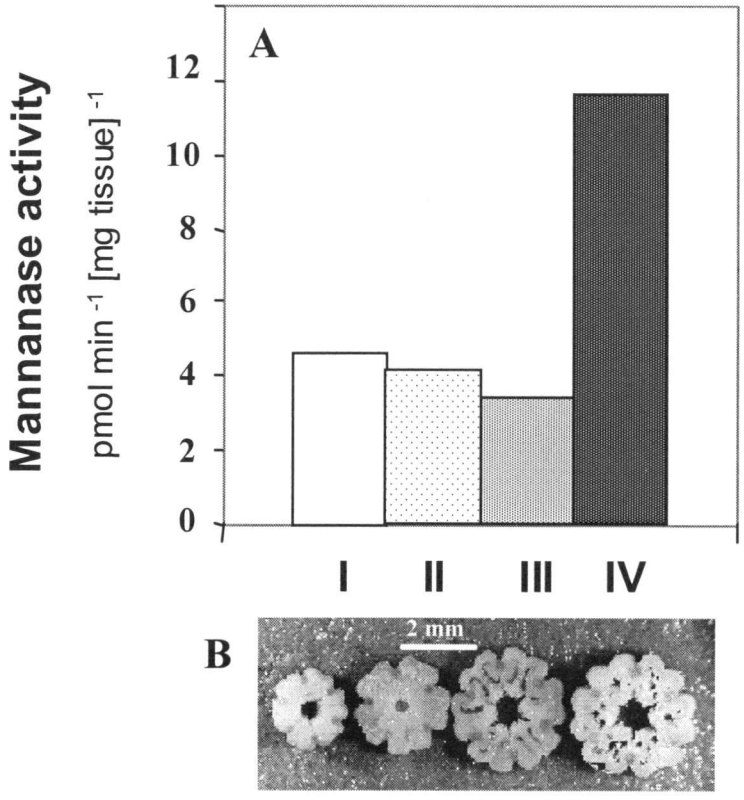

Figure 23. A. Mannanase activity in tomato anthers at different developmental stages. B. Cross-sections of four different stages of tomato anthers. Mature pollen was observed after stage IV.

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Dormancy and Germination of True Potato (*Solanum tuberosum* L.) Seeds: Characterization of Endo-ß-Mannanase Genes

Chapter 6

General Conclusions

Alvaro R. Monteros

This research dealt with mechanisms of seed dormancy and germination using true potato seeds (TPS) as a model system. Seed dormancy is a complex phenomenon regulated by multiple factors. Seed dormancy is due in part to immaturity or physiological inactivity of embryos in mature seeds (embryo dormancy). The physical and chemical properties of the outer cover of seeds, such as testa and endosperm, also influence dormancy (coat-imposed dormancy). In many instances a combination of embryo- and coat-imposed effects is observed.

Both embryo- and coat-imposed dormancy were observed in TPS (Chapter 2). Freshly harvested TPS showed very deep dormancy. De-tipping experiments clearly showed that mechanical resistance imposed by the endosperm cap inhibited radicle protrusion. However, de-tipping did not completely break deep dormancy at the optimal temperature (15 °C). Not all embryos of deeply dormant seeds showed radicle elongation after removal of the endosperm cap. This suggests that embryo dormancy also contributes to TPS dormancy in freshly harvested seeds. Embryo dormancy persisted despite several treatments. Biochemical and molecular biology research on the key enzymes and genes regulating embryo dormancy remain to be done. Identification and characterization of these factors will allow understanding the mechanisms of embryo dormancy and develop innovative technologies for dormancy breakage.

In contrast to embryo dormancy of freshly harvested TPS, the major constraint to germination in thermodormant 1-year-old TPS was mechanical resistance of the endosperm cap to radicle elongation. It appears that there was no

substantial degree of embryo dormancy in thermodormant seeds, because most de-tipped thermodormant seeds germinated. Endosperm weakening is probably the determinant factor in TPS germination at relatively high temperatures (25°C or above).

The results of this research suggest that one of the cell wall degrading enzymes, endo-β-mannanase maybe a key factor in endosperm weakening and induction of germination in TPS (Chapter 3). The expression of endo-β-mannanase is suppressed by continuous synthesis of ABA in thermodormant seeds. This conclusion is supported in that application of the ABA synthesis inhibitor fluridone, induced both mannanase expression and TPS germination under thermodormant conditions. The mechanisms of ABA synthesis and degradation are not yet fully understood. Further research on gene expression of ABA synthetic enzymes will address mechanisms by which ABA synthesis and degradation regulate dormancy levels in TPS and other seeds.

This research showed that endo-β-mannanases are also expressed in germinated seeds and anther tissues in potato and tomato flowers (Chapters 4 and 5). It is likely that endo-β-mannanase is involved in many physiological processes in plants, such as reserve mobilization during seedling establishment and pollen maturation in anthers, in addition to its important role in seed dormancy. This enzyme has been well characterized in seed germination processes in this and other research. However, the function of this enzyme in other events in plant physiology is very limited. Elucidating the involvements of endo-β-mannananses in cell wall

modifications and other phenomena in plants will contribute to our understanding of plant biology in the future.

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