SUPPRESSION OF PHOSPHOLIPASE Dα IN SOYBEAN

by

JUNG HOON LEE

B.S., Kyungpook National University, South Korea, 1999
M.S., Kyungpook National University, South Korea, 2001

AN ABSTRACT OF A DISSERTATION

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Abstract

Demands on value-added crops have been raised to improve agricultural, industrial, and economical value. Currently, transgene application is one of most effective methods to satisfy these demands. Success in herbicide-resistant soybean (*Glycine max* (L.) Merr.) has boosted genetic engineering to be used for biochemical, nutritional, cultural, and physiological improvements. The objectives of this study were to establish transgenic soybean lines with attenuated phospholipase Dα (PLDα) activity in the seed, test the alteration of fatty acid profiles affected by transgene and somaclonal variation, and evaluate the physiological alteration of transgenic lines by both transgene and somaclonal variation. To change fatty acid profile in soybean seed, we attenuated PLDα enzyme activity by an RNA interference construct using the PLDα gene sequence. Two transgenic soybean lines were established by particle inflow gun bombardment of co-bombarding pSPLDi and pHG1 transgenes, and evaluated for the presence and expression of transgenes thoroughly through the T_5 generation. PLDα-suppressed soybean lines were characterized by decreased PLDα enzyme activity and PLDα protein both during seed development and in mature seeds. The PLDα-attenuated transgenic lines, SW1-7-1-1 and SW1-7-1-2, contain 36% and 49% oleic acid in the filed and greenhouse evaluations, respectively, which are equivalent to the mid-oleic acid soybean lines improved by conventional breeding and mutagenesis. Phenotypic and genetic analysis of the transgenic lines suggested the possibility that the multi-copy transgene integration formed direct or indirect repeats by random ligation during integration and organization of transgenes in the soybean genome, and the transgene cluster with tandem repeats may consequently increase the probability of transgene silencing.

Various factors, such as high humidity and temperature, result in the loss of seed viability. Fayette seed stored for two months since harvest exhibited about 95% viability; however Fayette seeds stored for 33 months at room temperature and uncontrolled relative humidity become non-viable. PLDα-attenuated transgenic soybean seeds have been produced by transformation. PLD-suppressed transgenic soybean seeds have maintained viability when stored for 33 months at room temperature. Germination of transgenic seed stored for 33 months ranged from 30 to 50%. Increased leakage of electrolytes associated with the loss of viability was observed in null-transgenic and background seeds versus transgenic seed. The increase in electrolyte leakage may

have been induced by lipid peroxidation and free radical formation which can generate oxidative damage in the cell and subsequently decrease seed viability. Differences in the ultrastructure of cotyledon tissue were observed between PLDα-suppressed soybean and the background cultivar. The loss of viability in the background cultivar was consistent with observations of the plasma membrane being detached from the cell wall complex and disorganization of oil bodies.

Stresses caused by temperatures higher or lower than ambient are one of agricultural problems that reduce crop productivity in many areas and diverse species. To overcome the uncertainty of environmental fluctuations, efforts continue to improve high and low temperature tolerance in crops. PLDα-suppressed transgenic events were produced by antisense suppression driven by constitutive and seed-specific promoters using the particle inflow gun (PIG) bombardment method. Nine fertile transgenic events suppressed the expression of PLDα protein. PLDα enzyme activity in T_1 seed was observed to be reduced by 25 percent compared to the non-transgenic control. When soybean seedlings were exposed to lethal freezing temperature, increased electrolyte leakage associated with oxidative damage and biophysical changes were observed in non-transgenic soybean, whereas membrane stability and integrity were maintained in transgenic soybean seedlings. The early growth of PLDα-attenuated soybean seedlings was recovered from extreme heat-shock (45 °C) and freezing treatments (-8 °C). The disruption of the plasma membrane and organelles was observed in freeze-stressed non-transgenic control seedlings. On the other hand, the structures of the plasma membrane, oil bodies, and cell organelles in transgenic seedlings were partially sustained after enduring freezing and thawing stresses.

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Approved by:

Co-Major Professor
William T. Schapaugh

Co-Major Professor
Harold N. Trick

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CHAPTER 1 - Overall Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the major crops in the world, second to corn in total acreage and production in the United States (USDA-NASS, 2007). In 2006, it was forecast that soybeans were planted on 75.5 million acres (30.6 million hectares), producing a record 3.188 billion bushels (86.77 million metric tons) in the US. The total soybean crop value exceeded $ 20.1 billion dollars (forecast). US are a world leader in soybean exports, accounting for 42 percent of world's soybean trade. Soybean represented 57% of oilseed production in the world in 2006 and 38% of those soybeans were produced in the United States (USDA-ERS, 2007).

Two main products are processed from soybean; soybean oil and a dried residue after oil extraction known as meal. Soybean meal is the most important protein source for animal feed, accounting for 65% of protein supplies in the world (USDA-ERS, 2005). Soybean oil content represents about 20% of the total dry weight of the seed. Soybean oil is rich in phospholipids known as lecithin. The five major fatty acids in soybean are palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid. The fatty acids differ in the number of carbon atoms and degree of desaturation, which result in differences in nutritional value and their chemical properties. Soybean oil consists of 15% saturated and 83.5% unsaturated fatty acids. Average content of unsaturated fatty acid consists of 22% oleic acid, 54% linoleic acid, and 7.5% linolenic acid. It also contains the saturated fats consisting of 4% stearic acid and 10% palmitic acid (USDA, 1979).

Soybean oil accounts for about one-fourth of the worldwide edible oil production followed by palm oil, rapeseed oil, and sunflower seed oil (USDA-ERS, 2003). Vegetable oils on the market contain triacylglycerol molecules, which contain three acyl fatty acid chains and a single glycerol backbone. Raw soybeans processed by commercially and economically established extraction methods. The physicochemical properties of soybean oil enable it to be utilized not only in many edible applications, but also as a substitute for petroleum-derived materials, such as in the production of biodiesel (Metzger and Bornsheuer, 2006). Metabolic changes in oil composition have been accomplished via biotechnology in oil seed crop and model plants. The production of vegetable oils that contains altered fatty acid content and high-value fatty acid requires an understanding of the factors that mediate fatty acid metabolism and fatty acid flux in oil seed crops.

Modifying soybean oil composition is one of goals in soybean breeding programs (Burton, 1990). Based on the chemical and nutritional properties of soybean oil, changes in fatty acid profile in soybean seed to reduce saturated and polyunsaturated fat and increase monounsaturated fat are desirable to improve food and other applications of soybean oil (Wilson, 2004). Several novel fatty acid soybean genotypes have been established by conventional breeding, mutation breeding, and transgene application (Sandhu et al., 2007; Burton et al., 2005; Singh and Hymowitz, 1999). It is important that the fatty acid composition of genotypes containing novel fatty acid profiles be stable in different growing conditions (Primomo et al., 2002).

The quality and viability of seeds in storage is impacted by many environmental, biochemical, biological and genetic factors. The environmental factors contributing to the

quality and viability of soybean seed include time, temperature, microbial infection, and humidity. Conditions such as low temperature and humidity contribute to the preservation of the seed quality and viability during storage (Hutchinson, 1944; Justice and Bass, 1978).

Processes that occur drying seed aging include in membrane protein composition (Nowakowska and Rakowski, 2002), disruption of the nuclear envelope (Haithcock et al., 2005), protein degradation (Kumar et al.,1999), decreases in lipid content (Lin and Pearce, 1990), and oxidative stresses, as well as decreases in mRNA translation (Gidrol et al., 1990) and DNA replication capability. It has also been reported that increased oxidative damage and reduced levels of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and ascorbate peroxidase are key components of the aging process (Bailly et al., 1996; 1998).

Altered electrolyte leakage through the plasma membrane and increased membrane fluidity are inevitable events during long-term storage of soybean seed (Leinonen, 1998; Rakowski et al., 1998). Oxidation and peroxidation of lipid decrease the economic and nutritional value of soybean seed products through modifications in color, taste and odor (Nakayama et al., 1981). Understanding the impact of lipid degrading enzymes on the stability of the lipid membrane is an important component of elucidating the natural aging process (Devaiah et al., 2007). Among many lipid degrading enzymes, phospholipase D (PLD) may be involved in the first step of phospholipid degradation to generate phosphatidic acid (PA).

Phospholipase D enzymatic activity and PLD families

PLD is a hydrolytic enzyme which cleaves a phospholipid to phosphatidic acid and a head group (inositol, glycerol, ethanolamine, and choline). PLDs hydrolyze the P-O bond in the polar head groups of glycerophospholipids (Hanahan and Chaikoff, 1947). Glycerophospholipids such as phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC) are major substrates of PLD reaction (Qin and Wang, 2002). In addition, PLDs catalyze transphosphatidylation in which the phospholipid head group is exchanged. For example, PC can be converted to PS using L-serine as a substrate (Yang et al., 1967; Dawson, 1967).

Compared to mammalian PLD genes, plants possess a diverse family of PLD genes. Mammalian and yeast genomes contain two and one PLD gene, respectively, implying that PLD may be involved in the lipid signaling cascade (Elias et al., 2002). In *Arabidopsis* alone, 12 PLD genes have been cloned and separated in 6 classes based on sequence similarity (Wang, 1997; Qin and Wang, 2002; Wang, 2005). Plant PLDs can be divided into 2 subfamilies by conserved domains located on the N-terminus. Both subfamilies of PLD genes contain two HxKxxxxD (HKD) motives in the C-terminal region, which are essential for lipase activity. PLDζ and mammalian PLDs contain a phox homology (PX) domain and a pleckstrin homology (PH) domain characterized as lipid binding and linking to polyphosphoinositide signaling, whereas PLDα, -β, -γ, -δ, and -ε contain a C2 domain (van Leeuwen et al., 2004). C2 domains activated by Ca^{2+} cause changes in localization, resulting in soluble proteins binding to membranes. C2 domains are also involved in protein-protein interactions (Kopka et al., 1998). From phylogenetic data of protein structure, homology, and the presence of the PH and PX domains in two *Arabidopsis*

PLDζs, it is suggested that these proteins are more closely linked to yeast and mammalian PLDs than to other plant PLDs (Bargmann and Munnik, 2006).

Plant PLDs mainly catalyze the hydrolysis of PC, PE, PI and PG into PA and a free head group (Pappan et al., 1998). Characteristics of different classes of plant PLDs were fully examined *in vitro,* in terms of substrate selectivity, appropriate prerequisite of pH and catalytic agents, and stimulants for enzymatic activity. In addition, *Arabidopsis* PLDδ activity has been reported to be stimulated by oleate (Zhang et al., 2003). PLDs mainly differ in their dependence on Ca^{2+} and phosphoinositides (PI) for their activity as well as specific localization in the cell (Pappan et al. 2004; McGee et al. 2003).

Regulation of plant PLD activity is more complicated *in vivo*, as PLD is regulated by the cellular levels of Ca^{2+} and PIP_2 as well as by interacting with the α1-subunit of heterotrimeric G proteins (Lein and Saalbach, 2001). Actin and phosphorylation also have been suggested to regulate plant PLD activity *in vivo* (Kusner et al., 2003; Novotna et al., 2003).

Phospholipase D function

For many decades, PLD in plants was believed to be involved in merely catabolism and anabolism of phospholipids, such as the degradation of membrane lipid during senescence and necrosis. Recently, many other functions of PLD have been revealed in plants, animals, and microbes. In higher plants, PLD isoenzymes play different roles in membrane remodeling and cell signaling during development, stress response, programmed cell death, etc. (Zhang et al., 2003; Taylor and Low 1997). PLDα1, known as PC-PLD, is

the conventional type of PLD in plants and exhibits the highest activity under milli-molar concentrations of Ca^{2+}. PLDα, a subfamily of PIP_2-independent PLD among the membrane-binding and cytosolic PLDs, is relatively high expressed in a broad range of plant cell types. PLDα1 activity tends to decline as plants mature, where as soluble and membrane bound PIP_2- dependent PLD activities such as PLDβ and PLDγ showed peak activity at the end of seed maturation in *Brassica napus* seeds (Novotna et al., 2000; Pappan et al., 2004; Wang, 2005).

A well-known function of most lipases, including PLD, is to degrade lipid membranes. PLD activity can break down membrane lipid species such as PC, PE, and PG generating PA and a free head group. By accumulating PA in cell, the formation of hexagonal lipid particles results in the loss of cell membrane integrity and cell viability (Kooijman et al., 2003). It has been suggested that PLD may be involved in senescence, wounding and cell death by degrading membrane lipids (Fan et al., 1997).

Munnik and Musgrave (2001) proposed that PLDs function as a tether between membranes and proteins. Membrane tethering by PLD was hypothesized by discovering the presence of bridges between microtubules and the plasma membrane in tobacco cell. A phosphatidyl-PLD intermediate formed by the first step of transphosphatidylation was quickly deployed on membranes (Gardiner et al., 2001). The formation of the microtubule cytoskeleton is disorganized by treatment of n-butanol in tobacco cell suspensions (Dhonukshe et al., 2003). A phospholipase D antagonist 1-butanol affects root shape, root hair, and cotyledon morphology. PLDs are crucial enzymes to seedling development and microtubule formation (Motes et al., 2005). PLD was also proposed to be involved in

vesicular transport both by signaling and forming membrane during vesicle fission and fusion (Ktiskakis, 1998).

There is evidence that PA produced from membrane phospholipids serves as a secondary messenger molecule in plants (Cockcroft, 1996). PA is generated either indirectly by a two step action of PLC and diacylglycerol kinase or directly by PLD activity (Munnik, 2001). PLDs and PLD-derived PA are involved in numerous physiological processes in lipid metabolism, defense response, hormone action, cell development, and aging. PLD, as a cell membrane degrading enzyme, releases a free head group (mainly choline) and produces PA. PLD-derived PA and DAG kinase-derived PA as cell signaling molecules may induce several signaling changes in Ca^{2+} concentration and other targets like protein kinases, actin assembly and secretion. Signal transduction is the most successful strategy to overcome environmental hostilities. An elicitor perceived by a receptor can trigger modification of G protein in the plasma membrane. G protein activates PLD expression by an unknown downstream signal, and directly activates PLDβ and PLDγ. However, G protein does not activate PLDα, which hydrolyze N-acylphosphatidylethanolamine (NAPE) to N-acylethanolamine (NAE). Pathogen elicitor increases NAE14:0 levels in tobacco cell (Chapman et al., 1998), and NAE14:0 subsequently activates the expression of phenylalanine ammonia lyase (PAL) independent from other stimuli of pathogen. Another putative model for NAPE/NAE metabolism is that the downstream gene, nitric oxide synthase (NOS), is activated by PLDβ and PLDγ, perceiving pathogen elicitors (E1, E2, and E3) by G protein linked receptors (R1, R2, and R3), respectively. In animal cells, NAE triggers the accumulation of nitric oxide (NO)

which is involved in defense gene activation (Stefano et al., 1996). Recent research suggested that NO functions as a signal molecule to interact ROI-mediated defense response (Delledonne et al., 1998).

Wounding may cause translocation of PLDs to the plasma membrane, resulting in the influx of calcium ion into the cytoplasm. PLD mRNA and protein levels are not increased by wounding (Wang et al., 2000). Elevated calcium ion can trigger wound responsive gene expression and transient accumulation of jasmonic acid. PLDα by coordinating with PLA2 directly triggers NADPH oxidase to produce Reactive oxygen intermediates (ROI)-mediated defense gene activation or the release of free fatty acids and lysophospholipids by wounding. Free fatty acids from the chloroplast and lysophospholipids used for jasmonic acid biosynthesis can activate downstream genes for wounding and defense responses. The accumulation of free fatty acid in wounded castor bean leaves was associated with PLD-mediated hydrolysis of phospholipids (Ryu and Xang, 1998).

PLDs are also involved in ABA-induced stomata closure. ABA produces reactive oxygen species that contribute to the open plasma membrane Ca^{2+} channel. PLDs are regulated by the change in cytoplasmic Ca^{2+} concentration by ABA. Ca^{2+} -dependent PLDs produce PA, and then PA may inactivate K+ channels in the membrane, eventually leading to the closing of stomata (Jacob et al., 1999). ABI1 protein putatively works as a signal protein to inhibit ABA responses. ABA-dependent PLDα1-derived PA is associated with ABI1 protein bound to the plasma membrane. It is proposed that PA produced by ABA-

dependent PLDα1 activity inhibits the action of ABI1 protein by tethering in the plasma membrane so that the ABA response can occur (Zhang et al., 2004).

Welti et al. (2002) profiled changes of lipid species between wild-type and PLDα-deficient *Arabidopsis* with electrospray ionization tandem mass spectrometry (ESI-MS/MS) to elucidate the role of PLDα in cold and freezing injury. The amount of many lipid species which have two polyunsaturated acyl tails such as 36:6-PC, 36:5PE, 36:6-PE, 34:6-MGDG, and 36:6-DGDG were increased in both plants suffering from cold acclimation and freezing. Molecular species of PA, with the same fatty acid as those lost from PA, dramatically accumulated in freezing treatments, but this occurred to a lesser extent in plants in which PLDα1 was suppressed. This suggested that PC is the substrate of PLDα1 *in vivo* (Welti et al., 2002).

Enzymatic activity and function of PLDα in seeds

Recently, Devaiah et al. (2007) reported that seed viability and lipid stability were enhanced by suppressing PLDα in *Arabidopsis*. Synthesis of glycerophospholipids occurs in the endoplasmic reticulum (ER) and chloroplast in plants. During lipid biosynthesis, PI and PG are synthesized from PA in the ER and chloroplast, respectively. On the other hand, PE and PC are produced from DAG only in the ER. In the membrane, these glycerophospholipids are hydrolyzed by phospholipases and generate intermediates such as lysoPC, lysoPS lysoPE, lysoPG, lysoPI, PA, DAG, and free head groups. Lysophosphatidate synthesized from the acyl-CoA pool by acyltransferase is the precursor of PA in seeds. Membrane-associated PLD activity in seeds is the major anabolic pathway

related to membrane lipid degradation. Diacylglycerol cholinephosphotransferase is a key enzyme converting DAG to PC species. Triacylglycerol is synthesized from DAG with one additional enzyme step. PLD activity is the opposite-directional counterpart of triacylglycerol synthesis. The putative role of PLD in seeds is degrading membrane lipids and supplying diacylglycerol to produce storage lipid. PLDα is the pre-existent form of PLD in the plasma membrane and cytosol. Membrane bound PLDα activity tends to decline as plants mature. Soluble PLDα activity peaks at the end of seed maturation in *Brassica napus* seeds (Novotna et al., 2000). PLDβ and -γ are induced by wounding and other stress factors (van Leeuwen Wet al., 2004) and are relatively ubiquitous in plant tissue, including seeds.

In seeds, PA is a major source of DAG. DAG may be converted to various PC species. The soluble form of PLD activity dramatically increases at the end of seed maturation through germination (Novotna et al., 2000; Ryu et al., 1996). The soluble PLD activity is not only involved in the utilization of PC in the lipid metabolic pathway in the seed but is also detected in the hypocotyl.

Plant transformation using RNAi

The use of biotechnology to generate value added soybean with enhanced oil quality and abiotic stress tolerances appears promising. The transgenic approach to knock-out or knock-down intrinsic genes using RNA interference (RNAi) as means of effecting degradation of the targeted transcript has been investigated (Kerschen et al., 2004). Gene silencing was first recognized as co-suppression of endogenous gene and homologous gene

by counterparts of the RNA molecule (Napoli et al., 1990; van der Krol et al., 1990). While gene silencing was a well known phenomenon in plant and other organisms, the principal mechanisms of gene silencing had not been understood until Fire et al. (1998) discovered that double stranded RNA (dsRNA) functions as an inducer of post transcriptional gene silencing (PTGS) in *C. elegans*. RNAi-dependent gene silencing has been demonstrated in plants, animals, and fungi (Agrawal et al., 2003). The RNAi mechanism has been applied to reverse genetic studies in many organisms (Misquitta et al., 1999; Chuang and Meyerowitz, 2000). RNAi has also been used as a therapeutic agent against genetic disorders, cancer, and viral infection (Wall and Shi, 2003). The exploitation of RNAi has been extended to establishing transgenics with suppression of target endogenous genes in various organisms (Agrawal et al., 2003).

PLD study in soybean

Abousalham et al. (1995) purified soybean PLDα from a soybean cell suspension culture using hydrophobic affinity chromatography. The size of soybean PLD was estimated to be 92 kDa. The sequence of the 15 amino acid residues of the N-terminus is similar in sequence to cabbage and castor bean PLD. Like PLDs isolated from other plants, the activity of soybean PLD is affected by the concentration of Ca^{2+} at a specific pH (Abousalham et al., 1995). A full length soybean PLD homologous gene has not been cloned, although 45 different PLD expressed sequence tags (EST) from soybean are detectable in the NCBI nucleotide database. The most predictable benefit by reduced PLDα activity in food industry is the alteration of lecithin content in soybean. Lecithin, one type

of phospholipid abundant in soybean, is a substrate for PLD. Soybean has been used for producing lecithin as a byproduct during oil extraction. Soybean lecithin ranged from 1.48 to 3.08 percent of fresh weight of seeds. A high level of PLD enzyme activity was observed in dried peanut seeds (Heller et al., 1968), and total PLD activity in soybean cotyledon increase during seed development (Ryu et al., 1996). PLD activity in seeds has been recognized as contributing to the deterioration in quality of seeds during storage, handling, and processing (List et al., 1990; 1992; Nakayama et al., 1981). Recently, Devaiah et al. (2007) reported that seed viability and lipid stability were enhanced by suppressing PLDα in *Arabidopsis*. Reduction in PLDα activity in seeds holds great potential to improve not only the quality and quantity of soy oil and lecithin, but also the shelf-life of soybean seeds.

The overall objective of my dissertation was to produce value-added soybean genotypes by suppressing PLDα activity in the soybean seed. Detailed objectives were: 1) establish transgenic soybean lines with attenuated PLDα activity in the seed and test the alteration of fatty acid profiles affected by transgene and somaclonal variation, 2) improve seed viability by suppressing PLD activity in the seed, and 3) investigate low temperature and heat-shock tolerance in PLDα-suppressed soybean seedlings.

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CHAPTER 2 - Changes in fatty acid profile in PLDα-suppressed soybean seed

Abstract

PLD may be involved in the first step of phospholipid degradation. The objectives of this study were to establish transgenic soybean lines with attenuated PLDα activity in the seed, test the alteration of fatty acid profiles affected by transgene and somaclonal variation, and evaluate the physiological alteration of transgenic lines by both transgene and somaclonal variation. To change fatty acid profile in soybean seed, we attenuated PLDα enzyme activity by an RNA interference construct using the PLDα gene sequence. Two transgenic soybean lines were established by particle inflow gun bombardment of co-bombarding pSPLDi and pHG1 transgenes, and evaluated for the presence and expression of transgenes thoroughly through the T_5 generation. PLDα-suppressed soybean lines were characterized by decreased PLDα enzyme activity and PLDα protein both during seed development and in mature seeds. The PLDα-attenuated transgenic lines, SW1-7-1-1 and SW1-7-1-2, contain 36% and 49% of oleic acid in the field in the greenhouse evaluations, respectively, which are equivalent to the mid-oleic acid soybean lines improved by conventional breeding and mutagenesis. Genetic analysis of the transgenic lines suggested the possibility that the multi-copy transgene integration formed direct or indirect repeats by random ligation during integration and organization of transgenes in the soybean genome,

and the transgene cluster with tandem repeats may consequently increase the probability transgene silencing.

Introduction

Modifying soybean oil composition is one goal in soybean breeding programs (Burton, 1990). Based on the chemical and nutritional properties of soybean oil, changes in fatty acid profile in soybean seed to reduce saturated and polyunsaturated fat and increase monounsaturated fat are desirable to improve food and other applications of soybean oil (Wilson, 2004). Several novel fatty acid soybean genotypes have been established by conventional breeding, mutation breeding, and transgene application (Sandhu et al., 2007; Burton et al., 2005; Singh and Hymowitz, 1999). It is important that the fatty acid composition of genotypes containing novel fatty acid profiles is stable in different growing conditions (Primomo et al., 2002).

Phospholipase D (PLD) in plants had been observed to be involved in the catabolism and anabolism of phospholipids, such as the degradation of membrane lipids during senescence and necrosis. Recently, other functions of PLD have been revealed in plants, animals, and microbes enhanced by cloning PLD genes from different organisms. In higher plants, PLD isoenzymes play different roles in membrane remodeling, cell signaling during development, stress response, and programmed cell death (Zhang et al., 2003; Taylor and Low, 1997). PLDα1, known as PC-PLD, is the conventional type of PLD in plants and exhibits the highest activity under milli-molar concentrations of Ca^{2+}. PLDα, a form of PIP_2-independent PLD among the membrane-binding and cytosolic PLDs, is relatively high expressed in a broad range of plant cell types. PLDα1 activity tends to decline as plants mature, while soluble and membrane bound PIP_2- dependent PLD

activities such as PLDβ and PLDγ peaked at the end of seed maturation in *Brassica napus* seeds (Novotna et al. 2000; Pappan et al. 2004; Wang 2005).

Devaiah et al. (2007) reported that PLDα-knockout and knockdown *Arabidopsis* tended to decrease the fatty acid content of seeds. The total amount of 5 major fatty acids, palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid, in fresh weight were significantly decreased in *pld*α1-AS (antisense) and *pld*α1-KO (knockout) seeds. The effect of PLDα on fatty acid metabolism is still unclear. The high expression of PLDα1 increases deterioration of phospholipid membrane, and may be certainly involved in lipid cycling.

Abousalham et al. (1995) purified soybean PLD from a soybean cell suspension culture using hydrophobic affinity chromatography. The estimated size of soybean PLD was 92 kDa. The sequence of the 15 amino acid residues of the N-terminus is similar in sequence to cabbage and castor bean PLD. Like PLDs isolated from other plants, the activity of soybean PLD is affected by the concentration of Ca^{2+} at a specific pH (Abousalham et al., 1995). A full length soybean PLD homologous gene has not been cloned, although 45 PLD expressed sequence tags (EST) from soybean are detectable in the NCBI nucleotide database. Gm-r1030, a putative precursor RNA of PLD, is one such candidate discovered from an EST from immature soybean cotyledons.

The objectives of this study were: 1) to establish transgenic soybean lines with attenuated PLDα activity in the seeds, 2) to evaluate the physiological alteration of transgenic lines by both transgene and somaclonal variation, and 3) to examine any changes in soybean oil quality by suppressing PLDα activity in the seed.

Material and Methods

Plasmids construction for transformation

The 1.3 Kb partial sequence of soybean PLDα (SPLDα) was cloned by RT-PCR using degenerate primers from the castor bean PLD sequence (Wang et al., 1993; 1994). The plasmids used for soybean transformation via particle inflow gun (PIG) bombardment originated from pUC19 and the expression cassette were subcloned from pCong (4.1 Kb) harboring soybean β-conglycinin α subunit promoter and terminator unit (Doyle et al., 1986). A pSPLDanti (β-conglycinin promoter::SPLDanti) plasmid was constructed using 1.3 Kb partial sequence of SPLDα. SPLDα was digested with *Bam*HI and ligated between the β-conglycinin promoter and terminator in the antisense orientation. To construct pSPLDi, an 820 bp of SPLDα partial sequence was subcloned into pGEM-T Easy vector (Promega), and multi-cloning site which is not homologous through the plant kingdom were added at the 3'end of SPLDα fragment. The subcloned fragment digested by X*ba*I and S*al*I was ligated between the β-conglycinin promoter and SPLD anti sequence at the pSPLDanti vector. The vector pHG1 (Finer et al., 1991), containing the selectable marker gene hygromycin phosphotrasferase or HPT (1.0 Kb), was used in co-bombardment to provide hygromycin resistance to transgenic tissue. The HPT gene served as a selectable marker during transformation and regeneration (Fig. 2.1).

Plant material and tissue culture

Soybean cultivars 'Flyer' (Bernard et al., 1987) and 'Fayette' (Bernard et al., 1988) were grown in a greenhouse under 25 ± 2 °C/ 18 h day and 20 ± 2 °C/ 6 h night

photoperiod. Immature pods were excised at growth stage R5 and surface-sterilized with 1.05% sodium hypochlorite (NaOCl) and 0.02% Tween-20 for 15 min with agitation, and then followed by three washes with sterile distilled water in a hood. Methods for culture initiation and proliferation were as described by Finer (1988) and modified by Trick et al. (1997). Briefly, approximately three to six months after initiation of somatic embryo cultures on D40 medium, the embryos were proliferated on D20 medium, prepared for bombardment and then transformed using the particle inflow gun (Finer and McMullen, 1991). For co-bombardment, one microgram of pSPLDi plasmid and pHG1 plasmid were mixed in a 1:1 ratio. Putative transformed tissues were maintained on D20H7.5, D20H3, and D20H15 medium containing 7.5 mg/l, 3 mg/l, and 15 mg/l hygromycin, respectively. Throughout the selection process, hygromycin concentration was either increased or decreased depending upon the health of the tissue. After 3 to 6 months, putative transgenic clumps were tested by PCR for the presence of pSPLDi and HPT transgenes. Transgene positive tissues were then transferred and matured on M6 medium. After embryos matured to a 'torpedo' stage of development and an apical meristem was visible, embryos were desiccated for 1 to 2 days and rooted in vermiculite saturated with 1/2-strength OSM medium. Recovered plants possessing two trifoliate leaves were transplanted to peat moss, harden at 23 ºC under 24 hour light then transferred to 5 gallon pots and grown to maturity in the greenhouse under a 16-h photoperiod.

PCR, southern blot and northern blot analyses

Genomic DNA was extracted from young soybean leaves and immature seeds via a modified CTAB DNA extraction method by Saghai-Maroof et al. (1984). For PCR

reactions, 50 ng of genomic DNA in 5 µl aliquots was used as a template in a 50 µl PCR reaction containing 1X NH_4 buffer, 1.5 mM MgCl, 0.2 mM of deoxinucleotide triphosphate (dNTPs), 20 pmol of forward and reverse primer and 1.25 U *Taq* DNA polymerase (BioLab). Primer sequences are shown in Table 2.1.

For southern blot analysis, 20 µg of genomic DNA was digested overnight using the appropriate restriction enzyme (50 U) at the designated temperature. Quantification of DNA was performed using the Nanodrop ND1000 spectrophotometer (Nanodrop, DE USA). Digested DNA samples were separated by electrophoresis on a 0.8% TAE agarose gel and denatured with a 0.4 M NaOH solution for 12 to 16 hours. Using the semi-dry and wet capillary transfer methods, DNA was blotted onto Hybond N^+-XL membrane (Amersham Biochech Ltd., Piscateway, NJ).

A 1.2 Kb SPLDα fragment, a 1.0 Kb HPT fragment, and a 1.1 Kb β-conglycinin promoter fragment were used for probe synthesis, which were digested from pSPLDi and pHG1 plasmids. All DNA probes (50 ng) were labeled with [αP-32]dCTP (sp. ACT. 3000 *Ci*/mM) using the random priming method (Promega, WI, USA). A radioactive labeled probe was then purified by Nick column (Amersham), to remove the unlabeled [αP-32]dCTP and non-specific small fragments. The purified probe DNA was denatured at 95 ºC for 10 min and placed on ice for 10 min. The membrane was hybridized overnight with each DNA probe at 65 ºC in hybridization solution. After hybridization, the membrane was washed twice in 2X SSC with 0.1% SDS for 15 min and twice in 0.2X SSC with 0.01% SDS for 30 min at 65 ºC.

For the RNA analysis, total RNA was isolated from soybean tissues (>200mg dry weight) using the TRIZOL reagent (Invitrogen, Canada) extraction method. Fifteen μg of total RNA was separated on a 1.0% agarose gel containing 1.5% formaldehyde and transferred onto a nylon membrane using the semi-wet capillary method. The membrane was hybridized at 42 ºC in 10 ml of ULTRAhyb^{TM} Solution (Ambion, CA USA) with 1.1 Kb HPT probe containing a [αP-32]dCTP labeled with random priming and was washed twice in 1X SSC with 0.1% SDS for 15 min at 42 ºC and twice in 0.1X SSC with 0.01% SDS for 30 min at 42 ^{o}C. The hybridizing signals were detected using the Storm840 phosphorimager (Molecular Dynamics, CA USA).

RT-PCR and real time RT-PCR

To eliminate genomic DNA contamination, RNA samples were treated with 1 μl of RNase free RQ1 DNase (Promega, WI USA) for 20 min. Two μg total RNA was used for cDNA synthesis with the AMV reverse transcription system according to the manufacturer's protocol (Promega, WI, USA) with a random hexamer for amplifying transgene and oligo-dT primer for amplifying endogenous PLD mRNA. After cDNA synthesis, DNase free water was added up to a final volume of 100 μl. To amplify transgene transcript and endogenous PLD mRNA, five μl of aliquot was used for a template with individual primer set via 25 cycles of PCR reaction. The PCR condition was 95 ºC for 3 min as activating *Taq* polymerase for 1 cycle, and then 95 ºC for 45 s, 52 ºC for 30 s and following 72 ºC for 1 min 30 s for extending amplicons with 25 cycles.

To quantify gene expression PLD transcripts from various soybean seeds, 20 μl aliquots from reverse transcription were diluted in 80 μl of nuclease free water and used for

a template of real time RT-PCR following manufacturer's instruction (BIO-RAD, CA, USA). Primers for quantitative RT-PCR were designed by Beacon designer 6 (Premier Biosoft International, CA) with around 55 to 60 ºC Tm value. Primer sequences for realtime RT-PCR are listed in Table 2.1. The PCR reaction was performed in 50 µl of 1X iQ SYBR Green Supermix, 200 nM of each primer, cDNA template and sterile water via Bio-Rad iCycler (BIO-RAD, CA, USA). Each reaction had 3 biological replications with 2 technical replications. The gene expression fold was technically calculated by $2^{-\Delta\Delta CT=(CtGOI\ for\ Tr-CtGOI\ for\ CK)-(CtHKG\ for\ Tr-CtHKG\ for\ CK)}$. When PCR efficiencies for each primer set are not 100%, $[(1+E_{GOI})^{-\Delta CtGOI}]/[(1+E_{HKG})^{-\Delta CtHKG}]$ [Ct: threshold cycle; GOI: gene of interest; HKG: house keeping gene; Tr: cDNA samples from transgenic soybean; CK: cDNA samples from non-transgenic control; E: primer efficiency (%)] was substituted for ΔΔCt value.

Western blot analysis

Proteins were extracted from immature or mature soybean seed (Zhang et al., 2003). Total native proteins were incubated at 95 ºC for 15 min with SDS-PAGE loading buffer which was 100 µl of 50 mM Tris-HCL, pH 6.8, 10 mM DTT, 2% SDS, 0.01% bromophenol blue, and 10% glycerol. Twenty micrograms of denatured proteins then were fractionated by 10% SDS-PAGE gel on 100 V until dye reached the bottom of the PAGE gel. The membrane containing denatured proteins was blotted with *Arabidopsis* PLDα1 antibodies as described by Zhang et al. (2003). Briefly, gels were transferred to an Immobilon^{TM}-P transfer membrane (Millipore, Bedford, MA) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The

membranes were blocked with non-fat milk for 1 h and soaked in the first antibody overnight. The PLDα polyclonal antibody was diluted to 1:1000 in TBST (10 mM Tris, 140 mM NaCl and 0.05% Tween 20). The blots were washed in 1 X TBST 3 times, 5 min/wash. Goat anti-rabbit IgG (H+L) horseradish peroxide (HRP) conjugate (Bio-Rad) was used as the second antibody at a dilution of 1:1000 in TBST. The blots were incubated in a second antibody for 1 h and washed two times in TBST followed by one wash in TBS (10 mM Tris, 140 mM NaCl, pH 7.5). The protein bands recognized by the antibody were visualized with a HRP color development reagent (Bio-Rad).

PLD enzyme assay

Total protein was extracted by grinding immature and mature soybean seed with liquid N_2 chilled screw driver in 1.5 mL microcentrifuge tube with homogenization buffer containing 50 mM Tris-HCL, pH 7.,5, 10mM KCL, 1 mM EDTA, 0.5 mM phenyl methylsulfonyl fluoride, and 2 mM DTT at 4 ^{o}C (Fan et al., 1997). Protein concentration was measured using the Nanodrop ND1000 spectrophotometer. Aliquots of 20 µg of native protein were used for the PLD enzyme assay as described previously (Fan et al., 1997). Briefly, the reaction mixture contained 100 mM MES, pH 6.5, 25 mM $CaCl_2$, 0.5 mM SDS, 1% (v/v) ethanol, and 2 mM phosphatidylcholine (egg yolk) containing dipalmitoly glycerol-3-phosphate-(methyl-3H)-choline in a final volume of 200 µl. Substrate preparation, reaction conditions, and product separation were based on the procedure used by Wang et al. (1993). The release of free head group, ^{3}H-labeled choline, into the aqueous phase was quantified using a liquid scintillation counter (Beckman).

Transgenic soybean field and greenhouse experiment

Agricultural traits of PLDα-suppressed soybean lines were evaluated in 2005 by planting T_4 transgenic soybean lines derived from individual T_3 plants and in 2006 by planting T_5 transgenic soybean lines, bulked from the progeny tested in the T_4 generation. Non-transgenic check cultivars were planted along with the transgenic lines at the Kansas State University Ashland Agronomy Farm, Manhattan, Kansas in 2005 and 2006, respectively. Nine T_4 pSPLDi transgenic lines, two null genotypes (derived from a transgenic line, but has been lost the transgene through segregation), and two background cultivars were planted on 21 July 2005. In 2006, 9 T_5 lines of homozygous pSPLDi transgenic soybean plants, 2 null genotype lines, and 2 check cultivars were planted at the same location on 26 June. The experimental design in the 2005 field test was a randomized incomplete block design with 1 to 4 replications per entry and was a randomized complete block design with 3 replications per entry in 2006. The check cultivars included 'Fayette' and 'Flyer'. Weeds were controlled with pre-emergence herbicide application and hand weeding.

The center two rows of each plot were evaluated for plant maturity, plant height, lodging, 100-seed weight, seed quality and plot yield. Maturity was recorded as the date when 95% of pods had reached mature color. Height was measured in cm as the distance from the soil surface to the top of main stem. Lodging was rated on a scale of 1 to 5, where 1 equaled almost all plants erect, and 5 equaled almost all plants prostrate. Seed weight was measured as weight in grams per hundred seeds. Seed quality was rated according to a 1 to 5 score considering the amount and degree of wrinkling, defective seed coats, greenishness,

and moldy or rotten seeds, where 1 equaled excellent and 5 equaled poor. Plot yield was recorded as the weight in grams of seed from the center two rows of the plot.

Eight transgenic lines and two background lines were planted in greenhouse in Nov. 11th, 2006. Ten lines, 5 SW progeny lines, 3 SI progeny lines, and 2 checks, were grown in 10 gallon (CONVERT GALLONS TO METRIC) pots under 25 ± 2 °C/ 18 h day and 20 ± 2 °C/ 6h night photoperiod. Seed from five individual plants from each line were harvested in Mar. 20th, 2007 for evaluation.

Fatty acid, protein, and oil analyses by near infrared (NIR) method

Oil and protein contents were measured the using the nondestructive NIR method for whole grain analysis (American Association of Cereal Chemist (AACC) method 39-21, 1998). Fifteen to 20 seeds were used for oil and protein analysis. For fatty acid analyses, a 50 mg sample of clean seed from various generations was prepared by taking an equal volume or weight of seed from each replication. Seed samples were submitted to the USDA-ARS National Center for Agricultural Utilization Research, Peoria, Illinois. Fatty acid composition was analyzed by gas chromatography using a modified procedure from Christie's method (1989). Results from the fatty acid analyses were expressed as a percentage of the total fatty acid in the grain.

Statistical analysis

Segregation ratios (3:1) of SW and SI progeny were evaluated using the Chi-square test. Filed data were analyzed using SAS (SAS Institute Inc., Cary, NC) PROC mixed, and mean separation determined using Tukey or Tukey-Kramer (if unequal group sizes) test at

the $p<0.05$ and $p<0.01$ levels (Steel and Torrie, 1980). Analyses of variance of PLD enzyme activity and fatty acid content from the greenhouse experiment were analyzed using SAS PROC GLM. Means separated using the Tukey test at the $p<0.05$ and $p<0.01$ levels.

Results

Recovering soybean transformants

Soybean cultivars, Fayette and Flyer, were co-transformed with pHG1 and pSPLDi harboring RNAi structure of soybean PLD fragment (820 bp sense and 1300 bp antisense fragments) via particle inflow gun bombardment and regenerated under hygromycin selection (Trick et al., 1997). Presence of pSPLDi transgenes was confirmed in all transformed embryogenic calli by PCR throughout the regeneration procedures and all transgenic generations. Due to the presence of endogenous PLDα, PCR was performed by amplifying the 3' end of ß-conglycinin promoter fragment to loop fragment (820 bp) and loop fragment to the 5' end of ß-conglycinin terminator fragment. Primers from β-conglycinin promoter and terminator were alternatively used for PCR as an additional verification of the transgene integration. Amplicons from PCR reactions were of the predicted size from each of the positive transgenic events. One hundred forty-three putative tissues by various bombardments were recovered and tested using G.O.I and hygromycin PCR analyses (Appendix: Table A.1). Twelve transgenic soybean events were recovered and fully grown in the greenhouse. Two different transgenic events, SW and SI, were selected from twelve transgenic events based on the agronomic traits, such as fertility and yield, and the molecular analysis, such as PCR and southern blot analysis. SW and SI transgenic lines were derived from Fayette and Flyer, respectively. All T_0 individual transgenic lines were PCR positive for amplifying two different primer sets (Table 2.1). Nomenclature to describe the transgenic progeny used a prefix based on the name of transformant (either SW for Fayette, or SI for Flyer) followed by one or more numbers

separated by dashes corresponding to the seed harvested from an individual plant in a specific generation. The first number after the prefix indicated an individual plant in the T_0 generation, and the second number indicated the individual plant in the T_1 generation, etc.

Segregation of pSPLDi and pHG1 transgenes in T_2 transgenic lines

T_2 seeds harvested from SW1-3 and SI3-8 plants were investigated for the segregation of pHG1 transgenes by PCR. Forty-three cotyledons from SW1-3 and twelve cotyledons from SI3-8 were analyzed by PCR for the presence of pHG1 and pSPLDi transgenes. Nine out of 12 individuals from SI3-8 progeny and 30 out of 43 SW1-3 progeny were HPT PCR positive (Table 2.2). SW1-3 and SI3-8 lines were inherited transgenes as positive and negative in 3:1 ratio. Chi-square tests indicated that the integration sites of the transgenes were clustered or closely linked.

Southern blot analyses of T_0 to T_4 pSPLDi transgenic soybean lines

Southern blot analyses were executed for not only detecting the presence of transgenes, but also determining the copy number and integration pattern of exogenous PLDα. Southern blot analyses were performed using three different probes; a β-conglycinin promoter fragment, a partial SPLDα ORF fragment, and a HPT ORF fragment. Genomic DNA was digested with *Hin*dIII, digesting two sites in pSPLDi, which are at the 5' end of β-conglycinin promoter sequence and at the end of the PLD sense fragment, and double cuts in pHG1 (Fig. 2.1A). The hybridization pattern using a β-conglycinin probe showed that the β-conglycinin promoter was digested by *Hin*dIII at both ends. HPT hybridizing banding patterns confirmed the presence of HPT transgene in all T_0 SW individual plants

(Fig. 2.2A), these indicating that the pSPLDi and pHG1 plasmids were co-integrated into genomic DNA.

Polymorphic banding patterns were rarely detected from the T_0 to T_4 progenies of the same transgenic event using the *Hin*dIII digestion (Fig. 2.2 and 2.3). SW1-2-8-1, SW1-3-8-2, and SW1-3-8-3 lines showed a polymorphic single band with *Xba*I digestion (Fig. 2.4). The results implied that integration of the transgene is highly linked in clusters at a locus of the soybean genome. Two copies of intrinsic PLDα were detected from all background soybean cultivars, Fayette and Flyer with different restriction enzymes.

The hybridization pattern of transgene provided different arrangements of transgenic units. More than 20 integrated transgene copies were detected by *Hin*dIII digestion in T_4 transgenic SI lines (Fig. 2.3). Two strong band signals resulting from the accumulation of several transgenes were identical in the SI line at approximately 5 Kb and 2 Kb. A strong 5 Kb band might be expected by integrating the pSPLDi plasmid into the genomic DNA via a point in the 2 Kb band region. The presence of the 2 Kb band indicates that transgene integrating site is located within the 5 Kb band region (Fig. 2.5B). Twenty and 18 copy number of weak bands were counted from SW and SI, respectively, including 2 bands from endogenous PLDs in non-transgenic and control genotypes (Fig. 2.3). Based on the size of the transgene cassette and pCU19 backbone as well as the ratio of the transgene cassette to the entire plasmid, the number of functional transgenes was estimated at less than 3.5 and 2.5 from SW and SI transgenic lines, respectively.

The presence of four bands below 2 Kb in all T_4 SI lines could not be explained by the general integration model (Fig. 2.5A). Tandem direct repeat (DR) or indirect repeat (IR) integrations may have occurred at a soybean locus (Fig. 2.5C).

Changes in PLDα activity during seed development

To investigate the expression pattern of PLDα during seed development, PLDα activity was measured in soybean cotyledons at different stages of development. Two non-transgenic control soybean cultivars, Fayette and Flyer, were evaluated for PLDα enzyme activity from 11 to 40 days after fertilization (Fig. 2.6). Soybean PLD enzyme activity decreased throughout seed development in the control cultivars. Immature cotyledons, 11 days after fertilization (DAF) had a PLDα activity of about 20 nano mole per mg total protein. PLDα enzyme activity in the seed declined about 4 fold from 11 DAF to full maturity. PLDα activity per unit protein maintained higher levels during early seed development, and declined through late seed development. PLDα activity per cotyledon, however, increased throughout seed development, and peaked in the dry seed. These results were consistent with reported changes in PLDα activity and level of PLDα protein during seed development by Ryu et al. (1996).

PLD gene expression in PLDα-suppressed pSPLDi T_2 soybean lines, non-transgenic lines, and background cultivars

The presence of transgene in the T_2 generation was screened by planting T_2 seeds onto MS medium with 100 mg/L hygromycin for 10 days and selecting putative pSPLDi transgenic seedling based on hygromycin-derived necrotic symptoms. Seeds from T_2

transgenic soybean lines were collected at approximately 26 – 30 DAF and divided in three sections. Each section was used for either the DNA, RNA, or protein analyses. After investigating the presence of pSPLDi transgene by gDNA PCR with pSPLDi specific primer sets mentioned above, transgenic and non-transgenic soybean seeds were screened using the specific amplicon. Different levels of PLDα transcript were detected in immature cotyledons from pSPLDi transgenic and background cultivar soybean by RT-PCR. Fewer PLDα transcripts accumulated in immature cotyledons from SW1-7 line than in background soybean cultivar (Fig. 2.7A). Immunobloting with *Arabidopsis* PLDα1 antibody, decorating 92 *kD* PLDα protein, showed that different levels of PLDα accumulation were detected in transgenic, null transgenic, and background genotypes (Fig. 2.7B, C, and D). The size of the PLDα protein was consistent with previous research (Ryu et al., 1996). PLD enzyme activity in immature cotyledons of transgenic lines was observed to be 20 to 80% lower than activity in the background cultivars. Among all transgenic progenies, SI3-8-3 showed the lowest relative PLD activity of 21.5% compared to the background cultivar, Flyer. Decreases in PLDα enzyme activity and immunoblot analysis due to the reduction of PLDα transcript accumulation appeared to be caused by the presence of the pSPLDi transgene.

Suppression of PLD genes through T_5 SW and SI transgenic, non-transgenic, and background soybeans during seed development

RT-PCR amplifying two different regions of transgene and endogenous PLDα transcript showed different amplification results. All transgenic, null transgenic, and background cultivars exhibited an amplicon using RT-PCR with loop primers which anneal to both endogenous PLDα mRNA and loop fragment in pSPLDi transgene. PLD transcript,

however, was rarely detected using RT-PCR with pin primers which anneal to endogenous PLD mRNA, except in mature seed from SW1-2-8-1 and SI3-8-7-1 lines (Fig. 2.8A). This was in agreement with quantitative RT-PCR results (Fig. 2.8B). This apparent consistency on detecting PLDα-suppressed line might be applied to easy and prompt way for RNAi – mediated silencing study.

Immature and mature seed from T_5 transgenic soybeans were tested for PLDα enzyme activity and immunoblotting (Fig. 2.9 and 2.10). The suppression of PLDα activity was attenuated or not detected in T_5 transgenic soybean lines, compared to the suppression of PLDα activity in T_2 PLDα-suppressed lines.

Suppression of PLD genes in T_5 SW transgenic (SW1-2-8-1), null transgenic (SW1-7-1-5-N), and background soybeans during seed development

The transcript of HPT expression in T_5 transgenic, non-transgenic and background soybean lines observed by RNA blot analysis (Fig. 2.11A) suggested that pHG1 transgene has been stably inherited T_5 generation. Endogenous PLDα transcript was barely detected by RNA blotting analysis. To support northern blot analysis of HPT and endogenous PLDα, quantitative PCR was performed with primers which amplify 130 base pairs from the loop sequence to the double strand sequence which was supposed to be amplifying endogenous PLDα mRNA. The amount of endogenous PLDα in immature and mature SW1-2-8-1 transgenic soybean seeds was reduced 30 and 4.5 fold relative to the background cultivar, respectively. However, no suppression of PLDα transcript was detected in the null transgenic, SW1-7-1-5-N, seed (Fig. 2.11B and C). Reduced amounts of PLDα protein were accumulated and PLDα enzyme tended to exhibit reduced activity in SW1-2-8-1 seed

compared to SW1-7-1-5-N and background soybean seed (Fig. 2.11D, E and F). These results suggested that PLDα protein and enzyme activity was attenuated by the RNAi machinery, and the level of protein and PLDα enzyme activities in SW1-2-8-1 line were maintained as reduced levels during seed development.

Changes in agricultural traits and composition of five major fatty acids in PLD-suppressed soybean lines

Transgenic field tests of SW- and SI-derived progenies were performed in 2005 and 2006. Plant height of transgenic lines tended to be significantly shorter or taller than background cultivars, suggesting that the procedure of plant regeneration and recovery may affect stem elongation (Table 2.3). Few SI lines were similar in lodging and maturity compared to the null transgenic and background cultivars, SW progeny, however, tended to lodge more than the background cultivar. In SW lines and SW deriving null transgenic cultivar, maturity was observed to be retarded in 2006, whereas maturity in 2005 tended to earlier than the background cultivar. Seed weight and seed quality might be not affected by transgene, and plot weight was significantly different in lodging susceptible entries. Regardless of pod maturity, leaf maturity was observed to be retarded in SW and SI lines (not shown, appendix Fig. A.1).

Both protein and oil contents in SW and SI lines were observed to be lower than background cultivars (Table 2.5). Significant differences of protein content were found in SW1-3-8-8 lines compared to background cultivars. SW1-7-1-1, SW1-7-1-3, SW1-2-8-1, and SI3-8-2-1 lines were significantly decreased in oil content. In 2005, fatty acid composition was significantly different between SW lines and background cultivar, Fayette

(Table 2.4). Oleic acid content in SW lines was increased about 25 to 40 percent, and linoleic acid content was decreased 10 to 17 percent compared to the background cultivar. In contrast, an opposite shift in oleic acid and linoleic acid composition was observed in SI progenies. Oleic acid content in SI3-8-2-1 and SI3-11-6-1 was significantly lower than that in Flyer in 2005. However, polyunsaturated fatty acid contents were increased in all SI lines. In 2006, profiles of five major fatty acids in both SW and SI lines were not variable from null transgenic and background cultivars. Significant difference were found, however, in linolenic acid content from SW1-7-1-5-N and SW1-2-8-1 lines, indicating that transformation procedure may affect on changes of linolenic acid content.

Examing the common entries across the two years of field trials, sources of variation for compositional changes in stearic acid, oleic acid, linoleic acid, and linolenic acid were significant for entry and the entry by year interaction. The significant differences in palmitic acid content were found among years and entries, but the year by entry interaction was not significant. All sources of variation for agronomic traits except lodging rating showed significant year, entry, and year by entry interaction effects.

Oleic acid content of background lines was generally increased, and linoleic acid content was decreased in the greenhouse experiment. Interestingly, SW1-7-1-2 line exhibited the highest oleic acid, 49%, and the lowest linoleic acid, 32%. Most SW and SI lines showed a significant increase in linolenic acid content and a significant decrease in stearic acid content (Fig. 2.6).

SW1-7-1-2 exhibited lower linoleic acid content and higher oleic acid content than background cultivar, but there were no notable changes in saturated fatty acids (16:0 and

18:0) and linolenic acid (18:3). Seeds from T_4 progeny line, SW1-7-1-1, showed about 37% in oleic acid and 43% in linoleic acid, however, its background cultivar showed 25% in oleic acid and 52% in linoleic acid, which is average amount of individual fatty acid content in normal soybean cultivars. SW progeny line, SW1-7-1-2, showed mid-oleic acid content and low linoleic acid about 49% and 32% respectively, and exhibited to be 3% lower in palmitic acid and 2% lower in linolenic acid than those in background cultivar.

Discussion

Twelve independent transgenic soybean lines with pSPLDi and pHG1 were generated via co-bombardment using the particle inflow gun. Most transgenic events were fully fertile. Based on the background cultivar and pod setting ability, two elite lines, SW and SI, were chosen to further examine characteristics of PLD suppressed soybeans. By transferring endogenous DNA with particle inflow gun bombardment, more than 18 copies of the modified PLD transgene were integrated into both transgenic events. In T_1 seed, segregating ratio was best fit to 3:1, moreover banding patterns generated by different restriction enzymes were fairly consistent through the generations. Only two SW and SI progeny lines out of total 20 lines showed polymorphic banding patterns consisting of either an additional or deleted band using southern blot analysis. These results indicated that arrangement of pSPLDi and HPT transgenes within the host genome trace to two events. It has been reported that transgene locus is predominantly organized as transgene loci containing multiple copies of transgene in oat and rice (Pawlowski and Somers,1998; Kohli et al., 1998). The multimerization of transgene results in repeated transgene with direct or indirect orientation, and it process may occur either through extrachromosomal ligation or ligation during integration, suggesting that T-DNA repeats are formed by extrachromosomal ligation before integration (De Neve et al., 1997).

The total integration number of the transgene DNA was estimated based on RFLP with two different restriction enzymes, *Hin*dIII and *Xba*I. DNA hybridization patterns restricted by *Hin*dIII exhibited two strong bands which originated from two diverse fragments from 2 Kb of β-conglycinin promoter and SPLD sense region and 5 Kb of SPLD

antisense, terminator and pUC19 backbone region, respectively (Fig. 2.3A and B). These results supported the hypothesis that multimeriation of direct or indirect repeats transgene was formed by bombardment. The polymorphic banding pattern with double digesting enzyme can be used to predict putative numbers of functional transgene units. When assuming the probability of recombining site is equal to any type of sequence, estimated numbers of functional transgene unit were 3.5 in SW progeny lines. Although it is not obvious that recombination and methylation occurred in transgene loci, some of functional transgene units might suffer from transcriptional gene silencing (TGS) or posttranscriptional gene silencing (PTGS) as recombining heterochromatin region (Napoli et al., 1990; van der Krol et al., 1990). Particle bombardment has enabled soybean to be efficiently transformed with less genotypic limitations than *Agrobacterium*-mediated transformation, however, it produces transgenic events containing numerous copies of transgenes. The multiple tandem integration in many of the soybean lines may have resulted in gene silencing in the T_4 and T_5 generations (Fig. 2.5, 2.7, 2.8, and 2.10).

PLDα activity declines 4 fold as seeds mature (Fig. 2.6). Ryu et al. (1996) observed the same changes in PLD activity during soybean seed development. PLDα transcript was hardly detected during seed maturation using northern blot analysis.

Based on relative Ct values of PLD genes against soybean actin1 gene with real time RT-PCR in mature soybean seeds, Ct value for PLDα were lower around 14 cycles than that of soybean actin1. When we assume that primer efficiencies of PLDα and actin1 polymerase chain reaction are 2, quantitative RT PCR data suggest that PLDα mRNA levels theoretically were as low as 2^{-14} of actin1 mRNA in mature seeds (data not shown).

Co-suppression caused by pSPLDi transgene may suppress coincidently the accumulation of PLDß and PLDγ. Soybean EST clones, gi:21888889 and gi:17401471, are known as premature PLD cDNA from young soybean seedlings. Based on sequence homology with *Arabidopsis* PLDs, soybean ESTs, gi:21888889 and gi:17401471, showed sequence similarity to the 5'UTR region in *At*PLDβ and *At*PLDγ, respectively. Two homologous mRNAs to *At*PLDβ and *At*PLDγ were also suppressed in immature and mature seed of SW1-2-8-1 (Fig. 2.11B and D). Co-suppression on conserved domain, the HKD2 domain, presumably results in the suppression of PLDβ and PLDγ by RNAi mechanism.

The most visible difference in between non-transgenic soybean and PLD suppressed soybean was leaf senescence. Transgenic soybean lines had significant later leaf senescence (Appendix A Fig. A.1). In modified PLD transgene segregating lines, leaf drop was retarded by average of 6 to 8 days in the field during two years of testing, compared to control genotypes that had reached maturity. This delayed senescence may have been due to the PLD gene action reducing apoptosis or programmed cell death in the leaf senescence under certain condition. Somaclonal variation may have resulted in the leaf senescence. Also, poor seed and pod set on some genotypes may have disrupted normal plant senescence. Fan et al. (1997) showed that PLDα-deficient *Arabidopsis* exhibited normal growth and development. No evidence of retardation of natural senescence was observed in PLDα-ablated *Arabidopsis* plants. A high PLDα activity, which may be detrimental to membranes and hasten senescence, is controlled by changes in hormonal condition (Fan et al., 1997). It has been proposed that the PLDα play a role in senescence. In the detached leaf experiment with PLD*α*-deficient and wild type *Arabidopsis*, the aging process in PLD

α-deficient *Arabidopsis* tended to be similar to the wild type. In detached leaves, ABA and ethylene are well known to promote senescence. Detached leaves of PLDα-deficient *Arabidopsis* exhibited retarded phytohormone-promoted senescence, implying that PLDα may mediate ABA- and ethylene-promoted senescing events.

PLDα suppressed soybean putatively changes the lipid profile in seeds. Soybean is low in saturated fat with a content of about 15%, high in unsaturated fat with 61% polyunsaturated and 24% monounsaturated fat. The reaction of triacylglycerol synthesis in seeds might be altered branching point by suppressed PLDα activity in ER. PC is a major membrane phospholipid and substrate for the PLDα reaction. Biosynthesis of membrane phospholipid and storage lipids, TAG, shares many substrates and intermediates such as DAG, PA, and certain molecular species of PC, moreover, may be used both pathways as intermediates or final products. The difference between storage lipids and membrane lipids is the composition of fatty acid chain and location in the cell. Most of membrane phospholipids, mainly PC, consist of normal fatty acid chain, whereas TAG, storage form of glycerolipid, contains common fatty acid and unusual fatty acid chain. The substrate specificity of PLD leads to differentiate lipid metabolic pathway with alternative reaction to between PC containing common fatty acid and unusual fatty acids. To prove this concept, we need to construct membrane and storage lipid profiling maps first with non-transgenic soybean and PLDα-suppressed soybean. Second, we should know the substrate specificity of PLDα in soybean seeds by profiling membrane lipid species.

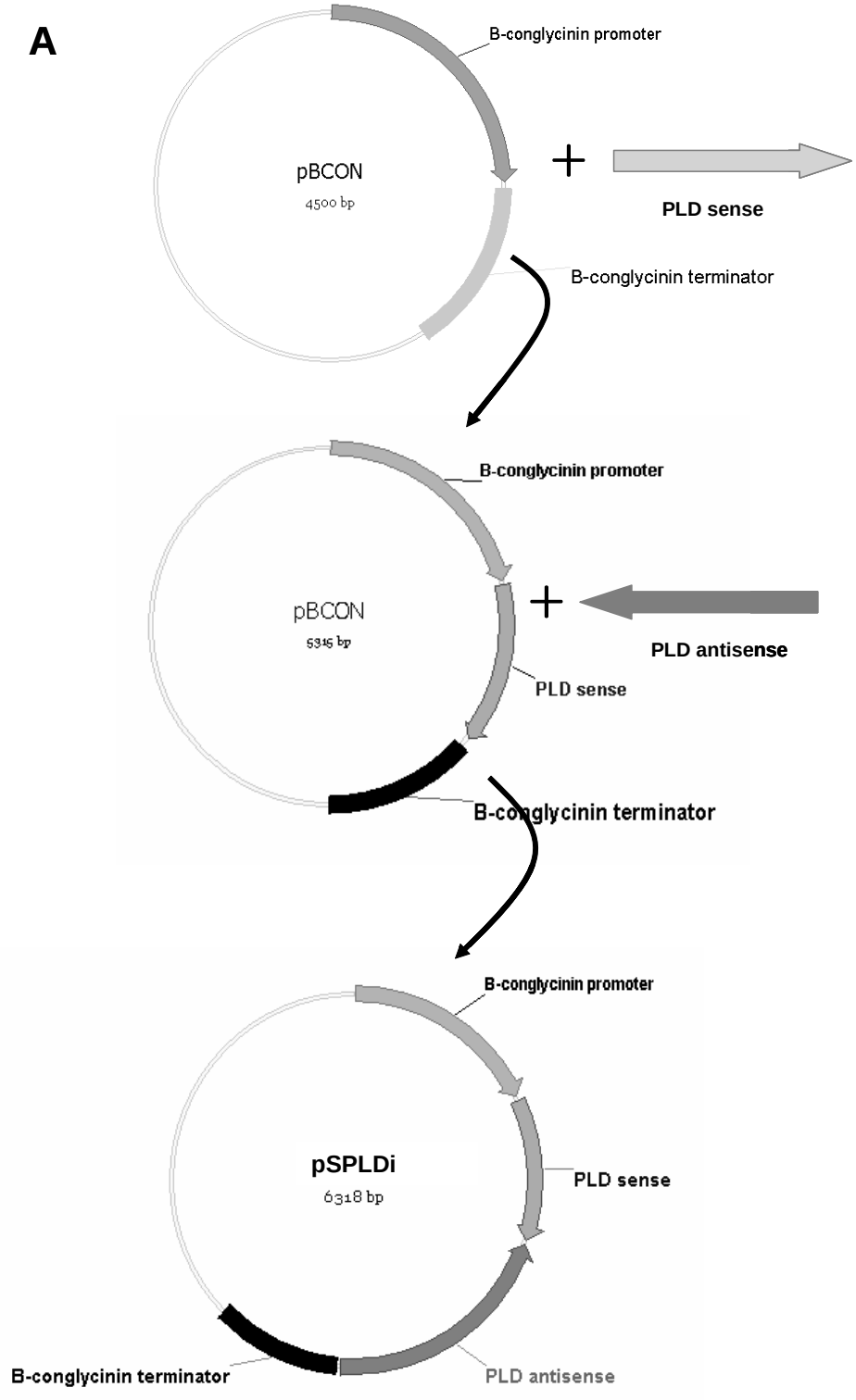
Biochemical changes from oleic acid to linoleic acid can be accomplished by microsomal fatty acid desaturase 2 (FAD2) and chloroplast-targeted fatty acid desaturases

(Ohlrogge and Browes, 1995). Two aspects of oleic acid content were observed in pSPLDi transgenic seeds. We cannot explain the discrepancy in oleic acid content through pSPLDi transgenic seeds at this moment. The alteration of oil composition is associated with environmental changes. It is important for soybean genotypes that contain novel fatty acid profile to possess stable fatty acid content and composition in different growing conditions (Primomo et al., 2002). Environmental influence on the fatty acid composition of soybean oil has been well studied in general soybean genotypes. Seed development at higher temperatures resulted in significant decreases in linoleic acid and linolenic acid contents, and a significant increase in oleic acid content (Howell and Collins, 1957; Dornbos and Mullen, 1992). Based on two weather data at Manhattan, Kansas obtained from KSU research and extension, temperature during seed development in 2006 was higher than that in 2005 (Data not shown). In 2005, compositional shifting on oleic acid to linoleic acid in SW progeny lines might result from high temperature during the seed development. Wilcox and Cavins (1992) reported that planting date for the cultivar Centry and two linolenic acid soybean lines affected the compositional changes in palmitic acid and linolenic acid. The result from this study is consistent with previous reports. We also cannot exclude the possible transgene silencing in the field study. Evidences existed of transgene silencing in T_4 generation (Fig. 2.8, 2.9, and 2.10). The genotype and transgene effects along with environmental influences need to be evaluated before drawing detailed correlation regarding changes in the membrane and storage lipids in transgenic soybean seeds.

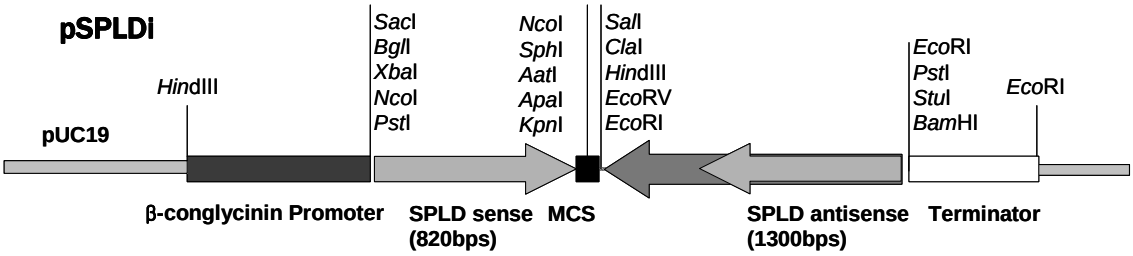
Observations in this study suggest that fatty acid profiles can be modified in PLDα-suppressed soybean lines generated by transformation, although phenotypic expression of those changes may be strongly influenced by the environment.

Figures and Tables

Figure 2.1 Schematic diagram of constructing vector used for transformation. (A); pSPLDi expression cassette containing the β-conglycinin promoter, PLD sense, PLD antisense, and β-conglycinin terminator. (B); Restriction map of pSPLDi plant expression vector. (C); pHG1 expression vector comprised of hygromycin phosphotransferase cassette and β-glucuronidase cassette under control of CaMV 35S promoter.


A
B-conglycinin promoter
pBCON
4500 bp
PLD sense
B-conglycinin terminator
B-conglycinin promoter
pBCON
5315 bp
PLD antisense
PLD sense
B-conglycinin terminator
B-conglycinin promoter
pSPLDi
6318 bp
PLD sense
B-conglycinin terminator
PLD antisense

B


pSPLDi
pUC19
HindIII
SacI
BglI
XbaI
NcoI
PstI
NcoI
SphI
AatI
ApaI
KpnI
SalI
ClaI
HindIII
EcoRV
EcoRI
EcoRI
PstI
StuI
BamHI
EcoRI
β-conglycinin Promoter
SPLD sense (820bps)
MCS
SPLD antisense (1300bps)
Terminator

C

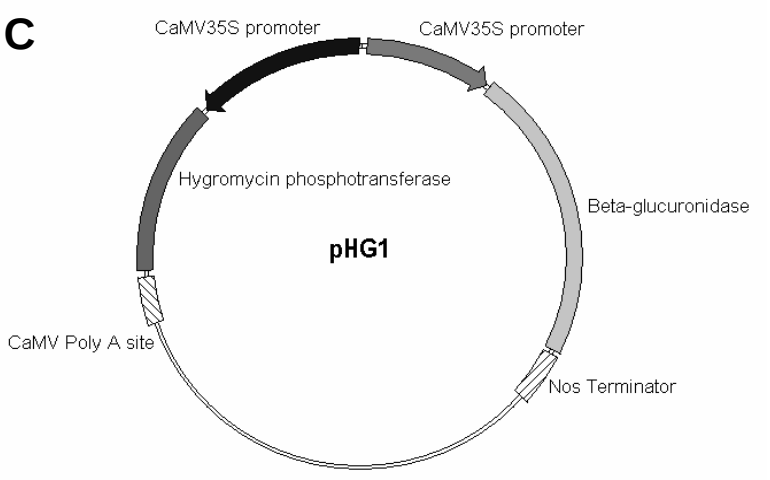

CaMV35S promoter
CaMV35S promoter
Hygromycin phosphotransferase
Beta-glucuronidase
pHG1
CaMV Poly A site
Nos Terminator

Figure 2.2 Southern blot analysis of PLDα-suppressed T_0 soybean lines with negative controls. Fifteen μg of genomic DNA was digested with *Hin*dIII. Digested genomic DNA from SW T_0 young leaves was hybridized with PLDα probe, beta-conglycinin promoter probe and hygromycin phosphotransferase probe. Each probe used for DNA-DNA hybridization was indicated on the right hand side bottom in the gel pictures. Arrows indicate soybean genomic copy of PLDα and β-conglycinin promoter.

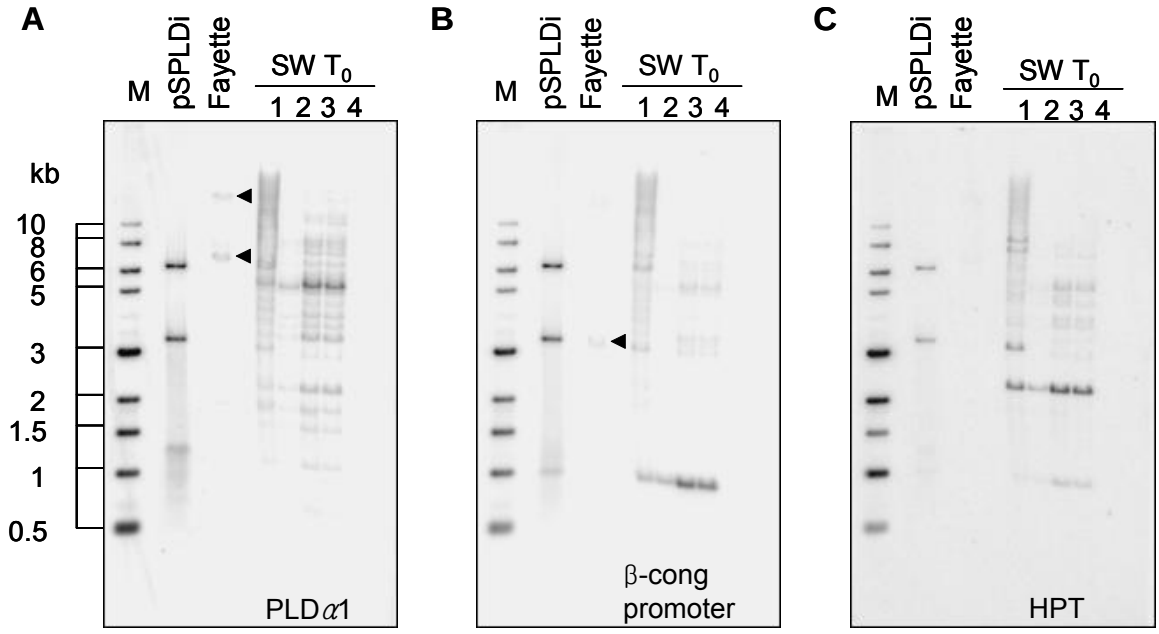


Figure 2.3 Southern blot analysis of T_4 PLDα- suppressed soybean lines with negative controls. Fifteen μg of genomic DNA was digested with *Hin*dIII. (A), (C), (D), and (E): Digested genomic DNA from SW T_4 and SI T_4 lines was separated by 0.8% agarose gel electrophoresis, transferred to nylon membrane, hybridized with PLDα and HPT probes, (B): Tree independent T_4 SW progenies and T_2 Juice (pSPLDanti) line were hybridized with PLDα and HPT probes. Left panel showed that number is based on kilo base pair corresponding to each size of molecular marker ladder (1Kb ladder). Arrow indicates different band patterns among transgenic lines.

A
Marker
pSPLDi
Fayette
SW1-7-1-3 a
SW1-7-1-3 b
SW1-7-1-3 c
SW1-7-1-3 d
SW1-7-1-3 e
SW1-3-8-7 a
SW1-3-8-7 b
SW1-3-8-7 b
SW1-3-8-7 c
SW1-3-8-8 d
SW1-7-1-2 a
SW1-7-1-2 b
kb
10
8
6
5
3
2
1.5
1
0.5
PLDa
B
Marker
pSPLDi
Jack
Fayette
SW1-3-8-7 a
SW1-3-8-6 a
SW1-3-8-6 b
SI3-8-2-2-N
Juice
SW1-7-1-5-N
PLDa
Marker
pSPLDi
Jack
Fayette
SW1-3-8-7 a
SW1-3-8-6 a
SW1-3-8-6 b
SW1-3-8-6 c
Juice
SW1-3-8-5 a
HPT
C
Marker
pSPLDi
Fayette
Flyer
SW1-7-1-4 a
SW1-7-1-4 b
SW1-7-1-4 c
SW1-7-1-4 d
SW1-7-1-6 a
SW1-7-1-6 b
SW1-7-1-6 c
SW1-7-1-6 d
SI3-4-3-1 a
SI3-4-3-1 b
SI3-4-3-1 c
SI3-8-1-2 a
kb
10
8
6
5
3
2
1.5
1
0.5
PLDa
D
kb
Marker
pSPLDi
Fayette
Flyer
SW1-7-1-7 a
SW1-7-1-7 b
SI3-11-6-1 a
SI3-11-6-1 b
SI3-11-6-1 c
SI3-11-6-1 d
SI3-11-6-1 e
SI3-8-1-1 a
SI3-8-1-1 b
SI3-8-1-1 c
SI3-8-1-1 d
10
8
5
3
2
1.5
1
0.5
PLDa
Marker
pSPLDi
Fayette
Flyer
SW1-7-1-7 a
SW1-7-1-7 b
SI3-11-6-1 a
SI3-11-6-1 b
SI3-11-6-1 c
SI3-11-6-1 d
SI3-11-6-1 e
SI3-8-1-1 a
SI3-8-1-1 b
SI3-8-1-1 c
SI3-8-1-1 d
HPT
E
Marker
pSPLDi
Fayette
Flyer
SW1-3-8-6 a
SW1-3-8-6 b
SW1-7-1-8 a
SW1-2-8-1 a
SW1-2-8-1 b
SW1-2-8-1 c
SW1-2-8-1 d
SW1-3-8-8 a
SW1-3-8-8 b
SW1-3-8-8 c
SW1-7-1-1 a
SW1-7-1-1 b
PLDa
Marker
pSPLDi
Fayette
Flyer
SW1-3-8-6 a
SW1-3-8-6 b
SW1-7-1-8 a
SW1-2-8-1 a
SW1-2-8-1 b
SW1-2-8-1 c
SW1-2-8-1 d
SW1-3-8-8 a
SW1-3-8-8 b
SW1-3-8-8 c
SW1-7-1-1 a
SW1-7-1-1 b
HPT

Figure 2.4 Southern blot analysis of nine T_4 transgenic progeny lines derived from SW1 were digested with *Xba*I and hybridized with PLDα probe. Left panel showed that number is based on kilo base pair corresponding to each size of molecular marker ladder (1Kb ladder). Arrow indicates different band patterns among transgenic lines.

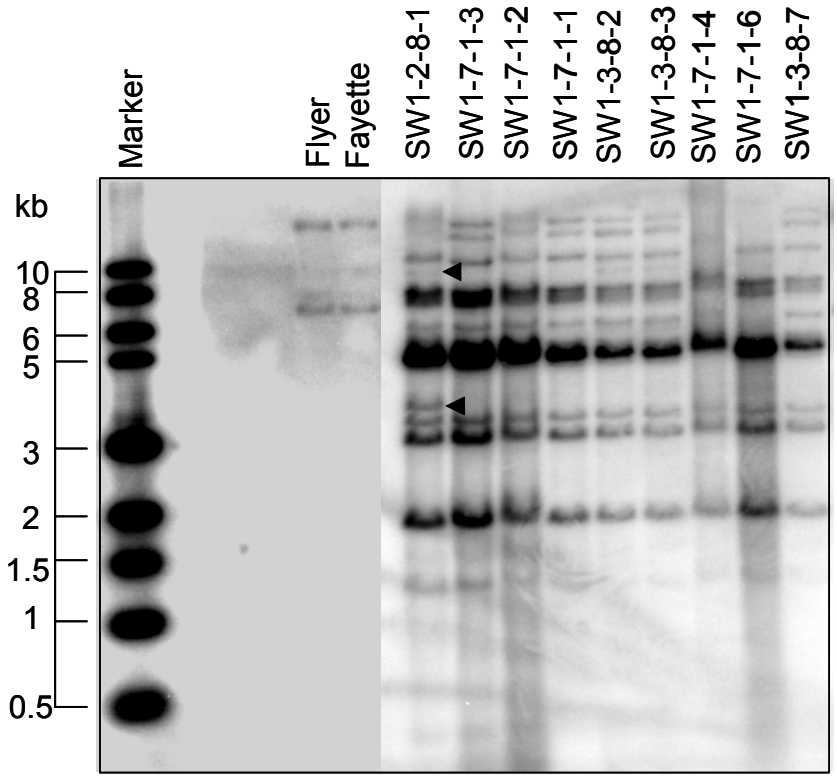


Figure 2.5 Southern blot analysis of T_4 SW and SI lines and possible integration pattern in T_4 SI lines. (A): Fifteen µg of genomic DNA was digested with *Hin*dIII and hybridized with PLDα probe. Left panel showed that number is based on kilo base pair corresponding each size of molecular marker ladder (1Kb ladder). Bands in the box exhibit unexpected bands among SI transgenic lines. (B): Restriction map of pSPLDi by *Hin*dIII digestion. Arrows in (A) indicate 2 Kb band and 5 Kb band. (C): Putative integration pattern of pSPLDi transgenes in soybean genome can only be explained by tandem integration model. The presence of small bands (>2 Kb) were produced by reverse repeated integration of pSPLDi transgene at the same locus of soybean genome.

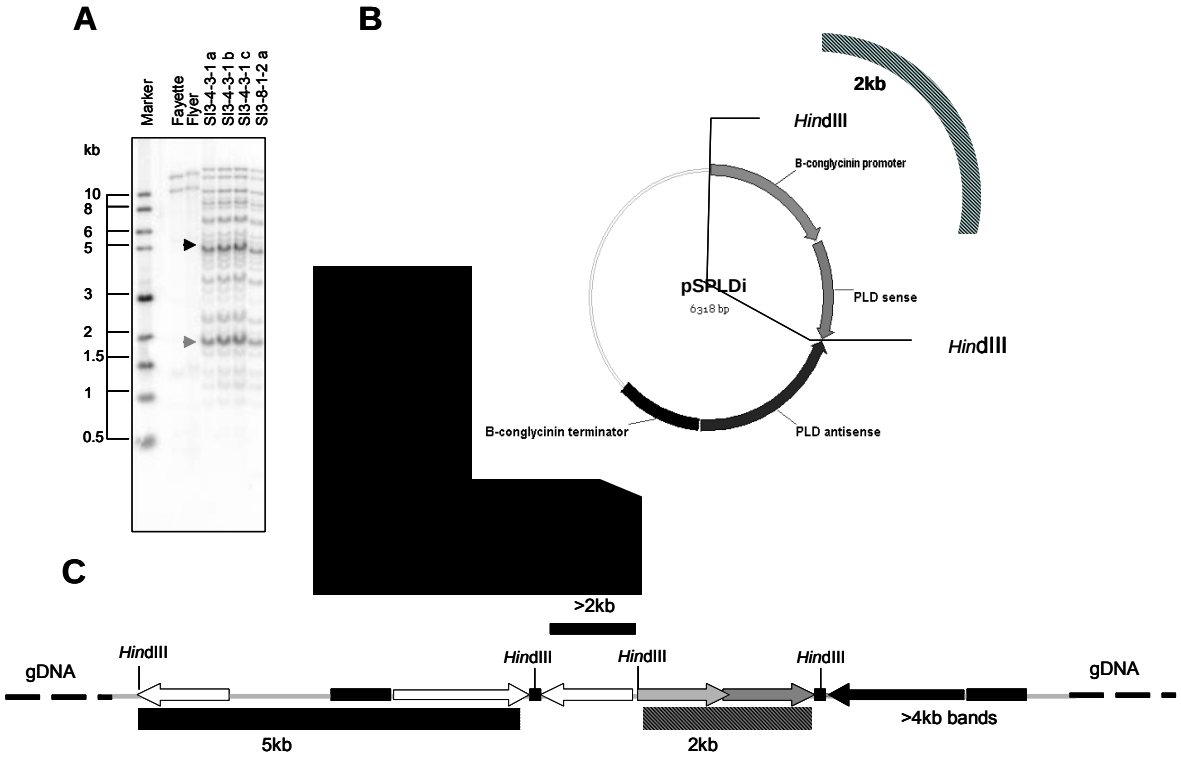


Figure 2.6 Changes in PLDα activities in 'Faytette' and 'Flyer' cotyledons during seed development. The PLDα enzyme assay utilized centrifugation at 10000Xg to collect supernatant. Data were expressed nmoles of phosphatidylcholine per minute reaction and mg of total protein. Error bar indicates ± SE of three biological replications with duplicated measurements.

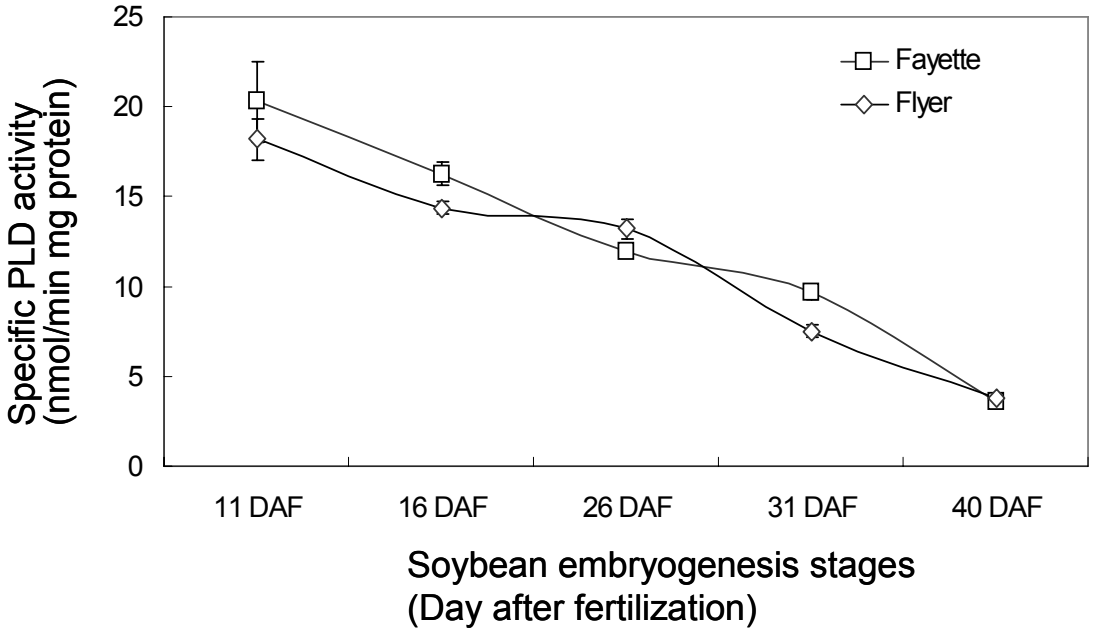


Figure 2.7 PCR, RT-PCR, immunoblot and enzyme assay of PLDα in the immature cotyledon form heterozygous T_1 seed from SW1, SW2, and SI3 lines. (A); Changes in PLDα mRNA in the immature cotyledon from SW1-7 and background cultivar 'Fayette'. One microgram of total RNA was used for reverse transcriptase reaction, and one twentieth of aliquot was taken for cDNA PCR with PLDα internal primer, and gel was stained with ethidium bromide after 25 cycles. (B), (C), and (D); PCR, immunoblot and enzyme assay of PLDα from SI 3, SW1, and SW2 lines respectively. Immature cotyledons were split with razor blade as three parts. The first part of tissue was used for PCR analysis to confirm the presence of modified PLDα transgene. Immunoblot analysis of PLDα was performed with *Arabidopsis* PLDα antibody, and PLDα proteins were decorated at 92 kD position based on the protein marker. PLDα enzyme assay was expressed nmoles of phosphatidylcholine min-1 mg-1 total protein. Error bar indicates ± standard deviation with three independent measurements.

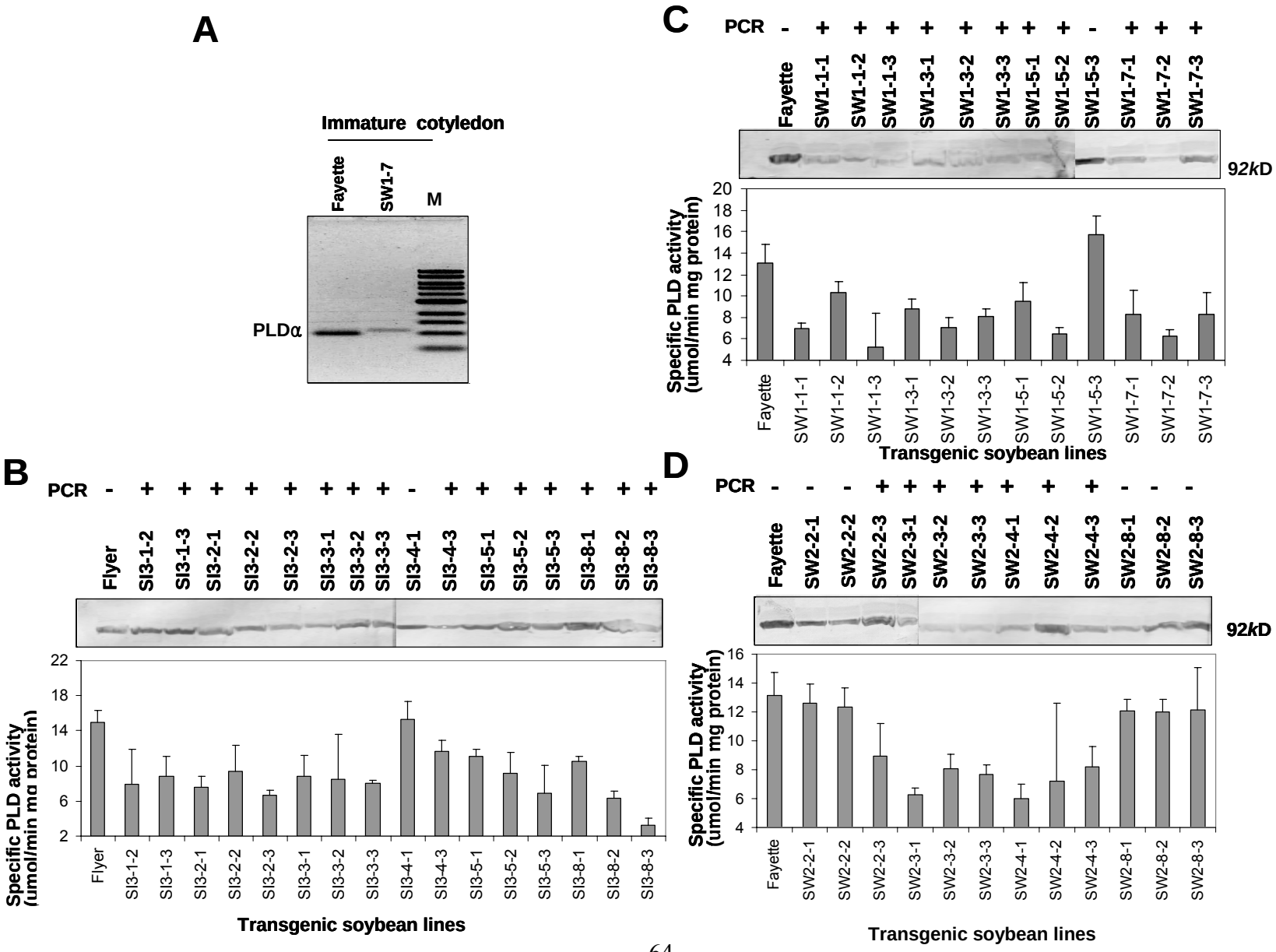

A
Immature cotyledon
Fayette
SW1-7
M
PLDα
C
PCR
Fayette
SW1-1-1
SW1-1-2
SW1-1-3
SW1-3-1
SW1-3-2
SW1-3-3
SW1-5-1
SW1-5-2
SW1-5-3
SW1-7-1
SW1-7-2
SW1-7-3
92kD
Specific PLD activity (umol/min mg protein)
Transgenic soybean lines
B
PCR
Flyer
SI3-1-2
SI3-1-3
SI3-2-1
SI3-2-2
SI3-2-3
SI3-3-1
SI3-3-2
SI3-3-3
SI3-4-1
SI3-4-3
SI3-5-1
SI3-5-2
SI3-5-3
SI3-8-1
SI3-8-2
SI3-8-3
Specific PLD activity (umol/min mg protein)
Transgenic soybean lines
D
PCR
Fayette
SW2-2-1
SW2-2-2
SW2-2-3
SW2-3-1
SW2-3-2
SW2-3-3
SW2-4-1
SW2-4-2
SW2-4-3
SW2-8-1
SW2-8-2
SW2-8-3
92kD
Specific PLD activity (umol/min mg protein)
Transgenic soybean lines

Figure 2.8 RT-PCR and realtime RT-PCR analysis of T_5 mature seed from SW1 and SI3 progeny lines. (A); RT-PCR analysis with alternative PCR primers (Table 2.1) for amplifying two different regions of transgene and endogenous PLDα mRNA based on the putative structure of PLDα double strand RNA. (B); Realtime RT-PCR analysis with various endogenous PLDs mRNA specific primers (Table 2.1). PCR primers of soybean PLDβ-like and γ-like were designed from soybean EST clones, gi:21888889 and gi:17401471, which showed sequence similarity to 5'UTR region in AtPLDβ and AtPLDγ, respectively.

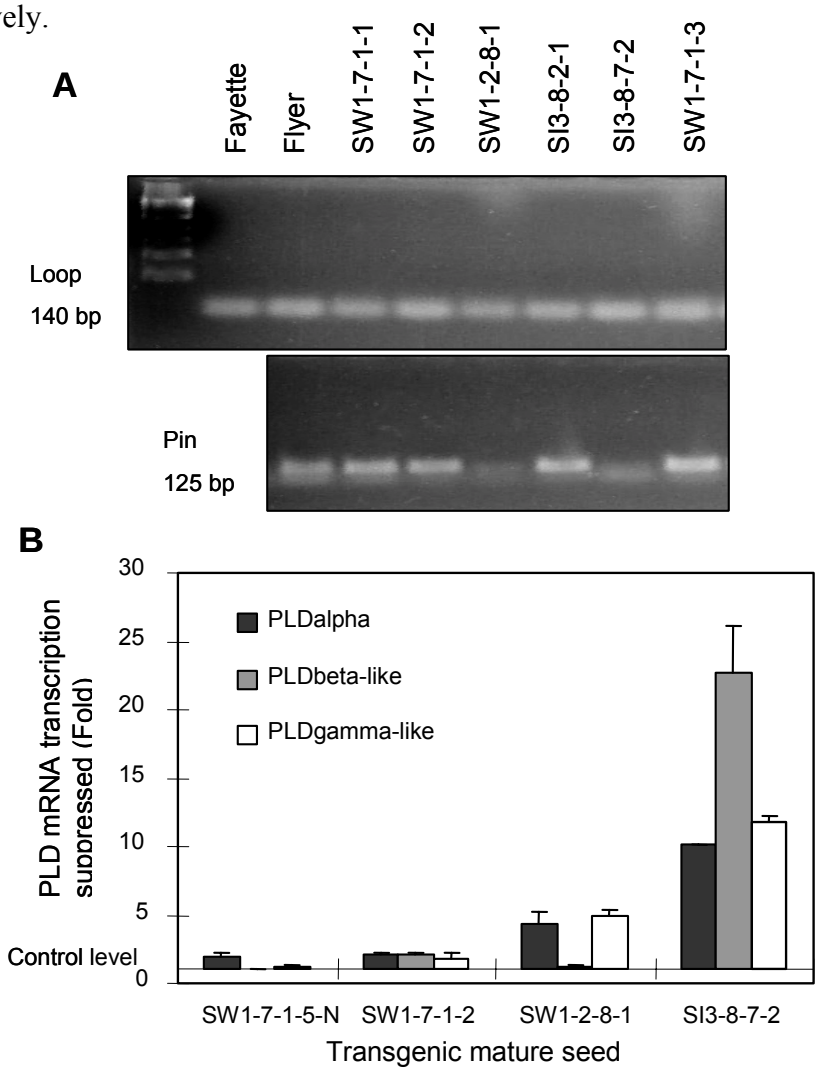


Figure 2.9 Changes in PLDα enzyme activity and the level of PLDα protein in the immature cotyledons from T_5 SW1 and SI3 progeny seed and background cultivars. (A) and (B); PLDα activities in immature cotyledons from T_5 transgenic lines. PLDα enzyme assay was expressed nmoles of phosphatidylcholine min^{-1} mg^{-1} total protein. (C); Immunoblot analysis of PLDα protein (92 *kD*) from immature cotyledon with polyclonal anti-*Arabidopsis* PLDα antibody. * indicates a significant difference using the Tukey test at $p<0.05$ and ** indicates a significant different at $p<0.01$ between non-transgenic background cultivar and transgenic lines. † and †† indicate a significant different at $p<0.05$ and $p<0.01$ between null transgenic lines and transgenic lines, respectively.

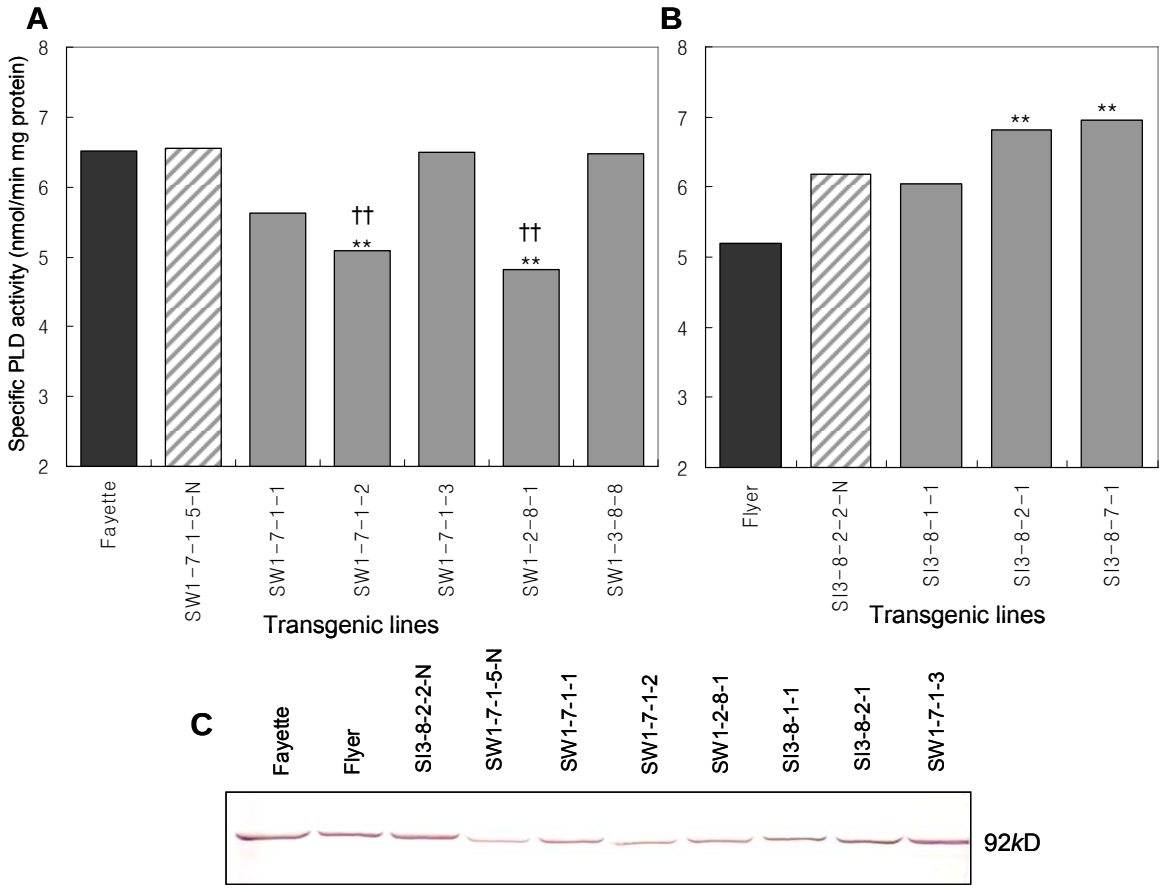


Figure 2.10 Changes in PLDα enzyme activity and the level of PLDα protein in the mature cotyledons from T_5 SW1 and SI3 progeny seed and background cultivars. (A) and (B); PLDα activities in mature cotyledons from T_5 transgenic lines. PLDα enzyme assay was expressed nmoles of phosphatidylcholine min^{-1} mg^{-1} total protein. (C); Immunoblot analysis of PLDα protein from mature cotyledon with polyclonal anti-*Arabidopsis* PLDα antibody. * indicates a significant difference using the Tukey test at $p<0.05$.

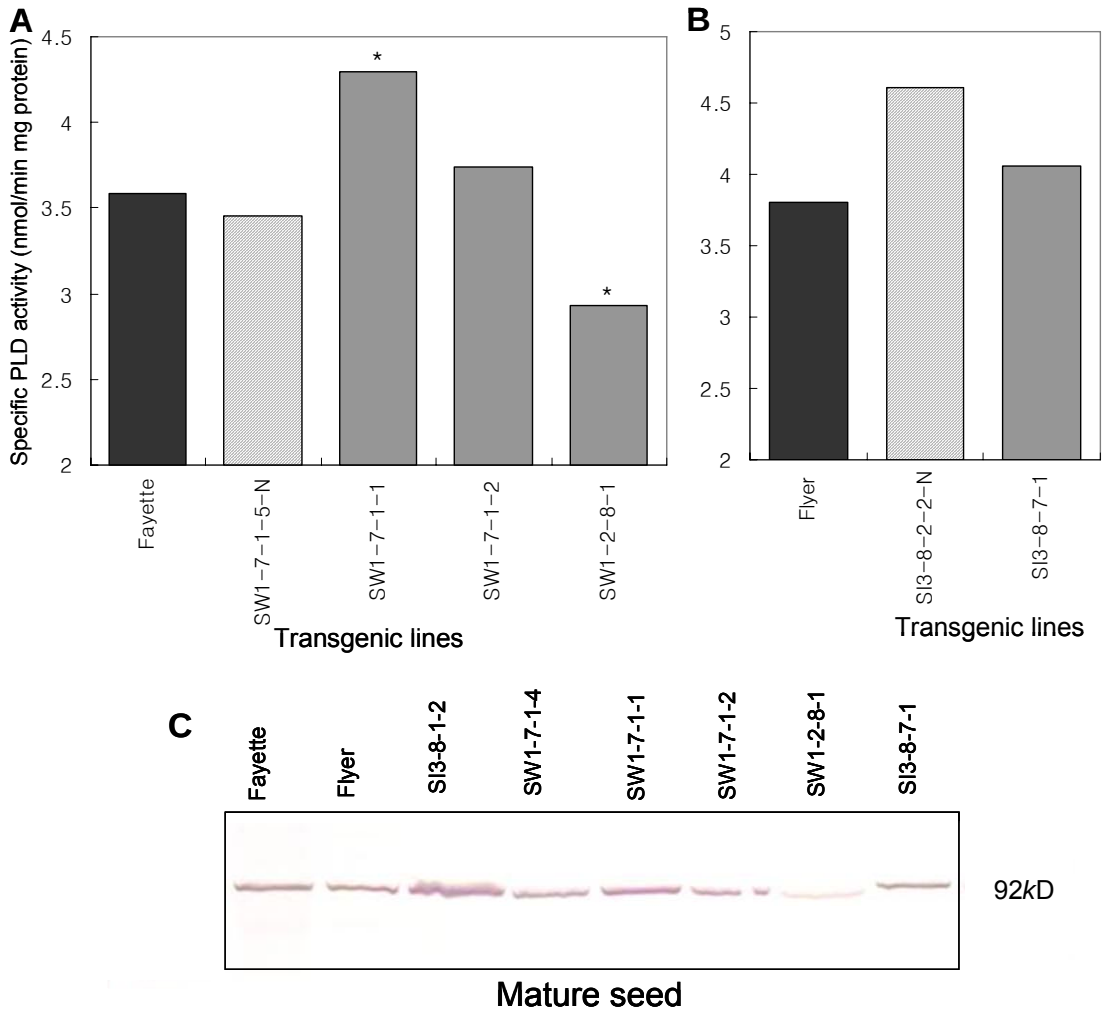


Figure 2.11 Changes in mRNA and PLDα protein in the immature and mature cotyledons from SW1 progenies and background cultivar 'Fayette'. SW1 progeny line, SW1-7-1-5-N, was characterized homozygous non-transgenic line segregated from T_2 progeny of SW1-7-1 by PCR and southern blot analysis. (A); RNA blot analysis of steady state levels for HPT in all transgenic soybean lines and background cultivar. Total RNA are from the immature soybean seeds that were background cultivar (Fayette), null transgenic line (SW1-7-1-5-N), and PLDα suppressed line (SW1-8-2-1). B and D; Quantitative PCR analysis of PLDα, β-like, and γ-like mRNAs from immature (B) and mature (D) cotyledon respectively. The levels of endogenous of PLDα, β-like, and γ-like were measured by real-time PCR from non-transgenic line (SW1-7-1-5-N) and PLDα suppressed line (SW1-8-2-1). Changes in PLD mRNA transcription were represented by the suppression fold against the level of PLD mRNA transcription in Fayette. PCR primers of soybean PLDβ-like and γ-like were designed from soybean EST clones, gi:21888889 and gi:17401471, which showed sequence similarity to 5'UTR region in AtPLDβ and AtPLDγ respectively. (C) and (E); Changes in PLDα activities in immature (C) and mature (E) cotyledons from non-transgenic line (SW1-7-1-5-N) and PLDα suppressed line (SW1-8-2-1). PLDα enzyme assay was expressed nmoles of phosphatidylcholine min^{-1} mg^{-1} total protein. Error bar indicates ±SE with three independent measurements. (F); Immunoblot analysis of cytosolic protein from immature and mature cotyledon with polyclonal anti-*Arabidopsis* PLDα antibody. Proteins (40 μg per lane) were separated in SDS-PAGE in 12% polyacylamide gel and PLDα protein then was visualized by alkaline phosphatase.

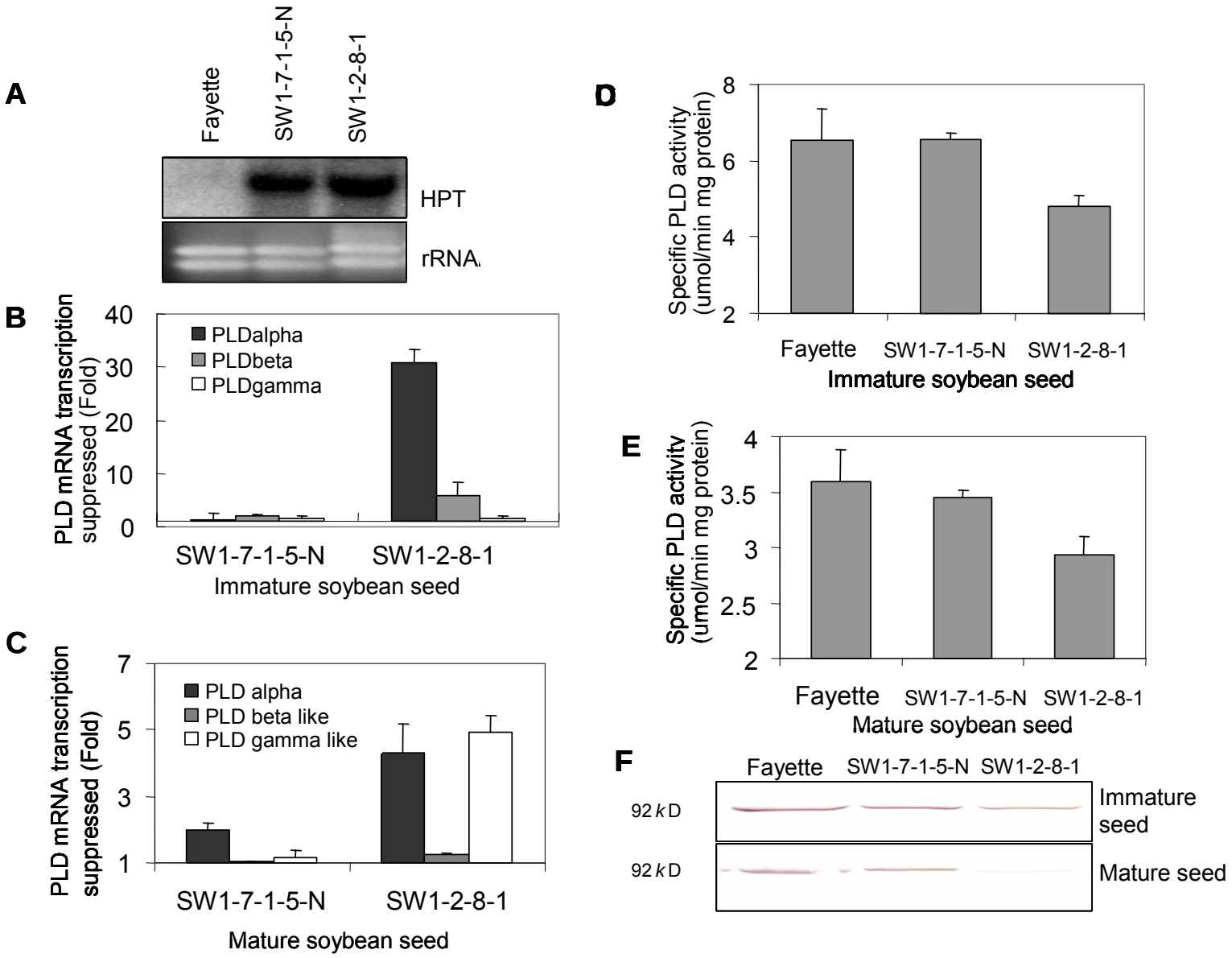

A
Fayette
SW1-7-1-5-N
SW1-2-8-1
HPT
rRNA
B
PLD mRNA transcription suppressed (Fold)
PLDalpha
PLDbeta
PLDgamma
SW1-7-1-5-N
SW1-2-8-1
Immature soybean seed
C
PLD mRNA transcription suppressed (Fold)
PLD alpha
PLD beta like
PLD gamma like
SW1-7-1-5-N
SW1-2-8-1
Mature soybean seed
D
Specific PLD activity (umol/min mg protein)
Fayette
SW1-7-1-5-N
SW1-2-8-1
Immature soybean seed
E
Specific PLD activity (umol/min mg protein)
Fayette
SW1-7-1-5-N
SW1-2-8-1
Mature soybean seed
F
Fayette
SW1-7-1-5-N
SW1-2-8-1
92 kD
Immature seed
92 kD
Mature seed

Table 2.1 Primer sequences used in this study

Primer name	Target gene	Primer Sequence (5' → 3')
SPLDsense	partial PLDα from soybean for realtime PCR	AGGTCAATGGATGGTGCTAGGG
SPLDanti	partial PLDα from soybean for realtime PCR	TCACTTTCTGGCTGGAGGAAGG
PLDiloopS	pSPLDi loop	CCA GCC AGA AAG TGA TGA ATG
PLDiloopA	pSPLDi loop	GCT CGG TGA CAT CTC CTT C
PLDidoubleS	pSPLDi double strand	CAG AGG GTG TTC CAG AAA GTG
PLDidoubleA	pSPLDi double strand	GGT TGC TCA GAA GGC TCA TAT C
BpldlikeF	PLDβ like	TTGGGCTGAACATACGGGTACG
BpldlikeR	PLDβ like	ACCGGGTACTTGAGCAGATGC
CpldlikeF	PLDγ like	AGGAACCAGAGAGCCTTGAATG
CpldlikeR	PLDγ like	AGAGGCTTCACTTTGCCCTTTG
SACTINF	ACTIN from soybean	TTA CAA TGA GCT TCG TGT TGA C
SACTINR	ACTIN from soybean	AAC ATA CAT GGC AGG CAC ATG
βCONGF	pSPLDi β-conglycinin promoter to loop	TAT TTC AAC ACC CGT CA
SQSPi1	pSPLDi β-conglycinin promoter to loop	CCA CAA TGA CAT GCG GAA ACC
SQSPi2	pSPLDi β-conglycinin terminator to loop	TTT CCG CAT GTC ATT GTG GTA
βCONGR	pSPLDi β-conglycinin terminator to loop	TAG CCC GAT ACT TTC CT
SPLDF	Soybean PLDα partial cDNA	GCA GAG ATG GAG AAA GCA
SPLDR	Soybean PLDα partial cDNA	CGT TAG AAT ACA GAT CCC
CaMV35SF	Hygromycin phosphotransferase	GCA CAA TCC CAC TAT CCT TCG CAA
HYGR	Hygromycin phosphotransferase	CTT CTA CAC AGC CAT CGG TCC AG

Table 2.2 Segregation analyses of the pSPLDi T_2 seeds derived from SW1-3 and SI3-8 lines as determined by HPT PCR.

Line	HPT PCR (+/-)[a]	X^2 value[b]	Best Fit[c]	*p*-value
SW1-3	30/13	0.63	3:1	0.428
SI3-8	9/3	0.00	3:1	1

[a] Segregation ratio of hygromycin phosphortransferase positive to negative plants for the pSPLDi transformants

[b] Chi-square value determined from the sum of {(observed – expected)^2 /expected}

[c] Mendelian segregation ratio determined from Chi-square values evaluated at α=0.05

Table 2.3 Agronomic traits of T_4 (2005) and T_5 (2006) pSPLDi transgenic soybean seeds from field experiment in 2005 and 2006. * indicates a significant difference of Tukey or Tukey-Kramer testes at $p<0.05$ and ** indicates a significant at $p<0.01$ between non-transgenic background cultivar and transgenic lines. † and †† indicate a significant different at $p<0.05$ and $p<0.01$ between null transgenic lines and transgenic lines, respectively.

Year	Entry[1]	Ht[2]	Lod[3]	Mat[4]	Sw[5]	Sq[6]	Plotwt[7]
2005	Fayette	34.0	1.8	Oct. 3rd	16.7	2.6	314.4
	SW1-7-1-5-N[a]	33.3	4.5**	Sep. 31st	16.0	3.8	65.5**
	SW1-3-8-8	22.7*††	5.1**	Sep. 31st	14.4	3.7	68.0**
	SW1-7-1-1	31.0	4.3**	Sep. 29th**	16.1	2.8	99.5**
	SW1-7-1-2	40.3*††	3.3**††	Oct. 4th†	19.0	3.0	135.5**
	SW1-7-1-3	28.2*	4.7**	Sep. 30th	16.4	3.5	48.2**
	Flyer	24.5	1.3	Oct. 3rd	16.9	2.8	275.5
	SI3-8-2-2-N[a]	19.5	1.0	Oct. 2nd	16.4	3.0	249.8
	SI3-8-1-1	16.9**	1.4	Oct. 6th	17.7	3.4	105.7**†
	SI3-8-7-1	21.8	1.3	Oct. 3rd	16.7	3.5	201.0
	SI3-11-6-1	23.5	1.0	Oct. 4th	18.8	3.8	179.5
2006	Fayette	26.0	2.3	Oct. 7th	13.5	3.0	657.0
	SW1-7-1-5-N[a]	17.3**	4.7**	Oct. 12th**	13.3	3.0	7.9**
	SW1-3-8-8	19.3	3.7*	Oct. 14th**	14.2	3.0	57.3**
	SW1-7-1-1	16.7**	4.0**	Oct. 9th	12.7	3.0	36.1**
	SW1-7-1-2	28.3†	3.3*†	Oct. 14th**	13.8	3.0	150.3**
	SW1-7-1-3	18.0**	3.7*	Oct. 8th	12.0	3.0	31.3**
	SW1-2-8-1	22.7†	2.3††	Oct. 13th**	12.6	3.0	18.0**
	Flyer	24.7	1.0	Oct. 10th	12.7	2.0	699.3
	SI3-8-2-2-N[a]	15.0*	1.0	Oct. 11th	12.9	3.0	449.0**
	SI3-8-1-1	14.0*	1.0	Oct. 11th	13.3	3.0	204.0**††
	SI3-8-7-1	15.0*	1.0	Oct. 12th	13.2	3.0	438.0*
	SI3-8-2-1	14.0*	1.0	Oct. 13th*	12.1	3.0	257.0**
	SI3-11-6-1	17.3	1.0	Oct. 11th	13.6	3.0	253.7**

[1]Nomenclature reflects transgenic lines and transgenic events

[2]Plant Height: the distance from soil surface to end of main stem (inch)

[3]Lodging rating: the range of rating 1 to 5

[4]Maturity date

[5]Seed weight: Gram per 100 seeds

[6]Seed quality: the range of rating 1 to 5

[7]Plot weight: total weight of seed harvested from two central rows of plot

[a] Null Transgenic soybean lines without pSPLDi transgenes from SW line and SI line

Table 2.4 Fatty acid composition of T_4 (2005) and T_5 (2006) pSPLDi transgenic soybean seeds from field experiment in 2005 and 2006. * indicates a significant difference at $p<0.05$ and ** indicates a significant at $p<0.01$ between non-transgenic background cultivar and transgenic lines. † indicates a significant different at $p<0.05$ between null transgenic lines and transgenic lines and †† indicates a significant different at $p<0.01$ (Tukey and Tukey-Kramer testes).

Year	Five major fatty acid composition (%)[2]					
	Entry[1]	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
2005	Fayette	12.5	3.6	25.6	52.0	6.3
	SW1-7-1-1	11.4*	3.3	36.1**	43.6**	5.6
	SW1-7-1-2	12.1	3.7	32.6	46.6	5.0*
	SW1-7-1-3	12.0	3.5	32.0	47.2	5.3
	Flyer	12.6	3.6	29.1	49.3	5.4
	SI3-8-7-1	13.1	3.4	25.3	51.5	6.8*
	SI3-8-2-1	13.3	3.4	24.0	52.3	7.0**
	SI3-11-6-1	13.4	3.3	24.0	52.8	6.5*
2006	Fayette	11.3	4.9	24.4	52.3	6.5
	SW1-7-1-5-N [a]	11.8	4.4	23.8	51.8	8.2
	SW1-3-8-8	9.9	4.3	23.5	54.7	7.6
	SW1-7-1-1	10.7	4.7	26.6	50.4	7.6
	SW1-7-1-2	11.3	4.7	25.9	50.4	7.6
	SW1-7-1-3	11.6	4.8	25.7	50.0	8.0
	SW1-2-8-1	11.8	4.2	24.1	51.5	8.5
	Flyer	11.3	5.0	25.4	50.2	8.1
	SI3-8-2-2-N [a]	11.3	4.5	27.3	49.8	7.2
	SI3-8-1-1	11.2	4.1	24.4	52.1	8.2
	SI3-8-7-1	11.7	4.1	23.2	52.3	8.9
	SI3-8-2-1	11.5	5.3	26.3	49.6	7.3
	SI3-11-6-1	12.2	3.9**	20.8**††	53.3	9.9*†

[1]Nomenclature reflects transgenic lines and transgenic events

[2]Five major fatty acid composition indicates palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3)

[a] Null Transgenic soybean lines without pSPLDi transgenes from SW line and SI line

Table 2.5 Protein content and oil content of T_5 pSPLDi transgenic soybean seeds from field experiment in 2006. N indicates null transgenic line. * indicates a significant difference at $p<0.05$ and ** indicates a significant at $p<0.01$ between non-transgenic background cultivar and transgenic lines. † indicates a significant different at $p<0.05$ between null transgenic lines and transgenic lines (Tukey and Tukey-Kramer testes).

Entry[1]	Protein content	Oil content
Fayette	45.7	16.8
SW1-7-1-5-N	44.4	16.4
SW1-3-8-8	43.2**	16.9
SW1-7-1-1	45.0	14.6**†
SW1-7-1-2	44.5	15.2
SW1-7-1-3	45.2	14.5**†
SW1-2-8-1	45.4	15.8*
Flyer	43.1	17.2
SI3-8-2-2-N	42.7	15.7
SI3-8-1-1	42.8	15.8
SI3-8-7-1	41.6	16.5
SI3-8-2-1	42.5	15.4*
SI3-11-6-1	43.2	17.3†

[1]Nomenclature reflects transgenic lines and transgenic events

Table 2.6 Fatty acid analysis of T_5 pSPLDi transgenic soybean seeds from greenhouse in 2006-2007 winter nursery. * indicates a significant difference at $p<0.05$ and ** indicates a significant at $p<0.01$ between non-transgenic background cultivar and transgenic lines (Tukey test).

Entries[1]	Five major fatty acid composition (%)[2]				
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Fayette	11.0	3.6	32.7	46.0	6.8
SW1-3-8-8	9.9**	3.2*	33.2	46.3	7.4
SW1-7-1-1	10.7*	3.1**	35.0	43.3*	7.8*
SW1-7-1-2	9.3**	3.3	49.3**	31.8**	6.3
SW1-7-1-3	11.1	3.0**	28.0	48.7	9.1**
SW1-2-8-1	10.7*	3.4	27.7	49.9	8.2**
Flyer	10.6	3.5	31.9	47.0	7.0
SI3-8-1-1	11.4	3.0**	26.1	48.6	11.0**
SI3-8-7-1	9.2**	2.3**	36.9*	41.6**	9.9**
SI3-8-2-1	9.6**	2.7**	37.2*	40.9**	9.6**

[1]Nomenclature reflects transgenic lines and transgenic events

[2]Five major fatty acid composition indicates palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3)

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CHAPTER 3 - Enhanced seed viability by suppressing phospholipase Dα in soybean seed

Abstract

Various factors, such as high humidity and temperature, cause the loss of seed viability. Fayette seed stored for two months following harvest exhibited about 95% viability, however Fayette seeds stored for 33 months at room temperature and uncontrolled relative humidity become non-viable. PLDα-attenuated transgenic soybean seeds have been produced by transformation. Germination of transgenic seed stored for 33 months ranged from 30 to 50%. Increased leakage of electrolytes associated with the loss of viability was observed in null-transgenic and background seeds versus transgenic seed. The increase in electrolyte leakage may have been induced by lipid peroxidation and free radical formation which can generate oxidative damage in the cell and subsequently decrease seed viability. Differences in ultrastructure of cotyledon tissue were observed between PLDα-suppressed soybean and the background cultivar. The loss of viability in the background cultivar was consistence with observation of the plasma membrane beding detached from the cell wall complex and the disorganization of oil bodies.

Introduction

Viability and seed quality of soybean seed in long-term storage depends on environment, biochemical, biological and genetic factors. Characterization of the aging process in seeds has noted changes in membrane protein composition (Nowakowska and Rakowski, 2002), disruption of the nuclear envelope (Haithcock et al., 2005), protein degradation (Kumar et al.,1999), decreases in lipid content (Lin and Pearce, 1990), oxidative stresses, as well as decreases in mRNA translation (Gidrol et al., 1990) and DNA replication capability. It has also been reported that increased oxidative damage and reduced levels of antioxidant enzymes such as superoxide dismuatse, catalase, and ascorbate peroxidase are consequences of the aging process (Bailly et al., 1996; 1998). Altered electrolyte leakage through the plasma membrane and increased membrane fluidity are inevitable events during long-term storage of soybean seed (Leinonen, 1998; Rakowski et al., 1998). Oxidation and peroxidation of lipids decrease the economic and nutritional value of soybean seed products through modifications in color, taste and odor (Nakayama et al., 1981). Understanding the impact of lipid degrading enzymes on the stability of the lipid membrane is an important component to elucidating the natural aging process (Devaiah et al., 2007).

Among many lipid degrading enzymes, phospholipase D (PLD) may be involved in the first step of phospholipid degradation to generate phosphatidic acid (PA). A well-known function of most lipases, including PLD, is to degrade lipid membranes, thus changing the structure of membrane lipids. PLD activity can break down membrane lipid species including PC, PE, and PG from phospholipid membranes resulting in PA and a free

head group. By accumulating PA in the cell, hexagonal phase$_{II}$ lipid particles form, causing the loss of cell membrane integrity and cell viability (Kooijman et al., 2003). It has been suggested that PLD may be involved in senescence and cell death by degrading membrane lipids (Fan et al., 1997). Recently, Devaiah et al. (2007) reported that seed viability and lipid stability were enhanced by suppressing PLDα in *Arabidopsis*. Modification of PLDα in seeds promises to improve storability of *Arabidopsis* seeds resulting from a decrease in oxidative stress and the degradation of membrane lipids.

PLD suppressed transgenic lines have been created in soybean. To better understand the role of PLDα in the aging process of seed, this study was conducted to investigate changes in viability and longevity of seeds in PLD suppressed transgenic soybean.

Material and Methods

Plant material, seed storage, germination test, and electrolyte leakage

Soybean cultivar, Fayette (Bernard et al., 1988), were co-transformed with pHG1 and pSPLDi harboring RNAi structure (820 bp sense and 1300 bp antisense fragments) via particle inflow gun bombardment and regenerated under hygromycin selection (see Chapter 2). T_1 soybean lines SW1-3, SW1-7, null transgenic line, SW1-5-N, and their background cultivar, Fayette were grown in a greenhouse under 25 ± 2 °C day and 20 ± 2 °C night temperature and a 16h light/8h dark photoperiod. Immature pods were excised at around 26 days after fertilization (DAF) for PCR, PLD enzyme assay and western blot analysis. Seeds were harvested from each T_1 line and the cultivar Fayette, and dried to 13% moisture. T_2 transgenic and Fayette seeds were stored in paper envelopes at 25 °C and 17% relative humidity for 33 months, and T_5 seeds derived from SW1-3 and SW1-7 lines were harvested and stored at the same condition for two months. Viability of transgenic, SW1-3 and SW1-7, and background cultivar seeds stored for 2 and 33 months was evaluated using standard germination test. Three replicating thirty seeds from each genotype were wrapped in 25 cm-long paper roll and drenched in water. The water level was maintained 5 cm below seeds lines in order to supply the same amount of water to individual seed. Germination test under water supply was assessed to count number of soybean sprout by 10 days.

Electrolyte leakage from 2 and 33 month stored seed was measured by modifying the method described by Fan et al. (1997). Briefly, three soybean seeds were agitated in 3 ml of 0.4 M mannose for 6 hours to induce ion leakage. Conductivity of the solution was

measured by YSI model 32 conductance meter (Scientific division, Yellow Springs Instrument Co., Inc) after vortexing the samples for 30 seconds. Total electrolytic concentration was determined after boiling samples for 15 min and cooling to 25 ºC. Electrolyte leakage was expressed as percentage of initial electrolytic concentration divided by total electrolytic concentration in the solution. Three replications of three seeds per replication were evaluated for each soybean line.

PCR analyses

Genomic DNA was extracted from young soybean leaves and immature seeds via a modified CTAB DNA extraction method by Saghai-Maroof et al. (1984). For PCR reactions, 50 ng of genomic DNA in 5 µl of aliquots was used as a template in a 50 µl PCR reaction containing 1X NH_4 buffer, 1.5 mM MgCl, 0.2 mM of deoxinucleotide triphosphate (dNTPs), 20 pmol of forward and reverse primers and 1.25 u *Taq* DNA polymerase (BioLab). The pSPLDi construct was amplified in a Hybaid PCR Exress Thermocycler (Hybaid, UK) using one cycle at 94 ºC for 5 min; 30 cycles at 94 ºC for 30 sec, 52 ºC for 60 sec, and 72 ºC for 90 sec; and a final cycle at 72 ºC for 7 min with forward βCONGF (5'-taa ttc aac acc cgt ca-3') and reverse SQSP1 (5'-cca caa tga cat gcg gaa acc-3'). PCR amplicon was run on 0.8% agarose gel with 1X TAE buffer at 50 volts. The gel was then stained in ethidium bromide (EtBr) solution for 10 min. The stained gel image was taken using the Kodak 1D^{TM} image Analysis Software (Eastman Kodak Company, New Haven, CT).

PLD enzyme assay

Total protein from T_2 lines was extracted using a chilled pestle to grind immature seed in a 1.5 mL microcentrifuge tube with liquid N_2 and a homogenization buffer containing 50 mM Tris-HCL, pH 7.,5, 10mM KCL, 1 mM EDTA, 0.5 mM phenyl methylsulfonyl fluoride, and 2 mM DTT at 4 ^{o}C (Fan et al., 1997). Protein concentration was measured with a Nanodrop ND1000 spectrophotometer (Nanodrop, DE USA). Aliquots of 20 µg of native protein were used for the PLD enzyme assay as described previously (Fan et al., 1997). Briefly, the reaction mixture contained 100 mM Mes, pH 6.5, 25 mM $CaCl_2$, 0.5 mM SDS, 1% (v/v) ethanol, and 2 mM phosphatidylcholine (egg yolk) containing dipalmitoly glycerol-3-phosphate-(methyl-3H)-choline in a final volume of 200 µl. Substrate preparation, reaction conditions, and product separation were based on a previously described procedure (Wang et al., 1993). The release of the free head group, ^{3}H labeled choline, into the aqueous phase was quantitated by liquid scintillation counter (Beckman).

Western blot analysis

Proteins were extracted from immature soybean seed (Zhang et al., 2003). Total native proteins were incubated at 95 ºC for 15 min with SDS-PAGE loading buffer which was 100 µl of 50 mM Tris-HCL, pH 6.8, 10 mM DTT, 2% SDS, 0.01% bromophenol blue, and 10% glycerol. Twenty micrograms of denatured protein were fractionated by 10% SDS-PAGE gel on 100 V until dye reached the bottom of the PAGE gel. The membrane containing denatured protein was blotted with PLDα antibodies as described in Zhang et al. (2003). Briefly, gels were transferred to an Immobilon^{TM}-P transfer membrane (Millipore,

Bedford, MA) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The membranes were blocked with non-fat milk for 1 h and soaked in the first antibody overnight. The PLDα polyclonal antibody was diluted to 1:1000 in TBST (10 mM Tris, 140 mM NaCl and 0.05% Tween 20). The blots were washed in 1 X TBST 3 times, 5 min/wash. Goat anti-rabbit IgG (H+L) horseradish peroxide (HRP) conjugate (Bio-Rad) was used as the second antibody at a dilution of 1:1000 in TBST. The blots were incubated in a second antibody for 1 h and washed two times in TBST followed by one wash in TBS (10 mM Tris, 140 mM NaCl, pH 7.5). The protein bands recognized by the antibody were visualized with a HRP color development reagent (Bio-Rad).

Major fatty acids analysis by gas chromatography

A 50 mg sample of clean T_2 seed is prepared by taking an equal volume or weight of seed from three replications. Seed samples were submitted to the USDA-ARS National Center for Agricultural Utilization Research, Peoria, Illinois. Fatty acid composition was analyzed using a gas chromatography modified procedure from Christie (1989). Results from the fatty acid analyses were expressed as percentage (%) of the total fatty acid in the grain.

Statistical analysis

Data collected from the fatty acid profiles, electrolyte leakage analyses, and PLD enzyme assays were examined by analysis of variance using SAS (SAS Institute Inc., Cary, NC) PROC general liner model (GLM) procedure. Means were separated using the LSD, Duncan's multi-range test, or Tukey test at the $p<0.05$ probability.

Transmission electron microscopy (TEM)

Soybean tissue was prepared for TEM and image capturing using a modified protocol as described by Boyle and Takemoto (1998). Briefly, soybean cotyledon tissues were immersion fixed in 2% paraformaldehyde (Ladd, Burlington, VT), 2% glutaraldehyde and 10% sucrose in 0.1 M sodium cacodylate buffer pH6.8 for 16 hours at room temperature (RT) with constant rotation. Samples were post-fixed with 2% osmium tetroxide and 10% sucrose in 0.1 M sodium cacodylate buffer pH 6.8. Fixed tissues were dehydrated in an ascending series of acetone with 50% - 100% at RT, embedded in Spurr's embedding media in gelatin capsules and polymerized at 65 ºC for 24 hours. Embedded soybean tissues were cut with glass knives on a Reichert Ultracut S microtome (Leica, Austria) and held on 100 nickel slotted grids. All images were captured using an Advanced Microscopy Techniques digital image capturing system (ATM, Chazy, NY).

Results

Changes in the level of PLDα protein and enzyme activity in T_1 soybean seeds, null-transgenic, and background cultivars

PLDα-suppressed soybean lines were generated by transferring pSPLDi and pHG1 plasmids via particle inflow gun bombardment (see Chapter 2). To confirm the suppression of PLDα in pSPLDi transgenic soybean seed, pSPLDi PCR, PLDα enzyme assay and western blot were performed using immature cotyledons of T_2 SW lines. Since endogenous PLD gene expression was suppressed by the RNAi machinery, the immature cotyledons from null-transgenic soybean and transgenic soybean showed different levels of PLDα protein (Fig 3.1). Approximately 92 *kD* of denatured soybean PLD protein was monitored in the soybean extract using a *Arabidopsis* PLDα1 antibody. The size of PLDα protein is consistent with previous research (Ryu et al., 1996).

The presence of the transgene in T_2 SW1 seeds was detected by PCR primers, specifically amplifying the pSPLDi construct. The difference in the level of PLDα protein and PLDα enzymatic activity was observed in pSPLDi transgenic seeds, suggesting that the changes in PLDα activity in transformants resulted from changes in PLDα protein content (Fig. 3.1). Null-transgenic segregating seed, SW1-5-N, showed no decrease in the level of PLDα protein and PLDα enzyme activity, whereas the PLDα activity in immature seeds from SW1-3 and SW1-7 lines was suppressed 40 to 50 percent below that of the background cultivar, Fayette. PLDα enzyme activity and western blot analysis were in agreement with pSPLDi transgene PCR data (Fig. 3.1).

Changes in PLD suppressed soybean seeds response to natural aging

To determine if PLDα activity affects soybean seed viability and germination, T_2 transgenic and the background cultivar seeds were evaluated electrolyte leakage of 2 and 33 month old seeds. The potential role of PLDα in seed viability for long period was investigated using germination test. The germination percentage of two-month-old soybean seeds from each genotype was approximately 95 percent (Fig 3.2A). No Fayette (control) seeds stored for 33 months had germinated after 10 days of imbibition. The germination of transgenic soybean lines, SW1-3 and SW1-7, were observed at 50 and 30%, respectively. As T_2 seeds were heterozygous transgenics, we performed PCR analysis to confirm the relationship between the presence of the transgene and seed viability in SW1-3 progeny line (Fig. 3.2B). Based on PCR and germination data, five out of 6 viable seeds were transgenic, whereas two out of 6 null-transgenic soybeans germinated. Results suggested that the viability of 33-month-old seed was associated with the suppression of PLDα.

Electrolyte leakage from null-transgenic and Fayette seeds was significantly greater than leakage from the transgenic lines (Fig. 3.2C). Leakage from SW1-3 and SW1-7 was around 14%, whereas leakage from Fayette seeds and null-transgenic soybean seeds was observed to be about 20% each. This lower electrolyte leakage from the PLD suppressed lines suggested increased membrane stability and therefore improved seed viability under long periods of seed storage compared to the non-transgenic seeds.

Changes in five major fatty acids in 2-month-old and 33-month-old PLD-suppressed soybean seeds

PLDα is the most widely distributed and abundant form among PLD isoenzymes in the plant cell (Devaiah et al., 2007). Enzymatic reaction of PLDs may share its preferable species of phospholipids with fatty acid desaturases such as FAD2 and FAD3 in the TAG biosynthetic pathway, consequently the degree of fatty acid desaturation may be elevated by suppressed PLD activity.

Palmitic acid composition in old soybean seed was significantly increased in both SW1-3 and background genotypes (Table 3.1). There were statistical differences in palmitic and oleic acid composition between aged and fresh non-transgenic seeds. Linolenic acid composition was significantly decreased in transgenic seed during long-term storage. On the other hand, linolenic acid composition in background genotype was increased during 33 month storage. Oleic acid composition in 33-month-old SW1-3 seeds decreased about 2%, and linoleic acid composition increased approximate 2%, whereas oleic and linoleic acid compositions from 33-month-old Fayette seeds decreased about 9%, and increased 10% of total fatty acid composition, respectively, proposed that the decomposition of fatty acids and oil content results from lipid peroxidation, and it affects on the viability of seeds (Bailly et al., 1998).

Ultrastructural changes in soybean cotyledon tissues response to natural aging

Transmission electron microscopy was used to investigate the ultrastructural changes in plasma membrane and cell wall complex of seed stored for 33-months from

PLDα-suppressed soybean and Fayette as well as fresh Fayette seeds (Fig. 3.3). Obvious changes of cell structure were detected by light microscopy with 100X magnification. The plasma membrane in 33 month old Fayette cotyledons was partially plasmolyzed and detached from cell wall (Fig. 3.3B). Plasma membrane detachment was observed in both transgenic and Fayette cotyledon tissues stored for 33 months, however plasma membrane in Fayette cotyledons was irregularly piled, resulting from cell dehydration (Fig. 3.3D, E, F, H and I). The presence of voids between the plasma membrane and secondary cell wall in Fayette cotyledons suggested that age-related changes during long-term storage resulted in the structural alteration of plasma membrane and eventually the loss of seed viability.

Discussion

Post-harvest degradation of phospholipids deteriorates the nutrient quality of oil during long-term storage of soybean seed (Wilson and McDonald, 1986; Bailly et al., 1996). PLD may be involved in the first step of phospholipid degradation. To preserve phospholipids in soybean seed for long-term storage, RNA interference under the control of a seed specific promoter, β-conglycinin α1 subunit promoter, was used to attenuate PLDα enzyme activity in soybean seed. Twelve independent transgenic soybean lines with pSPLDi and pHG1 were generated via co-bombardment using a particle inflow gun. Most transgenic events were fully fertile. Based on the background cultivar and pod setting ability of the transgenic progenies, an elite line, SW (Fayette background), was chosen to further evaluate characteristics of PLD suppressed soybean. Immature seeds of SW transgenic lines had lower levels of PLDα protein, and PLDα activity than null-transgenic and background cultivars.

Stored soybeans may undergo physical, physiological and chemical changes. Temperature, moisture, and time are critical factors impacting seed properties during storage. Changes in seed during storage include seed viability, grain color, moisture content, decomposition of phospholipids and free fat (Bailly et al., 1996; 1998), and protein degradation (Kumar et al., 1999). The natural and accelerated aging processes impact on biological membranes is reflected to the damage to the plasma and cell organelle membranes as well as deterioration in the nucleus envelope.

Fayette seeds stored for 33 months failed to germinate, whereas PLDα-suppressed transgenic seeds maintained 30 to 50% germination after the same duration of storage.

Bailly et al. (1996) reported a continuous efflux of electrolyte during the accelerated aging process of sunflower seeds. In this study, electrolyte leakage from 33-month-old Fayette seeds exceeded the leakage observed in PLDα-suppressed soybean seeds (Fig. 3.2C). Conventionally harvested and dried soybean seeds, containing 12% moisture, usually showed 9-12% electrolyte leakage shortly after harvest, depending on the quality of soybean.

Chapman and Robertson (1980) reported that levels of free fatty acid, mainly palmitic and linoleic acid, were increased during soybean seed storage under 18% relative humidity, demonstrating that the increase in fatty acid during storage results from lipid lipase and phospholipase activities. In this study, the level of palmitic acid was significantly increased in PLDα-suppressed transgenic and background genotypes during long-term storage. Oleic acid in the 33 month old Fayette seed was not significantly lower than the 2 month old seed for Fayette, whereas major changes in oleic and linoleic acid composition were monitored in Fayette seeds until 33 months (Table 3.1). Linolenic acid in SW1-3 seed was significantly decreased during long-term storage, however, there was no significant difference in oleic and linoleic acid composition in PLDα-suppressed soybean seeds.

Seed viability was improved by preserving cell shape and plasma membrane stability. Ultrastructural symptoms possibly related to age-induced membrane deterioration were observed in cotyledon tissues from 33-month-old Fayette soybean seeds. The loss of viability may have been the result of the degradation of plasma membranes and coalescence of lipid bodies. The oxidation and peroxidation of membrane phospholipid during aging processes may have contributed to this degradation (Bailly et al., 1996; 1998).

Farrant et al. (1988) suggested that the loss of structured water in the seed may lead to the loss of stability of structure of organelle and membrane, inducing the loss of viability. The loss of viability and increase in electrolyte leakage of sunflower seeds are positively correlated during natural aging process (Corbineau et al., 2002). This result may be caused by the membrane phospholipid deterioration, generating diacylglycerol by enzymatic or chemical hydrolysis of phospholipids. The interaction between enzymatic and non-enzymatic hydrolysis is still questionable. It has been suggested that high levels of PLDα1 activity result in the formation of PA which is a secondary messenger reflecting damage membrane and storage lipids in seeds (List et al., 1992). To test this hypothesis, we need to construct membrane and storage lipid profiling maps had to be constructed with background cultivars and PLDα-suppressed soybean.

PLDα1-suppressed and PLDα1-knockout *Arabidopsis* improved seed viability during the aging process and reduced deteriorating storage lipids (Devaiah et al. 2007). The storage lipids, mainly TAG, in soybean seeds are coated with a phospholipid monolayer to form a lipid body in the cytosol. High levels of PLD activity could hydrolyze the phospholipid monolayer, resulting in the degradation of the oil bodies after harvest, and deterioration of seed quality.

PLDα-ablated and PLDα-attenuated *Arabidopsis* exhibited enhanced the viability of seeds and stability of oil in the seeds (Devaiah et al., 2007). The PLD-mediated PA production could activate NADPH oxidase which generates H_2O_2 (Park et al., 2004). The decreased membrane integrity and the increased reactive oxygen species (ROS) encourage lipid peroxidation. Oxidative damage by lipid epoxidation and the generation of free

radicals has been associated with the loss of viability. Based on the findings that PLDα-suppressed soybean seed exhibited increased viability and lower electrolyte leakage than background cultivar, the degradation of the plasma membrane and oil bodies by the aging process is partially mediated by PLDα activity.

Figures and Tables

Figure 3.1 PLD enzyme activity and western blot analysis of PLDα in the immature cotyledon form heterozygous T_1 SW1 lines. A; PLDα enzyme assay of T_2 SW1 seeds. Immature cotyledons were split with razor blade as three parts. The first part of tissue was used for PCR analysis to confirm the presence of pSPLDi transgene. PLDα enzyme assay was expressed nmoles of phosphatidylcholine min^{-1} mg^{-1} total protein. Error bar indicates ±SE with three independent measurements. B; Immunoblot analysis of PLDα was performed with *Arabidopsis* PLDα1 antibody, and PLDα proteins were decorated at 92 *kD* position based on the protein marker. The presence of pSPLDi transgene was confirmed with G.O.I PCR. + indicates PCR positive of pSPLDi PCR, and – indicates PCR negative.

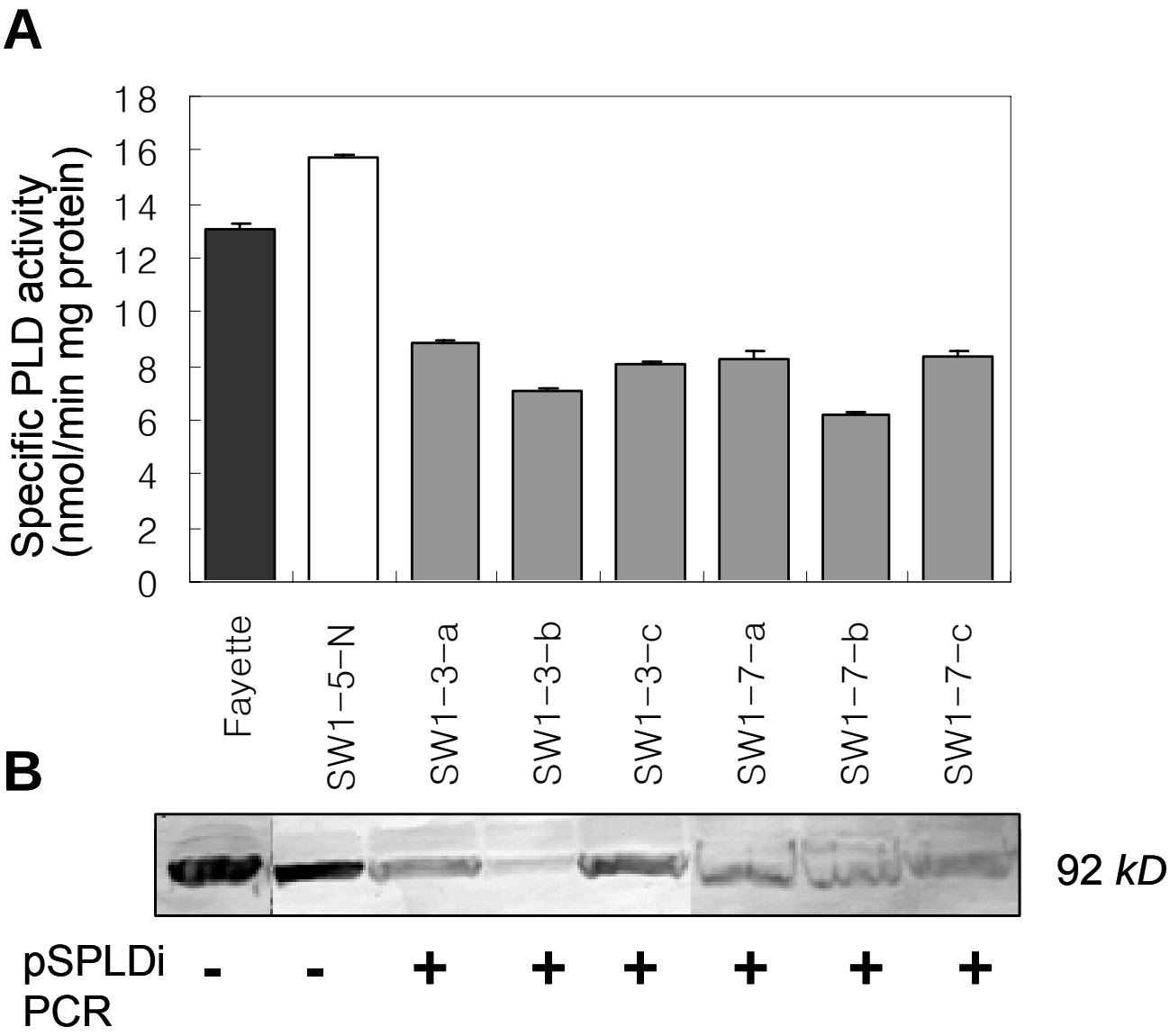
A
Specific PLD activity
(nmol/min mg protein)
18
16
14
12
10
8
6
4
2
0
Fayette
SW1−5−N
SW1−3−a
SW1−3−b
SW1−3−c
SW1−7−a
SW1−7−b
SW1−7−c
B
92 kD
pSPLDi - - + + + + + +
PCR

Figure 3.2 Effect of PLD suppression in seed viability of 33 months stored soybean seeds. Two T_2 SW1-3 and SW1-7 seeds were tested for germination, presence of PLD mediate transgene, and electrolyte leakage. A; Two-month-old and 33-month-old soybean seeds (10 seeds per replication) were placed on the wet paper roll for 10 days, and soybean sprouts were counted to calculate germination rate (%, No. of germinated soybean/ No. of total soybean X 100). Error bar means ±STDEV of three replications. B: PCR analysis for confirming the presence of pSPLDi transgene in soybean sprout from 33-month-old soybean seeds. C; Effect of pSPLDi transgene in electrolyte leakage of 33-month-old soybean seeds. Three seeds from SW1-3, SW1-7 and Fayette were tested ion leakage by conductivity meter, where positive (+) seed means transgene PCR positive and negative (-) seed represents transgene PCR negative. * indicates a significant difference at $p<0.05$ level between transgenic, null-transgenic, and background genotypes.

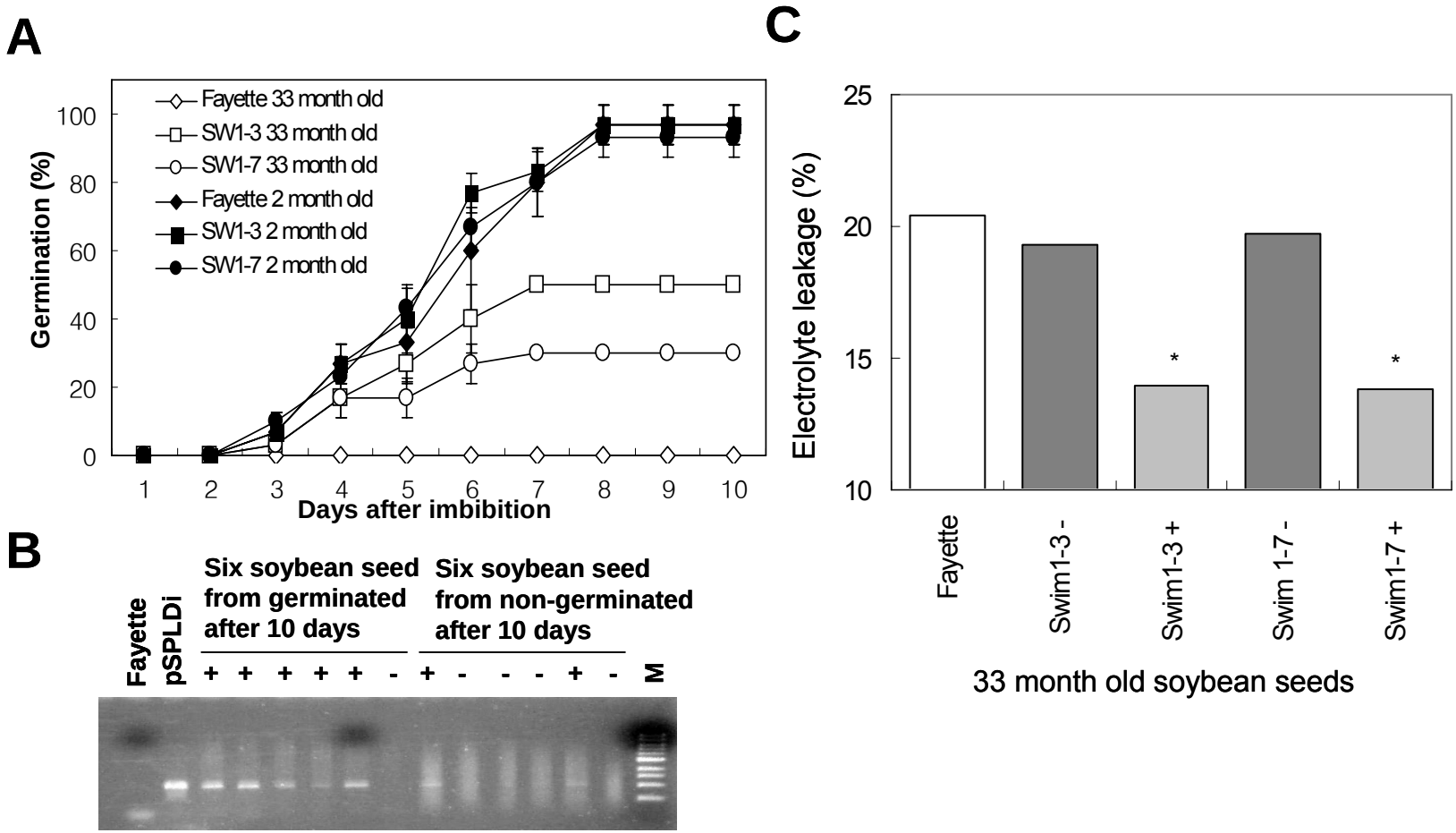
A
Germination (%)
Days after imbibition
Fayette 33 month old
SW1-3 33 month old
SW1-7 33 month old
Fayette 2 month old
SW1-3 2 month old
SW1-7 2 month old
B
Fayette pSPLDi
Six soybean seed from germinated after 10 days
Six soybean seed from non-germinated after 10 days
+ + + + + - + - - - + - M
C
Electrolyte leakage (%)
Fayette
Swim1-3 -
Swim1-3 +
Swim 1-7 -
Swim1-7 +
33 month old soybean seeds

Figure 3.3 Ultrastructure of mature cotyledon tissues and cell wall complex from 33-month-old soybean. Light and transmission electron microscopy showed 2-month-old Fayette (A, D, and G), 33-month-old Fayette (B, E, and H), and 33-month-old SW1-3-+ (C, F, and I) cotyledon tissues.

A; Cross section of cotyledon cells from 2-month-old Fayette seed with light microscopy.

B; Cross section of cotyledon cells from 33-month-old Fayette seed with light microscopy.

C; Cross section of cotyledon cells from 33-month-old SW1-3 seed with light microscopy.

D; Cross section of cotyledon cells from 2-month-old Fayette seed with transmission electron microscopy.

E; Cross section of cotyledon cells from 33-month-old Fayette seed with transmission electron microscopy.

F; Cross section of cotyledon cells from 33-month-old SW1-3 seed with transmission electron microscopy.

G; Closed view of cell wall complex from 2-month-old Fayette seed with transmission electron microscopy.

H; Closed view of cell wall complex from 33-month-old Fayette seed with transmission electron microscopy.

I; Closed view of cell wall complex from 33-month-old SW1-3 seed with transmission electron microscopy.

In (B, C, and D), white arrows indicate the sign of partial plasmolysis. Bars in A, B and C = 50 μm. Bars in D, E and F = 10 μm. Bars in G and H = 0.5 μm. Bar in I = 0.1 μm (Higher

magnification). PC, pectin-rich cell corner; ML, middle lamella; PW, primary walls; PD, plasmodesmata.

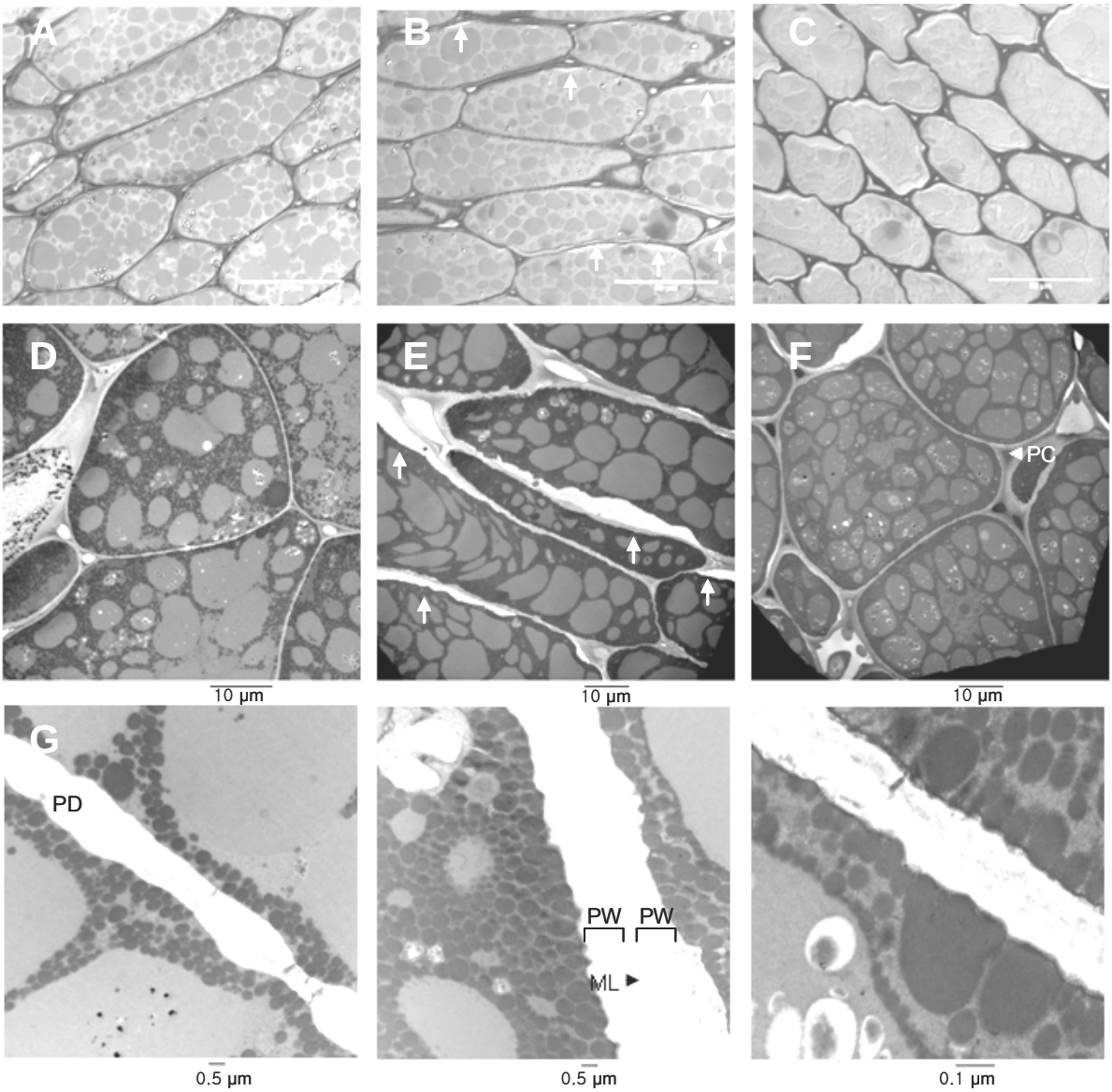

Table 3.1 Fatty acid analysis of 2-month-old and 33-month-old transgenic and Fayette seeds.

Entries[1]	Major fatty acid composition (%)[2]				
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
33 month old Fayette	12.5a	3.4ab	20.1b	56.8a	7.2a
33 month old SW1-3	11.2b	3.1b	31.5a	48.3ab	6.0b
2 month old Fayette	11.6b	3.6a	29.2ab	46.9ab	6.5ab
2 month old SW1-3-8-8	9.9c	3.2b	33.2a	46.3b	7.4a

Duncan's multi-range grouping at the α =0.05

[1]Nomenclature reflects genotypes and storage time

[2]Five major fatty acid composition indicates palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3)

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CHAPTER 4 - Freezing and heat-shock tolerance obtained from phospholipase Dα-attenuation in soybean seedlings

Abstract

Stresses caused by temperatures higher or lower than ambient are one of agricultural problems that reduce crop productivity in many areas and diverse species. To overcome the uncertainty of environmental fluctuations, efforts continue to improve high and low temperature tolerance in crops. Phospholipase Dα (PLDα)-suppressed transgenic soybeans were produced by antisense suppression driven by constitutive and seed-specific promoters using the particle inflow gun (PIG) bombardment method. Nine fertile transgenic events suppressed the expression of PLDα protein compared to the non-transgenic control. PLDα enzyme activity in T_1 seed was observed to be reduced by 25 percent. When soybean seedlings were exposed to lethal freezing temperature, increased electrolyte leakage associated with oxidative damage and biophysical changes were observed in non-transgenic soybean, whereas membrane stability and integrity were maintained in transgenic soybean seedlings. The early growth of PLDα-attenuated soybean seedlings recovered from extreme heat-shock and freezing treatments. The disruption of the plasma membrane and organelles was observed in freeze-stressed non-transgenic seedlings. On the other hand, the structure of the plasma membrane, oil bodies, and cell organelles in transgenic seedlings was partially sustained after enduring freezing and thawing stresses.

Introduction

Global warming and unexpected environmental fluctuations may result in crop loss. The increase in global temperature will mainly affect changes not only in the acreage of crop production but also the evaporating rate in the unit area (Reddy and Hodges, 2000; Hall, 2001). Reddy et al. (1997) suggested that a 5 ^{o}C increase in average global temperature will shorten the growth period based on the model experiment, and this will result in lower yields and poorer quality.

Plants have different tolerances to the freezing temperature, depending on species. The maximum tolerance to freezing in plants is an induced response to low temperature, but not constitutive. Recent studies on molecular, cellular, and biophysical responses to freezing-induced stress have contributed to understanding the mechanisms of freezing tolerance in plants (Uemura and Steponkus, 1999; Thomashow, 1998; Wang et al., 2006). Fundamental research on freezing-induced damage has focused on changes in membrane lipid profile, protein accumulation and intracellular concentrations of proline and sugar. Changes in membrane lipid profile have been attributed to induced low temperature stress (Welti et al., 2002; Li et al., 2004). Welti et al. (2002) elucidated the role of PLDα1 in cold and freezing injury by measuring changes in lipid species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), lyso phosphosphatidylethanolamine (lyso PE), and lyso phosphatidylcholine (lyso PC) between wild-type and PLDα1-deficient *Arabidopsis* using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Specific PC species

contribute to the stabilization of the plasma and chloroplast membranes. PLDα-ablated *Arabidopsis* showed significantly lower ion leakage than wild type *Arabidopsis* during freezing treatment and survived after recovering from low temperature stress. One molecular species of phosphatidic acid (PA), 34:6-PA, increased during freezing, but not during cold acclimation. Undetectable amounts of 34:6-PC existed in non-treated *Arabidopsis*. This implied that mechanisms, such as phosphorylation of diacylglycerol, are involved in the accumulation of 34:6-PA from MGDG and DGDG and other enzymes responsible for PE and PG (Welti et al., 2002).

Exposure to higher temperature mainly alters membrane function through modification of membrane fluidity and ordering (Maestri et al., 2002). Heat-shock stress treatments have been reported to increase lipid metabolic enzymes (Johnston et al., 2007), and shift the degree of saturation in ER membrane lipid (Grindstaff et al,. 1996). Increased fatty acid saturation is correlated with decreases in mobility and ordering of the membrane structure, and decreased unsaturated fatty acid is associated with increased mobility and ordering of the membrane structure (Wahid et al. 2007). It remains unclear whether higher or lower membrane fluidity is beneficial to heat-shock tolerance. Objectives of this research were to investigate low temperature and heat-shock tolerance in PLDα-suppressed soybean seedlings.

Material and Methods

Plasmids construction for transformation

A 1.3 Kb partial sequence of soybean PLDα (SPLDα) was cloned by RT-PCR using degenerate primers from Castor bean PLD sequence (Wang et al., 1993; 1994). The plasmids used for soybean transformation via particle inflow gun (PIG) bombardment originated from pUC19, and then expression cassette were subcloned from (pCong, 4.1 Kb) harboring soybean β-conglycinin α subunit promoter and terminator unit. pSPLDanti (β-conglycinin promoter::SPLDanti) plasmid was constructed with 820 bp of SPLDα partial sequence of SPLDα was digested with *Bam*HI and ligated in between β-conglycinin promoter and terminator as antisense orientation. To construct p35SSPLDanti, the CaMV35S constitutive promoter digested by *Hind*III and *Sac*I were ligated instead of β-conglycinin promoter on the pSPLDanti vector (Fig. 4.1A). The vector pHG1 (Finer et al., 1992) contained a selectable marker gene, hygromycin phosphotrasferase or HPT (1.0 Kb), were used co-bombardment to provide transgenic tissue to hygromycin resistance (Fig. 4.1B). The HPT gene was used as selective marker during transformation and regeneration as well as confirmation of the presence of transgenes by PCR.

Plant material and tissue culture

Soybean cultivar, Jack, was grown in a greenhouse under 25 ± 2 °C/ 18 h day and 20 ± 2 °C/ 6 h night photoperiod. Immature pods were excised at growth stage R5 and surface-sterilized with 1.05% sodium hypochlorite (NaOCl) and 0.02% Tween-20 for 15

min with agitation, and then followed by three washes with sterile distilled water in hood. Methods for culture initiation and proliferation were as described by Finer (1988) and modified by Trick et al. (1997). Approximately three to six months after initiation of somatic embryo cultures on D40 medium, the embryos were proliferated on D20 medium, prepared for bombardment and then transformed using the particle inflow gun (Finer and McMullen, 1991). For co-bombardment, one microgram of pSPLDi plasmid and pHG1 plasmid were mixed in a 1:1 ratio. Putative transformed tissues were maintained on D20H7.5, D20H3, and D20H15 medium containing 7.5 mg/l, 3 mg/l, and 15 mg/l hygromycin, respectively. Throughout the selection process hygromycin concentration was either increased or decreased depending upon the health of the tissue. After 3 to 6 months, putative transgenic clumps were tested by PCR for the presence of pSPLDi and HPT transgenes. Transgene positive tissues were then transferred and matured on M6 medium. After embryos matured to a "torpedo" stage of development and an apical meristem was visible, embryos were desiccated for 1 to 2 days and rooted in vermiculite saturated with ½-strength OMS medium. Recovered plants possessing two trifoliate leaves were transplanted to peat moss, harden at 23 ^{o}C under 24 hour light then transferred to 5 gallon pots and grown to maturity in the greenhouse under a 16-h photoperiod.

PCR and southern blot analysis

Genomic DNA was extracted from young soybean leaves and immature seeds via a modified CTAB DNA extraction method by Saghai-Maroof et al. (1984). For PCR reactions, 50 ng of genomic DNA in 5 µl aliquots was used as a template in 50 µl PCR

reaction containing 1X NH_4 buffer, 1.5 mM MgCl, 0.2 mM of deoxinucleotide triphosphate (dNTPs), 20 pmol of forward and reverse primer and 1.25 U Taq DNA polymerase (BioLab). Forward primer (5'-TCA CTT TCT GGC TGG AGG AAG G-3') and reverse primer (5'-TAG CCC GAT ACT TTC CT-3') were used to amplify modified PLDα transgene.

For southern blot analysis, 20 μg of genomic DNA was digested overnight using *Xba*I (50 U) at the designated temperature. Quantification of DNA was performed using the Nanodrop ND1000 spectrophotometer (Nanodrop, DE USA). Digested DNA samples were separated by electrophoresis on a 0.8% TAE agarose gel and denatured with a 0.4 M NaOH solution for 12 to 16 hours. Using the semi-dry and wet capillary transfer methods, DNA was blotted onto Hybond N+-XL membrane (Amersham Biochech Ltd., Piscateway, NJ).

A 1.2 Kb SPLDα fragment was used for probe synthesis, which were digested from pSPLDi. All DNA probes (50 ng) were labeled with [αP-32]dCTP (sp. ACT. 3000 *Ci*/mM) using the random priming method (Promega, WI, USA). A radioactive labeled probe was then purified by Nick column (Amersham), to remove the unlabeled [αP-32]dCTP and non-specific small fragments. The purified probe DNA was denatured at 95 ºC for 10 min and placed on ice for 10 min. The membrane was hybridized overnight with each DNA probe at 65 ºC in hybridization solution. After hybridization, the membrane was washed twice in 2X SSC with 0.1% SDS for 15 min and twice in 0.2X SSC with 0.01% SDS for 30 min at 65 ºC. The hybridizing signals were detected using the Storm840 phosphorimager (Molecular Dynamics, CA USA).

Western blot analysis and PLD enzyme assay

Proteins were extracted from immature or mature soybean seed (Zhang et al., 2003). Total native proteins were incubated at 95 ºC for 15 min with SDS-PAGE loading buffer which was 100 µl of 50 mM Tris-HCL, pH 6.8, 10 mM DTT, 2% SDS, 0.01% bromophenol blue, and 10% glycerol. Twenty micrograms of denatured proteins then were fractionated by 10% SDS-PAGE gel on 100 V until dye reached the bottom of the PAGE gel. The membrane containing denatured proteins was blotted with *Arabidopsis* PLDα1 antibodies as described by Zhang et al. (2003). Briefly, gels were transferred to an Immobilon^{TM}-P transfer membrane (Millipore, Bedford, MA) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The membranes were blocked with non-fat milk for 1 h and soaked in the first antibody overnight. The PLDα polyclonal antibody was diluted to 1:1000 in TBST (10 mM Tris, 140 mM NaCl and 0.05% Tween 20). The blots were washed in 1 X TBST 3 times, 5 min/wash. Goat anti-rabbit IgG (H+L) horseradish peroxide (HRP) conjugate (Bio-Rad) was used as the second antibody at a dilution of 1:1000 in TBST. The blots were incubated in a second antibody for 1 h and washed two times in TBST followed by one wash in TBS (10 mM Tris, 140 mM NaCl, pH 7.5). The protein bands recognized by the antibody were visualized with a HRP color development reagent (Bio-Rad).

Total protein was extracted by grinding immature and mature soybean seed with liquid N_2 chilled screw driver in 1.5 mL microcentrifuge tube with homogenization buffer containing 50 mM Tris-HCL, pH 7.,5, 10mM KCL, 1 mM EDTA, 0.5 mM phenyl

methylsulfonyl fluoride, and 2 mM DTT at 4 ^{o}C (Fan et al., 1997). Protein concentration was measured using the Nanodrop ND1000 spectrophotometer. Aliquots of 20 µg of native protein were used for the PLD enzyme assay as described previously (Fan et al., 1997). Briefly, the reaction mixture contained 100 mM MES, pH 6.5, 25 mM $CaCl_2$, 0.5 mM SDS, 1% (v/v) ethanol, and 2 mM phosphatidylcholine (egg yolk) containing dipalmitoly glycerol-3-phosphate-(methyl-3H)-choline in a final volume of 200 µl. Substrate preparation, reaction conditions, and product separation were based on the procedure used by Wang et al. (1993). The release of free head group, ^{3}H-labeled choline, into the aqueous phase was quantified using a liquid scintillation counter (Beckman).

Freezing, heat-shock treatment, and electrolyte leakage test

Six seeds of transgenic and background genotypes were germinated in 50 ml culture tubes with three replications with MS medium for each experiment (Musrashige and Skoog, 1962). Soybean seedlings were sprouted and grown in a growth chamber under a 16h photoperiod with a 25 º/19 ºC (day and night) temperature.

For the freezing treatment, 10-day-old soybean seedlings were placed at 4 ºC for 3 days to allow for cold acclimation, then the temperature was dropped from 4 to -8 ºC at 1 ºC per hour in the freezing chamber. After 2 hours at -8 ºC, three seedlings from each replication were collected for ion leakage test, and hypototyl and cotyledon from one seedling were excised for transmission electron microscopy. After the freezing treatment, the temperature was increased from -8 ºC to 25 ºC at 0.5 ºC per hour. Recovered plants were grown under 16 h-25 ºC /8 h-19 ºC (day and night) in a growth chamber for 10 days.

Control seedlings were grown under 16 h-25 ºC /8 h-19 ºC (day and night photoperiod/temperature) in a growth chamber for 23 days.

For heat-shock treatment, one-week-old soybean seedlings were exposed from 25 to 45 ºC without intermediated temperature acclimation in the growth chamber. After 2 hours at 45 ºC, three seedlings from each replication were collected for ion leakage test. After extreme heat-shock treatment, temperature was dropped from 45 ºC to 25 ºC at 5 ºC per hour. Soybean seedling were grown in 16 h-25 ºC /8 h-19 ºC (day and night) growth chamber for 10 days after heat-shock treatment. Control seedlings were grown under 16 h-25 ºC /8 h-19 ºC (day and night photoperiod/temperature) in a growth chamber for 17 days.

Electrolyte leakage from heat-shock and freezing treated three soybean seedlings from each genotype per replication was independently measured by modifying the method described by Fan et al. (1997), and three replications from each soybean line were tested. To evaluate electrolyte leakage, three replications from each soybean seedling from freezing and heat-shock stresses were tested. Briefly, soybean seedling was agitated in 3 ml of 0.4 M mannose for 6 hours to induce ion leakage. Conductivity of the solution was measured by YSI model 32 conductance meter (Scientific division, Yellow Springs Instrument Co., Inc) after vortexing the samples for 30 seconds. Total electrolytic concentration was determined after boiling samples for 15 min and cooling to 25 ºC. Electrolyte leakage was expressed as percentage of initial electrolytic concentration divided by total electrolytic concentration in the solution.

Transmission electron microscopy (TEM)

Soybean tissue preparation for TEM and image capturing were used by a modified protocol as described by Boyle and Takemoto (Boyle and Takemoto 1998). Briefly, soybean cotyledon and hypocotyl tissues were immersion fixed in 2% paraformaldehyde (Ladd, Burlington, VT), 2% glutaraldehyde and 10% sucrose in 0.1 M sodium cacodylate buffer pH6.8 for 16 hours at room temperature (RT) with constant rotation. Samples were post-fixed with 2% osmium tetroxide and 10% sucrose in 0.1 M sodium cacodylate buffer pH 6.8. Fixed tissues were dehydrated in an ascending series of acetone with 50% - 100% at RT, embedded in Spurr's embedding media in gelatin capsules and polymerized at 65 ºC for 24 hours. Embedded soybean tissues were cut with glass knives on a Reichert Ultracut S microtome (Leica, Austria) and held on 100 nickel slotted grids. All images were captured using an Advanced Microscopy Techniques digital image capturing system (ATM, Chazy, NY).

Major fatty acids analysis by gas chromatography

A 50 mg sample of clean T_3 seed is prepared by taking an equal volume or weight of seed from three replications. Seed samples were submitted to the USDA-ARS National Center for Agricultural Utilization Research, Peoria, Illinois. Fatty acid composition was analyzed using a gas chromatography modified procedure from Christie (1989). Results from the fatty acid analyses were expressed as percentage (%) of the total fatty acid in the grain.

Statistical analysis

Data collected from PLD enzyme assay, electrolyte leakage, and fatty acid analyses were examined by analysis of variance using SAS (SAS Institute Inc., Cary, NC) PROC general liner model (GLM) procedure. Means was separated using either LSD or Tukey tests at the level of $p<0.05$ Probability.

Results

Soybean transformation and southern blot analysis of PLD transgene of J- and T-lines

The combinations of pSPLDanti + pHG1 and p35SSPLDanti + pHG1 were co-bombarded into soybean cultivar Jack via particle inflow gun. Six and 7 events of fertile transgenic soybeans were recovered from pSPLDanti (J-line) and p35SSPLDanti (T-line), respectively. Hygromycin (100mg/L) was used as a selecting agent during the tissue culture and regeneration, and PCR analysis for the presence of HPT and SPLDanti transgenes through regeneration process and all transgenic soybean lines. PCR was performed with primers amplifying from the 5' end of PLDα antisense region to the 5' end of ß-conglycinin terminator due to the presence of endogenous PLDα in soybean genome. Amplicons from PCR reactions were of the predicted size from each of the transgenic events. All T_0 individual transgenic lines containing pSPLDanti+pHG1 were named using the prefix "J" and lines containing p35SSpldanti+pHG1 were named using the prefix "T". Homozygous transgenic lines were selected by both SPLDanti and HPT transgene PCR using T_2 immature cotyledon.

Southern blot analysis was used to detect the presence of transgenes and identify the copy number and integration pattern of exogenous PLD. DNA blotting restricted by single cut enzyme, *Xba*I, showed two and one transgene bands from T_1 TI and TH positive lines respectively (Fig. 4.2). Southern blot analysis of p35SSPLDi transgene was consistent with PCR results. A single copy of the pSPLDanti transgene in JU was confirmed by southern

blotting of the T_2 transgenic line (Fig 2.3B in chapter 2). Homogenous T_2 transgenic lines were selected based on both gene of interest and HPT PCR screening. The results indicated that the transgenes were stably inherited to the T_2 generation.

Changes of PLDα protein, activity, and fatty acid profile in T_3 transgenic seeds

The lower PLDα activity in the T_2 TI immature cotyledon was correlated with decreased amounts of PLDα protein (Fig. 4.3). These data suggested that the expression of antisense PLDα under control of CaMV35S promoter suppressed PLDα activity in transgenic seeds.

Changes of fatty acid composition were noted in T_3 pSPLDanti and p35SSPLDanti transgenic seeds (Table 4.1). TH line exhibited lower linoleic, palmitic, linolenic acid and higher oleic acid content than background cultivar. In contrast, the TI progeny had an oleic acid content of 21%, but it was not significantly lower than the check, Jack.

Freezing and heat-shock tolerance of PLDα-suppressed soybean

Sprouted soybean seedling from three T_2 pSPLDanti and three T_2 p35SSPLDanti transgenic lines were tested for heat-shock and freezing tolerance. Welti et al. (2002) reported that cold acclimation at 4 ºC for 3 days led to an increase in PLDα mRNA in wild-type *Arabidopsis*, subsequently elevating PLDα protein and activity. The freezing tolerance in transgenic and background soybean was characterized by membrane electrolyte leakage and survival and growth after recovery (Fig. 4.4). No Jack seedlings survived after freezing treatment. Approximately 42% electrolyte was leaked in Jack seedling. Few survivals were observed in PLDα-suppressed seedling after 10 days. On the other hand, all transgenic

seedling exhibited more tolerance to membrane leakage than background cultivar, ranged 13 to 20% electrolyte leakage.

To make comparisons between the heat-shock treated (25 ºC → 45 ºC) seedling and control (25 ºC), electrolyte leakage and growth after heat-shock treatment were measured (Fig. 4.5). Membrane leakage after heat-shock treatment was increased 2 to 4 percent in both transgenic and background seedlings. The heat-shock stress resulted in little damage to the plasma membrane. The growth of Jack seedlings had been stopped until 10 days after heat-shock, whereas all transgenic seedlings showed retarded growth. These result imply that PLDα is involved in heat induced stress. Johnston et al. reported that PLD activities were significantly increased after heat shock (Johnston et al., 2007).

Ultrastructural changes in PLDα-suppressed soybean seedling by freeze-induced stress

Transgenic and background seedlings being at -8 ºC for 2 hours were investigated for cellular changes. Obvious changes of cell structure by freezing treatment were observed in Fayette seedling cotyledon and hypocotyl cells (Fig. 4.6A to H). Freeze-damaged cotyledon and hypocotyls cell exhibited the disruption of plasma membrane and cell organelles. On the other hand, transgenic seedling, TI and JU, showed that the structure of plasma membrane and other cell organelles such as vacuole, lipid bodies, and protein bodies were less damaged than counterparts of background cultivar by freezing stress (Fig. 4.6 I to P). This result suggested that PLDα activity is involved in freezing-induced damages by degrading membrane phospholipid and ROS-mediated protein degradation.

Discussion

Fertile transgenic soybean plants were successfully developed to express antisense RNA homologous to soybean PLDα. The pSPLDanti and p35SSPLD transgenes inherited to the T_3 generation, suppressed the levels of PLDα protein and enzyme activity. Two transgenic lines, TI and TH, showed two and one transgene bands, respectively. Compared to the copy number of transgenes in pSPLDi transgenic, these events expressed relatively low copy number transgene. Particle bombardment has enabled soybean to be efficiently transformed with less genotypic limitations than *Agrobacterium*-mediated transformation, however, particle bombardment tends to produce transgenic soybean possessing numerous copies of transgene. The transgene co-suppression has been referred as lost in expression through several generations (Vaucheret et al., 1998; Henikoff, 1998). Multiple transgene integration with direct or indirect repeat results in the transgene silencing by methylation of the posttranscriptional gene silencing (PTGS) and transcriptional gene silencing (TGS). Previous research proposed that Agrobacterium-mediated transformation producing single or double copies of transgene is more stably inherited than multi copy transgenes by bombardment-mediated transformation (Chilton, 1993). Based on the southern blot analysis of TI and TH lines, single or double copies of transgene integration may be achieved by particle inflow gun bombardment with lower concentration of plant expression vector, p35SSPLDi (Fig 4.2). Adjusting the vector concentration can lead to fewer transgenic copies even by direct DNA transfer methods.

Results from the western blot and PLDα enzyme assays indicated endogenous PLDα enzyme activity in TI was suppressed by 25% compared to the background cultivar

(Fig 4.3). We produced transgenic soybean lines which attenuated PLDα activity from 80 to 20% by antisense and RNAi suppression. However, no PLDα-knockout soybean lines were observed from fifteen independent events.

Composition of four major fatty acids in some transgenic soybean lines was significantly different from that of background cultivar (Table 4.1). These results suggest that PLDα may be partially involved in fatty acid compositional changes in soybean seeds.

Membrane structure is damaged by freezing, and freezing damage leads to dehydration of the cell. The stability of membrane was characterized by electrolyte leakage and observation of the plasma membrane after freezing treatment. PLDα-suppressed soybean showed that plasma membrane and cell organelle membrane were maintained after freezing treatment. The plasma membrane of PLDα-suppressed soybean seedlings showed less electrolyte leakage than that of background genotype (Fig 4.4 and 4.6).

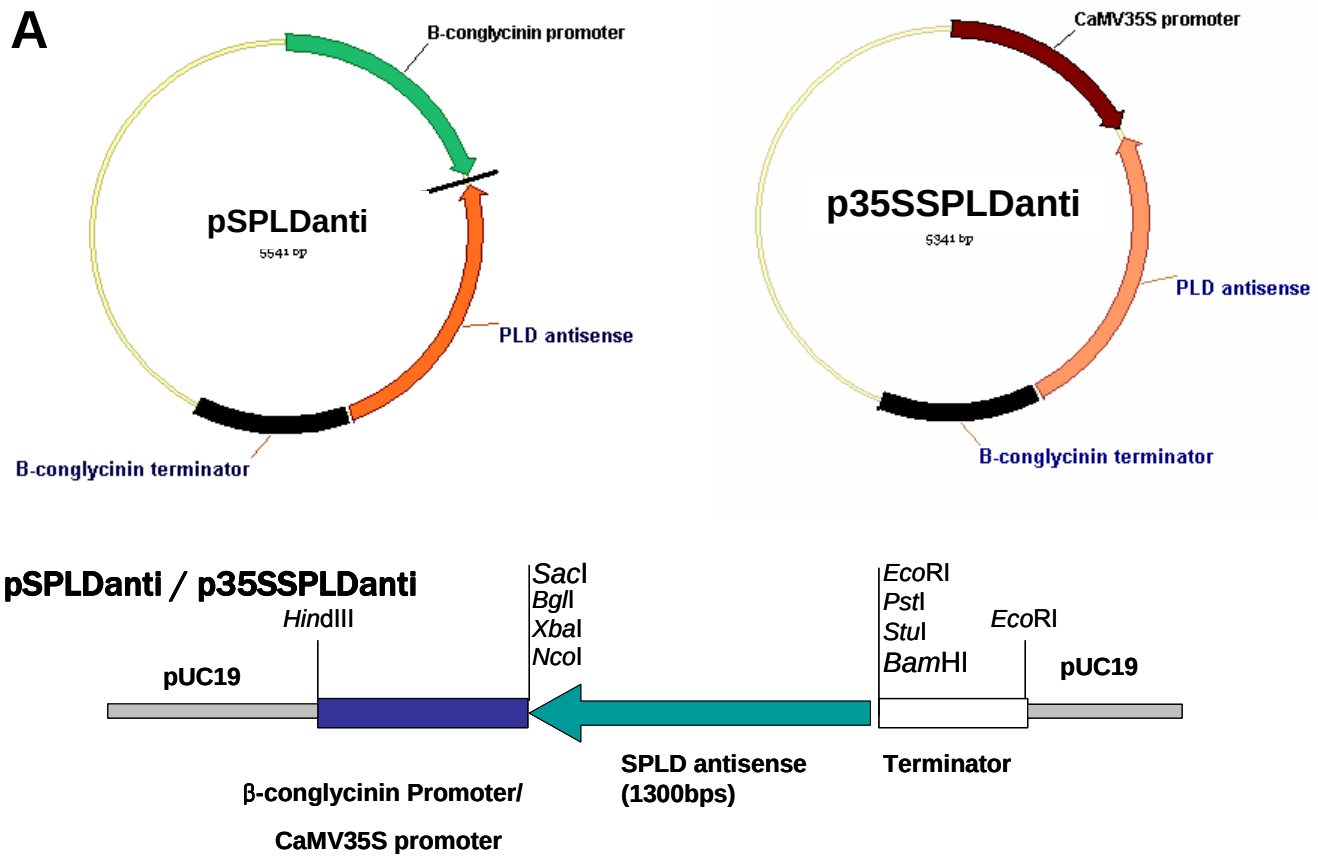
Heat shock stresses cause decline in relative growth rate and shoot dry weight in the early growth stages of maize and pearl millet (Ashraf and Hafeez, 2004). Early growth of PLDα-attenuated and background cultivars responded differently to heat shock stress. However, there was no major difference in electrolyte leakage after heat shock between the control and transgenic seedlings. The growth of the control seedlings was stopped after treatment, whereas all transgenic seedlings continued to grow, although at a retarded rate. The heat-shock stress resulted in little damage to the plasma membrane. Heat stress has been shown to loosen the chemical bonds of membrane molecules thereby increasing the fluidity of the biological membrane (Savchenko et al., 2002). The electrolyte leakage was, as an indication of cell membrane permeability, maintained after heat challenge in soybean

seedlings (Fig 4.5). Devaiah et al. (2007) suggested that PLDα-suppressed and –knockout plants showed a decrease in unsaturated fatty acids in lipid bilayer of *Arabidopsis*. PLDα-attenuated seedlings may be less sensitive to heat stress by preserving membrane fluidity and permeability.

Previous research demonstrated that membrane phospholipids degradation, oxidative damage, and seed deterioration were promoted by higher levels of PLDα activity (Samama and Pearce, 1993). Based on this study, PLDα-attenuated soybean might provide heat-shock tolerance to seedlings by reducing oxidative stresses. Moreover, PLDα-suppressed soybean seedlings demonstrated enhanced freezing tolerance by stabilizing the plasma membrane during cold acclimation.

Figures and Tables

Figure 4.1 Schematic diagram of constructing vector used for transformation. (A); pSPLDanti and p35SSPLDanti expression cassette containing PLD antisense sequence and β-conglycinin terminator under control of β-conglycinin promoter and CaMV 35S promoter respectively. (B); pHG1 expression vector comprised of hygromycin phosphotransferase cassette and β-glucuronidase cassette under control of CaMV 35S promoter.



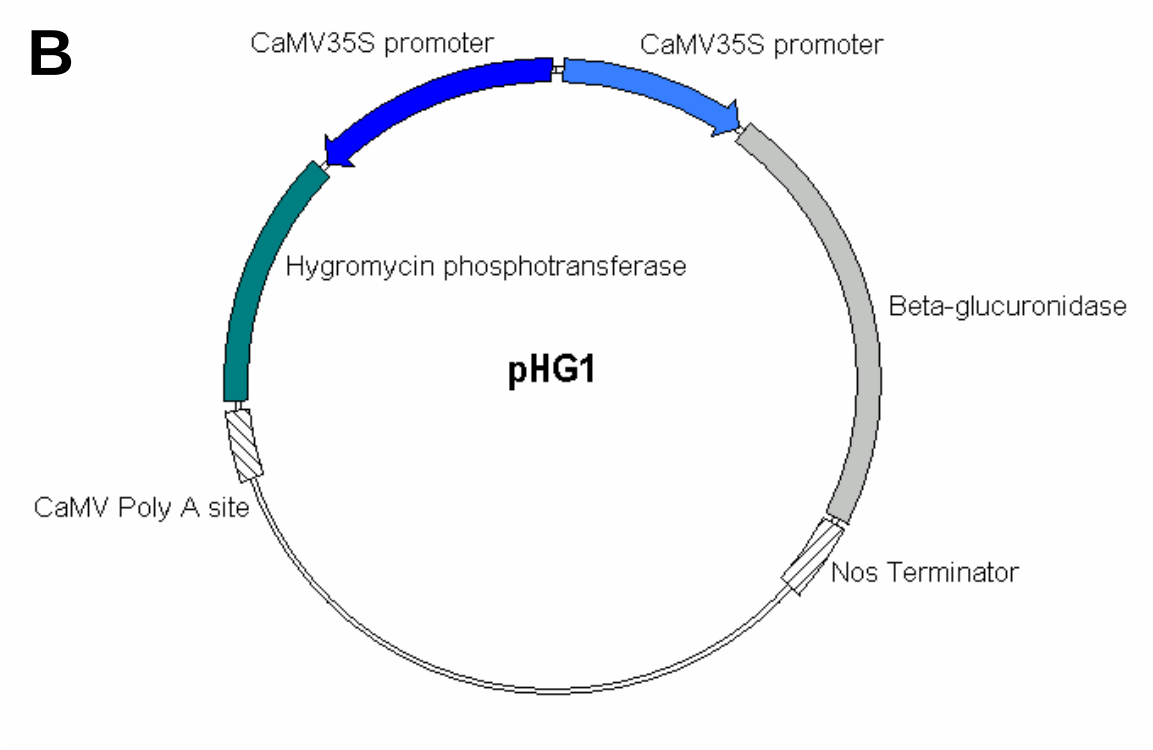

B
CaMV35S promoter
CaMV35S promoter
Hygromycin phosphotransferase
Beta-glucuronidase
pHG1
CaMV Poly A site
Nos Terminator

Figure 4.2 Southern blot analysis of p35SSPLDanti T_1 soybean lines and background cultivar Jack. Fifteen ug of genomic DNA was digested with *Xba*I and hybridized with PLDα probe. Left panel showed that number is based on kilo base pair corresponding to each size of molecular marker ladder (1Kb ladder). Bands in the box exhibit transgene band patterns in TI + and TH + lines. PCR positive (+) and negative (-) in transgenic lines were indicated by + or – after transgenic name.

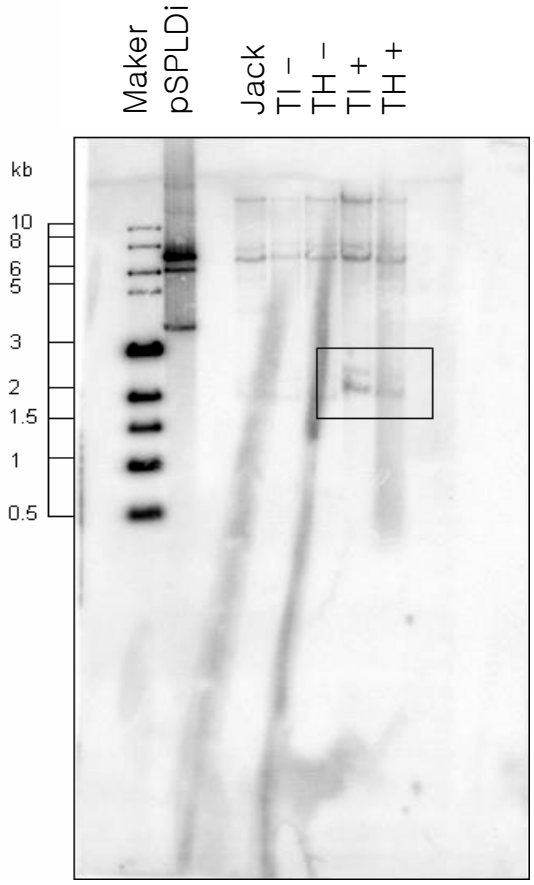


Figure 4.3 Changes in PLDα protein and enzyme activity in mature cotyledons from T_2 TI and background cultivar Jack. (A); PLDα enzyme assay was expressed nmoles of phosphatidylcholine min^{-1} mg^{-1} total protein.* indicates significant difference of mean by LSD test (α=0.05). (B); Immunoblot analysis of cytosolic protein from mature cotyledon was performed with polyclonal anti-*Arabidopsis* PLDα antibody. Proteins (40 μg per lane) were separated in SDS-PAGE in 12% polyacylamide gel and PLDα protein then was visualized by alkaline phosphatase.

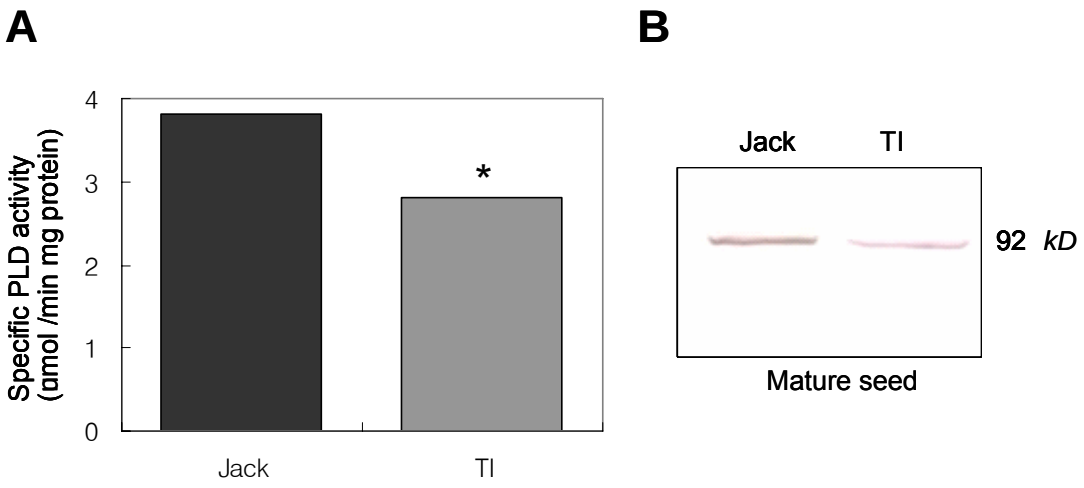


Figure 4.4 Acquired freezing tolerance by PLDα-suppression in soybean seedlings. Soybean seedling of six T_2 transgenic lines were exposed to freezing temperature (-8 ^{o}C) after cold acclimation for 3 days. (A); Changes in electrolyte leakage in pSPLDanti and p35SSPLDanti transgenic seedling after being at - 8 ^{o}C for 2 hours and non-treated. Error bar means ±LSD (α=0.05). (B); Increased freezing tolerance in transgenic soybean seedlings. Six-day-old soybean seedling had been placed - 8 ^{o}C for 2 hours by dropping 0.5 ^{o}C/ 1 hour after cold acclimation for 3 days, and recovered by increasing 1 ^{o}C/ 1 hour to 25 ^{o}C, then grown for 10 days in 25 ^{o}C growth chamber. PLDα-suppressed seedlings, TD and TI, survived after temperature recovery.

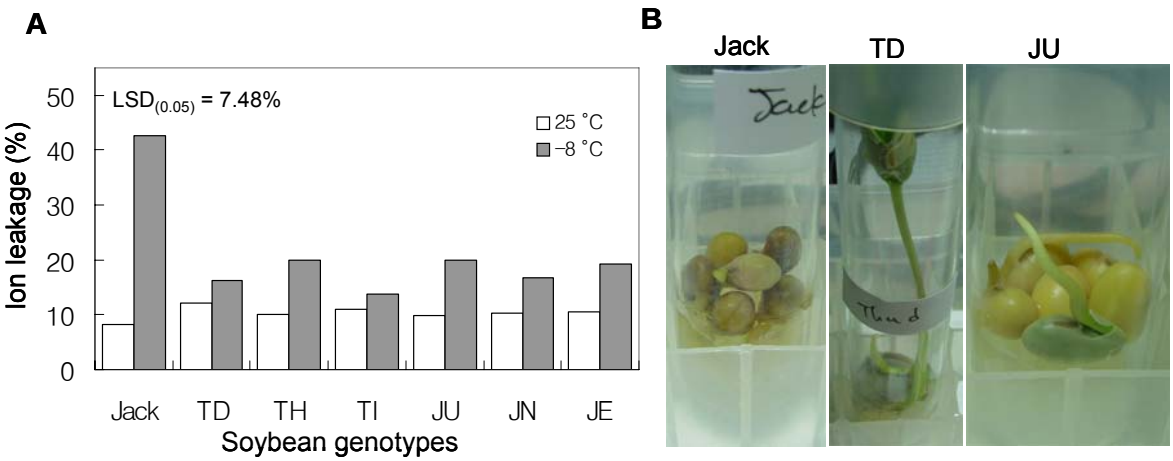


Figure 4.5 Enhanced heat-shock tolerance in PLDα-suppressed soybean seedlings. Soybean seedlings of six T_2 transgenic lines were challenged to heat stress at 45 ^{o}C without acclimation at higher temperature. (A); Changes in electrolyte leakage after heat-shock treatment in transgenic and Jack seedlings. Error bar means ±LSD (α=0.05). (B); Increased heat-shock tolerance in transgenic soybean seedlings. One-week-old soybean seedlings had been placed 42 ^{o}C for 2 hours without acclimation at higher temperature, and then grown for 10 days in 25 ^{o}C growth chamber.

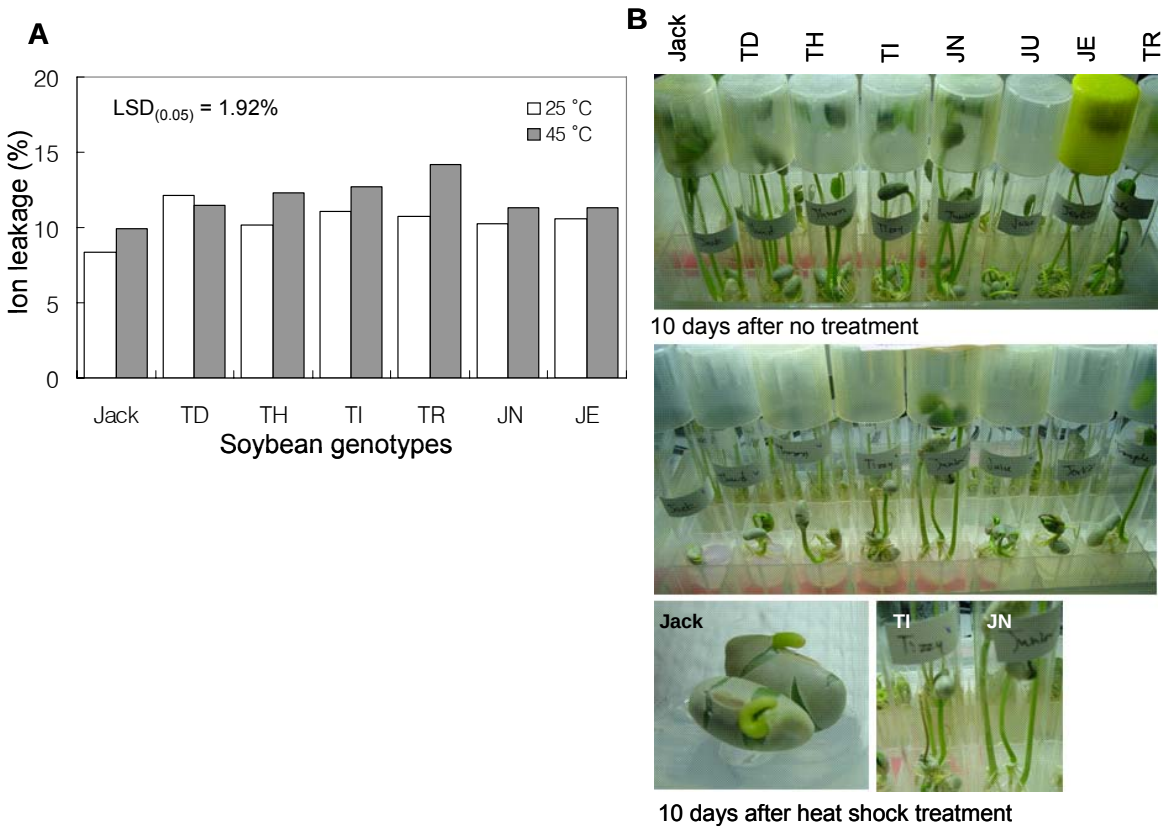


Figure 4.6 Ultrastructure of cotyledon and hypocotyl cells from freezing treated background soybean (Fayette) and transgenic soybean lines. Transmission electron microscopy showed Fayette cotyledon (A and B), Fayette hypocotyl (C and D), freezing treated Fayette cotyledon (E and F), freezing treated Fayette hypocotyls (G and H), freezing treated TI (p35SSPLDanti) cotyledon (I and J), freezing treated TI (p35SSPLDanti) hypocotyls (K and L), freezing treated JN (pSPLDanti) cotyledon (M and N), and freezing treated JN (pSPLDanti) hypocotyls (O and P) tissues.

A; Cross section of cotyledon cells Fayette with transmission electron microscopy.

B; Closed view of cell wall complex from Fayette cotyledon with transmission electron microscopy.

C; Cross section of hypocotyl cells from Fayette with transmission electron microscopy.

D; Closed view of cell wall complex from Fayette hypocotyl with transmission electron microscopy.

E; Cross section of cotyledon cells from freezing treated Fayette with transmission electron microscopy.

F; Closed view of cell wall complex from freezing treated Fayette cotyledon with transmission electron microscopy.

G; Cross section of hypocotyl cells from freezing treated Fayette with transmission electron microscopy.

H; Closed view of cell wall complex from freezing treated Fayette hypocotyl with transmission electron microscopy.

I; Cross section of cotyledon cells from freezing treated TI (p35SSPLDanti) with transmission electron microscopy.

J; Closed view of cell wall complex from freezing treated TI (p35SSPLDanti) cotyledon with transmission electron microscopy.

K; Cross section of hypocotyl cells from freezing treated TI (p35SSPLDanti) with transmission electron microscopy.

L; Closed view of cell wall complex from freezing treated TI (p35SSPLDanti) hypocotyl with transmission electron microscopy

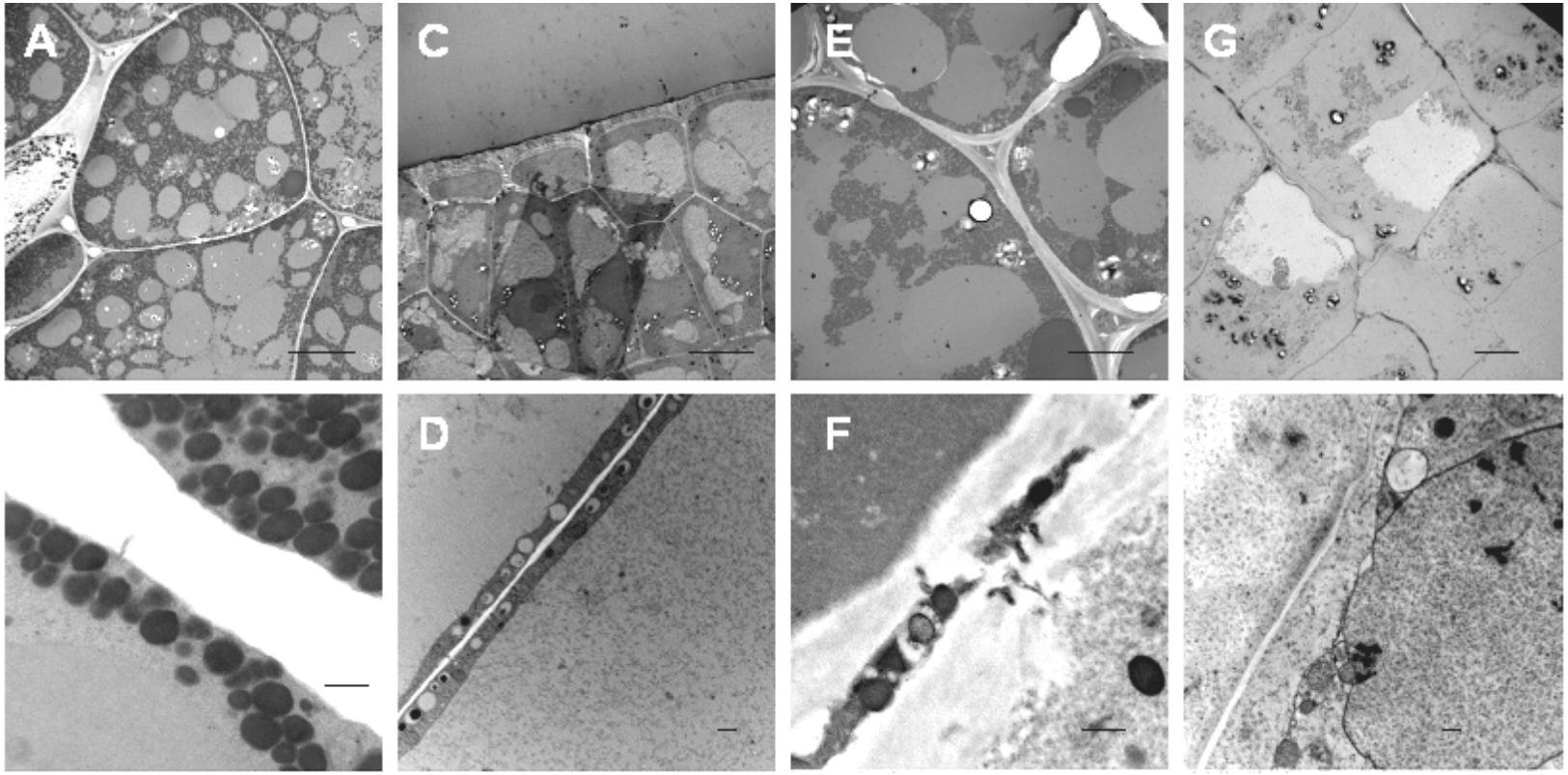
M; Cross section of cotyledon cells from freezing treated JN (pSPLDanti) with transmission electron microscopy.

N; Closed view of cell wall complex from freezing treated JN (pSPLDanti) cotyledon with transmission electron microscopy.

O; Cross section of hypocotyl cells from freezing treated JN (pSPLDanti) with transmission electron microscopy.

P; Closed view of cell wall complex from freezing treated JN (pSPLDanti) hypocotyl with transmission electron microscopy

Bars in A, C, E, G, I, K, M, and O = 10 μm. Bars in B, D, F, H, J, L, N, and O = 0.5 μm.


A
C
E
G
D
F

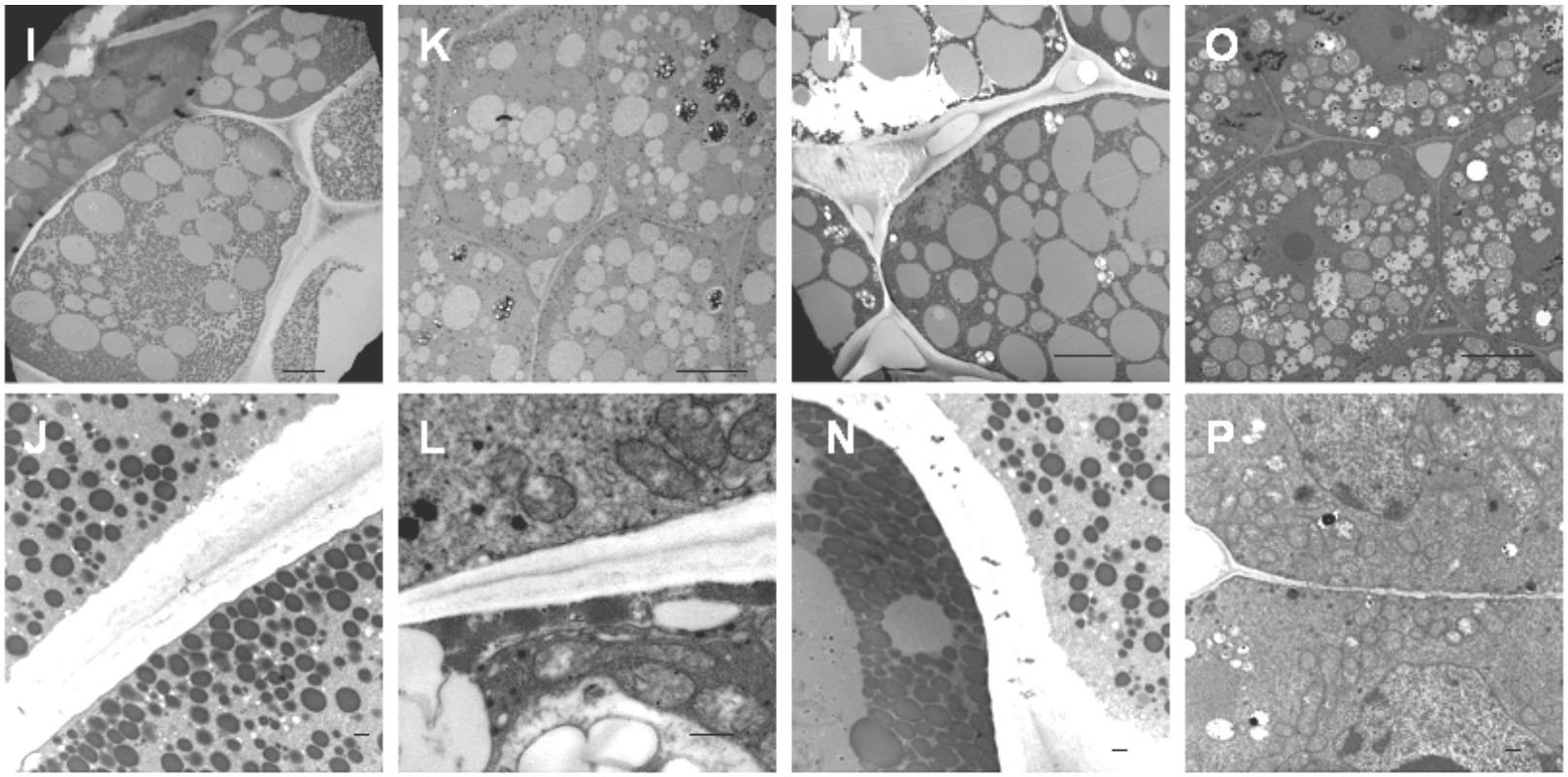

I
K
M
O
J
L
N
P

Table 4.1 Fatty acid analysis of T_3 pSPLDanti and p35SSPLDanti transgenic soybean seeds from greenhouse in 2006-2007 winter nursery.

Entries[1]	Major fatty acid composition (%)[2]				
	Palmitic acid	**Stearic acid**	**Oleic acid**	**Linoleic acid**	**Linolenic acid**
Jack	11.3a	3.8ab	25.1bc	52.4ab	7.5ab
TH[a]	9.3b	3.2b	47.7a	34.3c	5.5c
TR[a]	10.7ab	2.9b	28.8bc	50.7ab	6.9bc
TI[a]	11.4a	2.9b	20.9c	56.1a	8.7a
JU[b]	10.2ab	3.1b	37.9ab	42.3bc	6.5bc
JN[b]	10.9ab	4.5a	33.2bc	45.2bc	6.1c

Tukey grouping at the α=0.05 level

[1]Nomenclature reflects transgenic lines and transgenic events

[2]Five major fatty acid content indicates palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3)

[a]Transgenic lines contain p35SSPLDanti construct

[b]Transgenic lines contain pSPLDanti construct

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Appendix A- Supplementary Data

Figure A.1 Different leaf senescence observed in T_4 transgenic segregating line, SI3-8-3-3. The presence of pSPLDi transgene in individual plants was confirmed by PCR analysis. Leaf senescence in PCR positive plants was delayed for 6 to 8 days compared to PCR negative individuals.

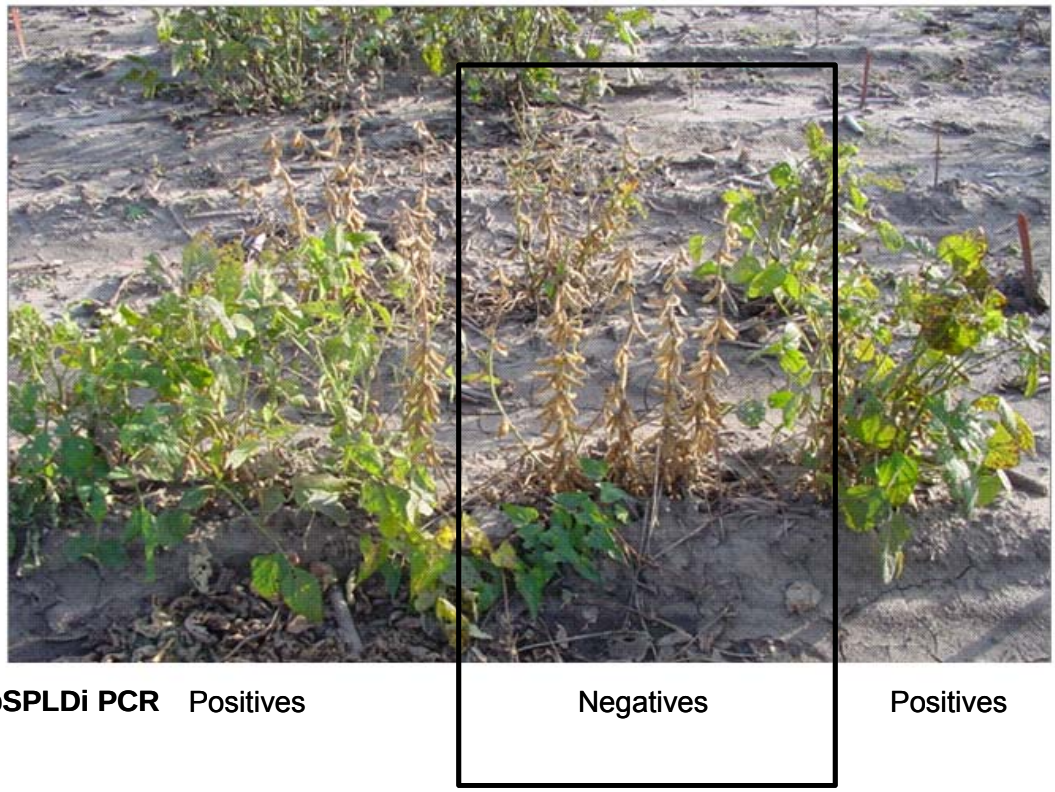

Table A.1 Molecular analysis of transgenic soybean analyzed in this study**.**

Name[1]	Plasmids[2]	Hyg[3]	G. O. I.[4]	S.B.[5]	Cultivar[6]
65 Gnome	pSPLDi:pHGI	-	n.a.[7]	n.a.	Jack01E8
69 Dwarf	pSPLDi:pHGI	+/-	n.a.	n.a.	Jack01E8
71 Sophia	pSPLDi:pHGI	-	n.a.	n.a.	Jack01E8
71 Silence	pSPLDi:pHGI	n.a.	n.a.	n.a.	Flyer01MM8
71 Salome	pSPLDi:pHGI	-	n.a.	n.a.	Jack01E8
72 Sizzle	pSPLDi:pHGI	+/-	n.a.	n.a.	Jack01E8
72 Swelter	pSPLDi:pHGI	-	n.a.	n.a.	Jack01E8
72 Slumber	pSPLDi:pHGI	-	n.a.	n.a.	Jack01E8
72 Splash	pSPLDi:pHGI	-	n.a.	n.a.	Fayette01MM9
72 Silo	pSPLDi:pHGI	-	n.a.	n.a.	Fayette01MM9
72 Swami	pSPLDi:pHGI	-	n.a.	n.a.	Fayette01MM9
72 Summer	pSPLDi:pHGI	-	n.a.	n.a.	Fayette01MM9
78 Star	pSPLDi:pHGI	-	n.a.	n.a.	Jack01MM9
78 Sky	pSPLDi:pHGI	-	n.a.	n.a.	Chap01E9
78 Sunny	pSPLDi:pHGI	-	n.a.	n.a.	**Flyer01MM8**
78 Sorbet	pSPLDi:pHGI	-	n.a.	n.a.	Chap01E9
78 Slow	pSPLDi:pHGI	-	n.a.	n.a.	Chap01E9
78 Spade	pSPLDi:pHGI	+/-	n.a.	n.a.	Chap01E9
78 Symphony	pSPLDi:pHGI	-,+/-	n.a.	n.a.	Jack01MM9
78 Sonya	pSPLDi:pHGI	-	n.a.	n.a.	Jack01E8
78 Swine	pSPLDi:pHGI	-	n.a.	n.a.	Jack01E8
78 Simian	pSPLDi:pHGI	-	n.a.	n.a.	Jack01E8
78 Sow	pSPLDi:pHGI	-	n.a.	n.a.	Jack01E8
78 Super	pSPLDi:pHGI	-	n.a.	n.a.	Jack01MM9
78 Sofa	pSPLDi:pHGI	-	n.a.	n.a.	Jack01MM9
78 Sigma	pSPLDi:pHGI	-	n.a.	n.a.	Jack01MM9
78 Socks	pSPLDi:pHGI	-	n.a.	n.a.	Jack01MM9
78 Slyer	pSPLDi:pHGI	-	n.a.	n.a.	Flyer01MM8
78 Strubbi	pSPLDi:pHGI	-	n.a.	n.a.	Jack01MM9
78 Shelly	pSPLDi:pHGI	-	n.a.	n.a.	Chap01E9
78 Sherry	pSPLDi:pHGI	-	n.a.	n.a.	Chap01E9
78 Swinney	pSPLDi:pHGI	-	n.a.	n.a.	Chap01E9
78 Savory	pSPLDi:pHGI	-	n.a.	n.a.	Jack01E8
78 Saloon	pSPLDi:pHGI	-	n.a.	n.a.	Jack01E8
86 Sag	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
86 Squirt	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
86 Salute	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
86 Slide	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
86 Soup	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
86 Score	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
86 Secret	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
86 Skip	pSPLDi:pHGI	-	n.a.	n.a.	Flyer02MM3
86 Spoil	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
86 Swim	**pSPLDi:pHGI**	**+**	**+**	**+**	**Fayette02E3**
86 Slurry	pSPLDi:pHGI	**-**	n.a.	n.a.	Jack02MM2
86 Sump	pSPLDi:pHGI	**-**	n.a.	n.a.	Jack02MM2
86 Stash	pSPLDi:pHGI	**-**	n.a.	n.a.	
86 Singer	**pSPLDi:pHGI**	**+**	**+**	**+**	**Flyer02MM3**
86 Siemens	pSPLDi:pHGI	**+**	**-**	n.a.	**Flyer02MM3**
86 Syngenta	pSPLDi:pHGI	**-**	n.a.	n.a.	Flyer02MM3

Table A. 1 Molecular analysis of transgenic soybean analyzed in this study.

(Continued)

Name[1]	Plasmids[2]	Hyg[3]	G. O. I.[4]	S.B.[5]	Cultivar[6]
86 Suzuki	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
86 SBC	pSPLDi:pHGI	-	n.a.	n.a.	Flyer02MM3
86 Sanka	pSPLDi:pHGI	-	n.a.	n.a.	Flyer02MM3
86 Sam's	pSPLDi:pHGI	+	-	n.a.	**Flyer02MM3**
86 Scholls	pSPLDi:pHGI	-	n.a.	n.a.	Flyer02MM3
86 Sunbeam	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
86 7up	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
86 Subaru	pSPLDi:pHGI	-	n.a.	n.a.	Jack02MM2
102 Saffron	pSPLDi:pHG1	-	-	n.a.	Jack03E4
102 Salsa	pSPLDi:pHG1	-	-	n.a.	Jack03E4
102 Sarong	pSPLDi:pHG1	-	-	n.a.	Jack03E4
102 Saunter	pSPLDi:pHG1	-	n.a.	n.a.	Jack03E4
102 Saga	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
102 Salami	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
102 Sabbath	pSPLDi:pHG1	-	-	n.a.	Jack03E4
102 Sash	pSPLDi:pHG1	-	-	n.a.	Flyer03MM4
102 Satyr	pSPLDi:pHG1	-	-	n.a.	Flyer03MM4
102 Sachet	pSPLDi:pHG1	-	n.a.	n.a.	Jack03E4
102 Straw	pSPLDi:pHG1	-	-	n.a.	Jack03E4
102 Sip	pSPLDi:pHG1	-	-	n.a.	Jack03E4
102 Strato	pSPLDi:pHG1	+	-	n.a.	**Jack03MM4**
102 Sphere	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
102 Skirt	pSPLDi:pHG1	-	-	n.a.	Jack03E4
102 Sink	pSPLDi:pHG1	-	-	n.a.	Jack03E4
102 Satire	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
102 Safari	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
102 Saccharin	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
102 Sift	pSPLDi:pHG1	+/-	-	n.a.	Jack03MM4
102 Scald	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
102 Scour	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
102 Slap	pSPLDi:pHG1	+/-	-	n.a.	Jack03E4
102 Six	pSPLDi:pHG1	+/-	-	n.a.	Jack03E4
102 Sky	pSPLDi:pHG1	+	-	n.a.	**Jack03MM4**
102 Scarf	pSPLDi:pHG1	+/-	-	n.a.	Flyer03MM4
102 Scrap	pSPLDi:pHG1	+/-	-	n.a.	Flyer03MM4
102 Scram	pSPLDi:pHG1	+/-	-	n.a.	Flyer03MM4
102 Scrawl	pSPLDi:pHG1	-	-	n.a.	Flyer03MM4
102 Salad	pSPLDi:pHG1	+/-	-	n.a.	Flyer03MM4
102 Stanza	pSPLDi:pHG1	-	-	n.a.	Flyer03MM4
102 Saute	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
102 Supper	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
102 Scab	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
102 Seep	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
102 Sail	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
102 Saddle	**pSPLDi:pHG1**	+	n.a.	n.a.	**?03MM4**
102 Sauna	pSPLDi:pHG1	-	n.a.	n.a.	Jack03E4
102 Sojourn	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4

Table A. 1 Molecular analysis of transgenic soybean analyzed in this study.

(Continued)

Name[1]	Plasmids[2]	Hyg[3]	G. O. I.[4]	S.B.[5]	Cultivar[6]
102 Salver	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
102 Socialism	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
102 Safflower	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
102 Sacrifice	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
108 Satin	pSPLDi:pHGI	-	n.a.	n.a.	Jack03MM4
108 Salient	**pSPLDi:pHGI**	**+**	n.a.	n.a.	**Jack03MM4**
108 Slack	pSPLDi:pHGI	-	n.a.	n.a.	Jack03MM4
108 Simmer	pSPLDi:pHGI	-	n.a.	n.a.	Jack03MM4
108 Slushie	pSPLDi:pHGI	-	n.a.	n.a.	Jack03MM5
108 Snowman	pSPLDi:pHGI	-	n.a.	n.a.	Jack03MM5
108 Snowman	pSPLDi:pHGI	-	n.a.	n.a.	Jack03MM5
111 A Sybill	pSPLDi:pHG1	-	-	n.a.	Jack03E4
111 A Stinky	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03E4
111 A Sappy	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03E4
111 B Swish	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
111 B Squash	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
111 B Spud	**pSPLDi:pHG1**	**+/-**	-	n.a.	Jack03MM4
111 C Sweetie	**pSPLDi:pHG1**	**+**	**+**	n.a.	**Jack03MM4**
111 C Slowly	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
111 C Sponge	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
111 B Sappy	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
111 C Sardine	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
111 D Sunset	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
111 D Stockdale	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03MM4
111 E Shrub	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03MM4
111 E Sumac	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03MM4
111 E Spike	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03MM4
111 F Shield	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03MM4
111 F Sandy	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03MM4
111 F Sue	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03MM4
111 F Satchel	pSPLDi:pHG1	**+**	**-**	n.a.	**Jack03MM4**
111 F Sack	pSPLDi:pHG1	-	-	n.a.	Jack03MM4
111 F Sophie	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM4
111 Slide	**pSPLDi:pHG1**	**+**	**+**	n.a.	**Jack03MM5**
111 Shine	pSPLDi:pHG1	-	-	n.a.	Jack03MM5
111 Shame	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03MM5
111 Sympathy	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03MM5
111 Scream	pSPLDi:pHG1	n.a.	n.a.	n.a.	Jack03MM5
111 Salt 'n Pepper	**pSPLDi:pHG1**	**+**	**+**	n.a.	**Jack03E4**
111 Sonny	pSPLDi:pHG1	**+**	-	n.a.	**Jack03E4**
111 Selena	pSPLDi:pHG1	-	-	n.a.	Jack03E4
111 Springstein	pSPLDi:pHG1	-	-	n.a.	Jack03E4
111 Snoop	pSPLDi:pHG1	-	n.a.	n.a.	Jack03E4
111 Sable	pSPLDi:pHG1	-	n.a.	n.a.	Jack03E4
111 Suzy-Q	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM5
111 Sage	pSPLDi:pHG1	-	n.a.	n.a.	Jack03MM5
115 Jupiter	pSPLDanti:pHG1	n.a.	n.a.	n.a.	Jack03W5

Table A. 1 Molecular analysis of transgenic soybean analyzed in this study.

(Continued)

Name[1]	Plasmids[2]	Hyg[3]	G. O. I.[4]	S.B.[5]	Cultivar[6]
115 Jinx	pSPLDanti:pHG1	n.a.	n.a.	n.a.	Jack03W5
115 Junior	**pSPLDanti:pHG1**	**+**	**+**	**+**	**Jack03MM5**
115 Jackson	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM5
115 Jingle	**pSPLDanti:pHG1**	**+**	**+/-**	n.a.	**Jack03MM5**
115 Juice	**pSPLDanti:pHG1**	**+**	**+**	**+**	**Jack03MM5**
115 Jam	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03W5
115 Jackal	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03W5
115 Jackpot	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03W5
115 Jelly	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03W5
115 Jade	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03W5
115 Joke	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03W5
115 Josh	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03W5
115 Jim	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Joyce	**pSPLDanti:pHG1**	**+**	**+**	n.a.	**Jack03MM4**
115 Jackie	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 John	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Julia	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Jazz	**pSPLDanti:pHG1**	**+**	**+**	n.a.	**Jack03W5**
115 Juniper	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03W5
115 Jasmine	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03W5
115 Junction	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03E4
115 Jaywalk	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03E4
115 Jitters	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM5
115 Journey	**pSPLDanti:pHG1**	**+**	**+**	n.a.	**Jack03MM5**
115 Jerk	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Jet	pSPLDanti:pHG1	**+/-**	n.a.	n.a.	**Jack03MM4**
115 Julep	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Jocund	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Jacknife	pSPLDanti:pHG1	**+**	**-**	n.a.	**Jack03E4**
115 Jump-start	pSPLDanti:pHG1	**+**	**-**	n.a.	**Jack03E4**
115 Jug	pSPLDanti:pHG1	**+**	**-**	n.a.	Jack03MM4
115 Judo	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Juggle	pSPLDanti:pHG1	**+**	-	n.a.	**Jack03MM4**
115 Judge	**pSPLDanti:pHG1**	**+**	**+**	n.a.	**Jack03MM5**
115 Jury	**pSPLDanti:pHG1**	**+**	**+**	n.a.	**Jack03MM5**
115 Jungle	**pSPLDanti:pHG1**	**+**	**+**	n.a.	**Jack03MM5**
115 Judicial	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM5
115 Judgement	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM5
115 Jumble	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Jog	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03W5
115 Jaunt	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03W5
115 Jacob	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Junta	pSPLDanti:pHG1	-	n.a.	n.a.	Flyer03MM4
115 Jujube	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM5
115 Junk	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM5
115 Jay	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM5

Table A. 1 Molecular analysis of transgenic soybean analyzed in this study**.**

(Continued)

Name[1]	Plasmids[2]	Hyg[3]	G. O. I.[4]	S.B.[5]	Cultivar[6]
115 Jaw	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM5
115 Jabber	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM5
115 Jilt	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Jib	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Jargon	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Jettison	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Jigsaw	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
115 Jubilee	pSPLDanti:pHG1	-	n.a.	n.a.	Jack03MM4
116 Jot	pSPLDanti:pHGI	-	n.a.	n.a.	Chap03E5
116 Jodhpurs	pSPLDanti:pHGI	-	n.a.	n.a.	Chap03E5
116 Jingoism	pSPLDanti:pHGI	-	n.a.	n.a.	Chap03E5
117 Taffy	p35SSPLDanti:pHG1	-	n.a.	n.a.	Jack04E1
117 Tactic	p35SSPLDanti:pHG1	-	n.a.	n.a.	Jack04E1
117 Teamster	p35SSPLDanti:pHG1	-	n.a.	n.a.	Jack04E1
117 Tepee	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Test	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Transit	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Trawl	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Tress	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Tower	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Trite	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Tractor	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Tinsel	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Talon	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Twitch	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Twit	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Tandem	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Traipse	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Tivoli	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
117 Tetanus	p35SSPLDanti:pHG1	-	n.a.	n.a.	Jack04E1
117 Tenpin	p35SSPLDanti:pHG1	-	n.a.	n.a.	Jack04E1
117 Trample	**p35SSPLDanti:pHG1**	-	**+**	n.a.	Jack04E1
117 Tipsy	p35SSPLDanti:pHG1	-	n.a.	n.a.	Jack04Ei
118 Tunic	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
118 Thorn	**p35SSPLDanti:pHG1**	**+**	**+**	**+**	**Jack04E1**
118 Tramp	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
118 Teak	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
118 Toy	p35SSPLDanti:pHG1	**+**	**-**	n.a.	**Jack04E1**
118 Trim	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
118 Thymine	p35SSPLDanti:pHG1	**+**	**+**	**+**	**Jack04E1**
118 Toxic	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
118 Taq	p35SSPLDanti:pHG1	-	-	n.a.	Jack04MW1
118 Tungsten	p35SSPLDanti:pHG1	-	-	n.a.	Jack04MW1
118 Tragic	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
118 Trance	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
118 Thermos	p35SSPLDanti:pHG1	-	-	n.a.	Jack04MW1

Table A. 1 Molecular analysis of transgenic soybean analyzed in this study**.**

(Continued)

Name[1]	Plasmids[2]	Hyg[3]	G. O. I.[4]	S.B.[5]	Cultivar[6]
118 Transom	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
118 Toddle	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
118 Tizzy	**p35SSPLDanti:pHG1**	**+**	**+**	**+**	**Jack04E1**
118 Thud	**p35SSPLDanti:pHG1**	**+**	**+**	**+**	**Jack04E1**
118 Tinsel	p35SSPLDanti:pHG1	-	n.a.	n.a.	Jack04E1
118 Tinder	p35SSPLDanti:pHG1	-	-	n.a.	Jack04E1
118 Travail	**p35SSPLDanti:pHG1**	**+**	n.a.	n.a.	**Jack04E1**
118 Tyro	**p35SSPLDanti:pHG1**	-	**+**	n.a.	Jack04E1
118 Triangle	**p35SSPLDanti:pHG1**	-	**+**	n.a.	Jack04E1
118 Topo	p35SSPLDanti:pHG1	-	n.a.	n.a.	Jack04MW1
119 Jacket	pSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
119 Jersey	pSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
119 Jalopy	**pSPLDanti:pHGI**	**+**	n.a.	n.a.	**Jack04MW1**
119 Jejune	pSPLDanti:pHGI	-	n.a.	n.a.	Chap04E1
119 Jerkin	**pSPLDanti:pHGI**	**+**	**+**	n.a.	**Jack04E1**
119 Jasper	pSPLDanti:pHGI	-	n.a.	n.a.	Chap04E1
119 Jasmine	pSPLDanti:pHGI	-	n.a.	n.a.	Chap04E1
119 Jiffy	pSPLDanti:pHGI	-	n.a.	n.a.	Chap04MW1
119 Jukebox	pSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
119 Jumbo	pSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
119 Jimmy	pSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
119 Jack	pSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
120 Telluride	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
120 Toronto	p35SSPLDanti:pHGI	-	-	n.a.	Jack04MW1
120 Tempe	p35SSPLDanti:pHGI	-	-	n.a.	Chap04MW2
120 Toad	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
120 Timid	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04MW1
120 Tiara	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04MW1
120 Toga	**p35SSPLDanti:pHGI**	**+**	n.a.	n.a.	**Jack04E1**
120 Tangent	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04MW1
120 Tail	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
120 Thwart	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
120 Toil	p35SSPLDanti:pHGI	-	n.a.	n.a.	Chap04MW2
121 Toll	p35SSPLDanti:pHGI	-	-	n.a.	Chap04MW2
121 Tonic	p35SSPLDanti:pHGI	-	-	n.a.	Chap04MW2
121 Totem	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
121 Toil	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
121 Taco	**p35SSPLDanti:pHGI**	-	**+**	n.a.	Jack04E1
121 Tooth	p35SSPLDanti:pHGI	-	**+**	n.a.	Jack04E1
121 Transform	**p35SSPLDanti:pHGI**	-	**+**	n.a.	Jack04E1
121 Time	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
121 Tiny	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
121 Town	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
121 Turtle	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
121 Tomato	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
121 Take-out	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1

Table A. 1 Molecular analysis of transgenic soybean analyzed in this study**.**

(Continued)

Name[1]	Plasmids[2]	Hyg[3]	G. O. I.[4]	S.B.[5]	Cultivar[6]
121 Train	p35SSPLDanti:pHGI	-	n.a.	n.a.	Chap04E1
121 Tuna	**p35SSPLDanti:pHGI**	-	**+**	n.a.	Chap04MW2
121 Torque	p35SSPLDanti:pHGI	-	-	n.a.	Jack04MW1
121 Turkey	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
121 Trendy	**p35SSPLDanti:pHGI**	-	**+**	n.a.	Jack04E1
121 Tylenol	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
121 Turquoise	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
121 Tour	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E1
121 Turnip	p35SSPLDanti:pHGI	-	n.a.	n.a.	Chap04E1
121 Target	p35SSPLDanti:pHGI	-	n.a.	n.a.	Chap04E1
121 True	**p35SSPLDanti:pHGI**	-	**+**	n.a.	Jack04E1
121 Tycoon	p35SSPLDanti:pHGI	-	n.a.	n.a.	Chap04E1
122 Tawny	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E2
122 Tense	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E2
122 Trifle	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E2
122 Tulip	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
122 Topaz	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
122 Trout	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
122 Trend	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E1
122 Twill	p35SSPLDanti:pHGI	-	**+**	n.a.	Jack04E2
122 Tyke	p35SSPLDanti:pHGI	-	-	n.a.	Jack04E2
122 Totter	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E2
122 Thermal	p35SSPLDanti:pHGI	-	n.a.	n.a.	Chap04MW2
122 Tun	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E2
122 Tub	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E2
122 Trap	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E2
122 Thick	p35SSPLDanti:pHGI	-	n.a.	n.a.	Chap04MS2
122 Trioval	p35SSPLDanti:pHGI	-	n.a.	n.a.	Jack04E2

[1]Nomenclature represents bombardment number and transformant name.

[2]Combination of plant expression vectors used in this study.

[3]PCR analysis with hygromycin phosphotransferase primers.

[4]PCR analysis with pSPLDi, pSPLDanti, and p35SSPLDanti primers.

[5]Southern blot analysis with PLDα probe.

[6]Background cultivars used for transformation.

[7]Analysis has not been performed.

Appendix B- Symbols and Abbreviation

°C	degrees Celsius
µl	micro liter
HPT	hygromycin phosphotransferase
bp	base pair
PLD	Phospholipase D
GUS	*β* - glucoronidase
h	hour
kD	kilo Dalton
PIG	Particle Inflow Gun
SDS	Sodium Dodecyl Sulfate
SSC	0.3 M Sodium citrate, 0.3 M NaCl (pH 7.0)
TAE	40 mM Tris, 1mM EDTA (pH 8.0)
TE	10 mM Tris HCL (pH 8.0), 1 mM EDTA (pH 8.0)
Taq	*Thermus aquaticus*
dCTP	Deoxycytidine triphosphoate
RQ1 DNase	RNA Qualified DNase
Oligo-dT	Oligo-deoxythymine
PCR	Polymerase Chain Reaction
G.O.I	Gene of interest
HKG	House keeping gene
SDS-PAGE	SDS - polyacrylamide gel electrophoresis
TAG	Triacylglycerol
PA	Phosphatidic acid

(continued)

RT-PCR	Reverse transcriptase polymerase chain reaction
DTT	dithiothreitol
TBST	Tris-buffered saline tween-20
HRP	Horseradish peroxidase
NIR	Near infrared
DR	Direct repeat
IR	Indirect repeat
DAF	Day after fertilization
PIG	Particle inflow gun
ROI	Reactive oxygen intermediates
SOD	Superoxide dismutase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine