THE EFFECT OF INDOLE ACETIC ACID, ABSCISIC ACID, GIBBERELLIN AND KINETIN ON THE EXPRESSION OF ARF1 GTP BINDING PROTEIN OF PEA (*Pisum sativum* L. cv. Araka)

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ABSTRACT

THE EFFECT OF INDOLE ACETIC ACID, KINETIN, GIBBERELLIN AND ABSCISIC ACID ON THE EXPRESSION OF ARF1 GTP BINDING PROTEIN OF PEA (*Pisum sativum* L. cv. Araka)

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ADP Ribosylation Factor 1 (ARF1) is a universal small GTP binding protein which has an important role in vesicular trafficking between endoplasmic reticulum and Golgi. ARF1 is a basic component of Coat Protein I (COPI) vesicles which have functions in both formation of coatomer complex and recruitment of cargo proteins. In this study, the expression ARF1 was analyzed in pea (*P. sativum* L. cv. Araka) grown at different developmental stages. Because of the differential hormonal levels at corresponding stages, the effects of hormones on ARF1 expression were also studied.

The results of present research show that ARF1 expression in embryos and 2 days grown plants after germination is lower when compared to 6 days grown plants. In order to see the hormonal effect, 3 weeks old plants were supplied with 50µM of each hormone for 3 times on alternate days. Protein extraction, cell fractionation,

Western blot was carried out and immunoblot analysis was conducted with *At*ARF1 polyclonal antibodies.

It was shown that, in pea shoots, abscisic acid and gibberellin increases the inactive GDP bound ARF1 by hydrolyzing ARF-GTP through activating ARF-GTPase activating protein (ARF-GAP) or partially inhibiting ARF-Guanine Nucleotide Exchange Factor (ARF-GEF). In roots, ARF-GDP (cytosolic fraction), ARF-GTP (microsomal fraction) and total amount of ARF1 (13.000 x g supernatant fraction) were down regulated by ~11, ~19 and ~11 fold respectively with the application of gibberellin; and by ~11, ~7 and ~3 fold respectively with the application of abscisic acid; when compared to control plants. These results indicate the importance of plant hormones in the regulation of ARF1 in pea.

Keywords: ADP Ribosylation Factor 1, ARF-GTPase Activating Protein, ARF-Guanine Nucleotide Exchange Factor, plant hormones, protein expression, *Pisum sativum*, pea

ÖZ

İNDOL ASETİK ASİT, KİNETİN, GİBERELLİN VE ABSİSİK ASİTİN GTP BAĞLAYAN ARF1 PROTEİNİNİN BEZELYEDEKİ (*Pisum sativum* L. cv. Araka) ANLATIMI ÜZERİNE ETKİSİ

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ADP Ribozilasyon faktörü 1 (ARF1) endoplazmik reticulum ve Golgi arasındaki veziküler taşımada önemli rolü bulunan evrensel bir küçük GTP bağlayan proteindir. ARF1, kaplama proteini 1 (COP1) veziküllerinin temel bir bileşeni olup; hem kotamer yapısının oluşumunda hem de kargo proteinlerin toplanmasında görev yapar. Bu çalışmada, bezelyenin (*P. sativum* L. cv. Araka) değişik erken gelişim evrelerinde ARF1 anlatımı incelenmiştir. Bitki hormonlarından indol asetik asit, kinetin, gibberellin ve absisik asidin değişik erken büyüme evrelerinde görülen ARF1 protein anlatım farkı üzerine etkisi araştırılmıştır.

ARF1 anlatımının embriyo ve çimlenmeden sonra ikinci gün örneklerinde, çimlenmeden sonra 6. Gün ile karşılaştırıldığında daha düşük olduğu gösterilmiştir. Bu farkın hormonal temellerini anlamak amacıyla, 3 hafta yetiştirilmiş bezelye bitkilerine 3 kez günaşırı her bir hormondan 50 µM uygulanmıştır. Protein eldesi, hücre fraksiyonlarının hazırlanması, Western blot ve *At*ARF1 poliklonal antikoru ile immünoblot analizleri gerçekleştirilmiştir.

Yapılan çalışmalar, Absisik asit ve giberellinin bezelye gövdesinde inaktif, GDP bağlı ARF oranını; ARF-GTPaz aktive edici proteinini (ARF-GAP) aktive ederek veya ARF Guanin nükleotid değişim faktörünü (ARF-GEF) kısmen inhibe ederek; yükselttiğini göstermektedir. Bu çalışma ile, Giberellin uygulaması sonucunda, toplam ARF1 miktarında (13.00 x g süpernatan fraksiyonu) 11 kez, sitozolik fraksiyonda (ARF1-GDP) 19 kez ve mikrozomal fraksiyonda (ARF1-GTP) 11 kez daha düşük anlatım yaptığı belirlenmiştir. ARF 1 protein anlatımında, ABA uygulaması sonucunda; 13.000 x g süpernatan fraksiyonunda 11 kez, sitozolik fraksiyonda 7 kez ve mikrozomal fraksiyonda 3 kez olmak üzere daha düşük anlatım tespit edilmiştir. Bu sonuçlar, bitki hormonlarının bezelyede ARF1 regülasyonundaki önemini göstermektedir.

Anahtar kelimeler: ADP Ribozilasyon faktörü 1, ARF-GTPaz aktive edici protein, ARF Guanin nükleotid değişim faktörü, bitki hormonları, protein anlatımı, *Pisum sativum*, bezelye

To My Beloved Mother and Father

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ABBREVIATIONS

ABA	Abscisic acid
AP	Adaptor protein
ARF	ADP Ribosylation factor
BFA	Brefeldin A
CCV	Clathrin Coated vesicles
COPI	Coat Protein I
COPII	Coat Protein II
DTT	Dithiotreitol
ER	Endoplasmic Reticulum
ERGIC	ER-Golgi Intermediate Compartment
GA3	Gibberellin
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GGPP	Geranylgeranyl Diphosphate
GPP	Geranyl Diphosphate
GTP	Guanosine triphosphate
IAA	Indole acetic acid
IPP	Isopentenyl pyrophosphate
MEP	Methyl Erythritol Phosphate
MVA	Mevalonate
PM	Plasma membrane
PMSF	Phenylmethylsulfonylfloride
SAR	Secretion Associated RAS

SDS	Sodium Dodecyl Sulfate
SDS PAGE	Sodium dodecyl sulfate Polyacrylamide Gel electrophoresis
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
TGN	Trans Golgi Network

CHAPTER 1

INTRODUCTION

In all eukaryotic cells, translation of the proteins starts at the cytosolic ribosomes. Secretory proteins or the ones which has post translational modifications are directed to secretory pathway which starts from ER, by the aid of a signal sequence. (Van Vliet *et al.*, 2003) The proteins which are directed to ER are transported to their final destinations after proper folding. The connection between subsequent compartments of the secretory pathway is mediated by vesicles (Nickel and Wieland, 1997).

*ARF*s are important regulators of vesicular transport. They control the formation of several different types of coated vesicles. These include COP I-coated vesicles that mediate intra-Golgi and Golgi-to- ER retrograde transport, as well as clathrin-coated vesicles containing the adaptor complexes AP-1 and AP-3, which carry cargo from the Golgi to the endocytic pathway (Balch *et al.*, 1992; Memon, 2004).

There are several studies on *ARF* and its regulation in plants. *ARF* had been first identified in plants in *Arabidopsis thaliana* (Regad *et al.*, 1993). *ARF* in pea was first identified in 1993 (Memon *et al.*, 1993) and it was shown that *ARF* plays important role in retrograde transport in plants (Memon, 2004). Light (Memon et. al, 1995) and developmental stage (Koyobashi *et al.*, 2001) dependent regulation of ARF1 were revealed previously. The relation of ARF1 with Guanine Nucleotide Exchange Factor (GEF) and its role in auxin transport was studied by several different groups (Geldner *et al.*, 2003). In this study, the effect of

phytohormones auxin (Indole acetic acid, IAA), cytokinin (kinetin), gibberellin (GA) and abscisic acid (ABA) on the expression of ARF1 in pea (*P. sativum* L. cv. Araka) was examined.

1.1 Pea

Pea (*P. sativum* L.) is a self pollinated annual horticultural crop. It is a cool season crop, planted in winter. It belongs to the division Magnoliophyta, class Magnoliopsida, order Fabales, family *Fabaceae*, subfamily *Faboideae* and tribe *Vicieae*. It has 30-150 cm long, weak, round, and slender stems. Its leaves are alternate, pinnate with 1-3 pairs of leaflets and a 1.5 - 6 cm long ovate or elliptic terminal branched tendril leaflets (www.hort.purdue.edu/newcrop/cropfactsheets/pea.html#Botany).

Figure 1.1 Pea (*P. sativum* L.)

Peas are cultivated for the fresh green seeds, tender green pods, dried seeds and foliage. Green peas are eaten cooked as a vegetable, and are marketed fresh, canned, or frozen while ripe dried peas are used whole, split, or made into flour. Pea is the second mostly cultivated legume, in the world with 20.6%, between 1996 – 2000 after beans (31.6%). The main countries in pea production are France, Canada, China, Germany, India, Russia, Ukraine, Australia, England and USA (Gül and Işık, 2002). In Turkey, pea production was 122.000 tons in 2005 where it was the third mostly produced legume vegetable (www.tuik.gov.tr). Some of the varieties used in Turkey are Sultani, Araka, Grey giant, Sprinter, Lancet, Safir tafto, Hada, Mira and Zenith (www.tarim.gov.tr)

P. sativum has been cultivated for thousands of years. The sites of cultivation have been described in southern Syria and southeastern Turkey, and some argue that the cultivation of peas with wheat and barley seems to be associated with the spread of Neolithic agriculture into Europe (www.wikipedia.org).

Pea has a remarkable vitamin and mineral value: 0.3 mg thiamine (23% USR), 65µg/100g Folic acid (16% RDA), 0.2 mg vitamin B6 (15% USR), 2.1 mg niacin (14% USR), 0.1 mg riboflavin (7% USR), 108 mg phosphorus (15% USR), 1.5 mg iron (12% USR), 1.2 mg zinc (12% USR), 33 mg magnesium (9% USR), with 244 mg potassium (5% USR) and 25 mg calcium (3% USR). Rich vitamin C (40 mg/100g), which comprises 67% United States daily recommendations for adults (USR) and dietary fiber (5.1 mg/100mg) content, makes pea a valuable vegetable. In addition to these it is beneficial because of its low calorie (80 kcal/100g dried seed), fat (0.4 g/100g dried seed) and cholesterol content. Besides its nutritive value, it has an agricultural value of nitrogen fixation with the help of its symbiont, *Rhizobium leguminosorum* via formation of nodules (www.wikipedia.org).

Garden pea (*Pisum sativum* L.) is a diploid plant with 2n=14 chromosomes. It is a classical plant for genetic studies because of its short life span and self compatibility properties.

1.2 GTP Binding Proteins

GTPases are molecular switches and timers that function via conformational changes resulting from the binding and hydrolysis of GTP by intrinsic activities (Bourne *et al.*, 1991; Wittinghofer, 1998). They are inactive as GDP bound species because of reduced affinity for downstream effectors. GTPases are activated by exchange of guanosine diphosphate (GDP) for GTP, a process mediated by various regulatory factors such as heptahelical receptors (G protein-coupled receptors, GPCRs) or guanine nucleotide exchange factors (GEFs). GTPases are found to be highly conserved from yeast to mammals. In view of their important regulatory function, it is not surprising that they play an important role in many plant processes, too (Bischoff *et al.*, 1999). G proteins can be broadly classified into two structurally distinct groups: heterotrimeric G – proteins and small GTP binding proteins (Lehninger, 2000).

Heterotrimeric G proteins are composed of three different subunits (Gα, Gβ, Gγ). They comprise a large gene family mediating a vast array of signaling processes in all eukaryotes, serving as a bridge between heptahelical G protein coupled receptors and effectors such as phospholipases, adenylate cyclases, phosphodiesterases, ion channels and protein kinases (Lehninger, 2000).

Small GTP-binding proteins (G proteins) are monomeric G proteins with molecular weights of 20–40 kDa. They regulate a wide variety of cell functions as biological timers that initiate and terminate specific cell functions and determine the periods of time for the continuation of the specific cell functions. They furthermore play key roles in not only temporal but also spatial determination of specific cell functions

1.3 Small GTP Binding Proteins

1.3.1 Classification

Small GTP-binding proteins (G proteins) exist in eukaryotes from yeast to human and constitute a superfamily consisting of more than 100 members. The members of this superfamily are structurally classified into at least five families: the Ras, Rho, Rab, *ARF/SAR*, and Ran families (Takai *et al.*, 2001). Ras GTPases regulate cell proliferation in yeast and mammalian systems. Members of the Rho GTPase family (i.e. Rho/Rac/Cdc42 proteins) control actin reorganization and signal transduction pathways associated with MAP kinases. The Rab and *SAR*1/*ARF* GTPase families function in distinct steps of membrane trafficking, whereas Ras-related nuclear protein (Ran) GTPases regulate transport of proteins and RNA across the nuclear envelope during the G1, S, and G2 phases of the cell cycle and microtubule organization during the M phase (Vernoud *et al.*, 2003; Takai *et al.*,2001). Vernaud *et al.* (2003) described 93 small GTP-binding proteins in Arabidopsis. These GTPases were classified within four of the five small GTPase families: with 57 Rab GTPases; 21 *ARF* GTPases; 11 Rho GTPases; and 4 Ran GTPases. Interestingly, Arabidopsis does not contain any Ras GTPases that can be identified based on phylogenetic analysis, perhaps reflecting unique mechanisms for control of cell signaling during development in plants (Vernoud *et al.*, 2003).

1.3.2 Mechanism of Action

Many upstream regulators and downstream effectors of small G proteins have been identified, and modes of activation and actions have gradually been elucidated (Takai *et al.*, 2001).

According to the structures of small G proteins, they have two interconvertable forms: GDP-bound inactive and GTP-bound active forms (Takai *et al.*, 2001). Physiological control of these GTPase "switches" occurs through association of the GTPase with accessory proteins, termed guanine nucleotide exchange factors (GEFs), that catalyze the conversion of the small GTP-binding protein to their GTP bound "active" conformation. In their "active" state, small GTPases interact with various downstream "effector" proteins that perform the diverse cellular functions controlled by this class of regulatory molecules. Inactivation occurs through either the intrinsic ability of the small GTP-binding protein to hydrolyze GTP to GDP + Pi, or through association with another set of accessory proteins, GTPase-activating proteins (GAPs), which stimulate this hydrolytic activity. Upon hydrolysis of GTP, the small GTP-binding protein is returned to the "inactive" state and is ready to begin the cycle again (Vernoud *et al.*, 2003).

1.4 Vesicular Transport in Plant Cells

Protein delivery to the cell surface through the endomembrane system is a common feature of eukaryotic cells. All proteins start to be translated in free cytosolic ribosomes and some are directed to ER with a specific amino acid signal – Lysine-Aspartic acid, Glutamic acid, Leucine (KDEL). All secreted proteins and integral proteins of the plasma membrane complete their translation at the endoplasmic reticulum (ER) and are inserted into or translocated across the ER membrane (Jürgens, 2002). Newly synthesized proteins are directed to the right compartment from ER via *secretory pathway*. The transport of these proteins is carried out by special coated vesicles that bud from a donor membrane and fuse with a target membrane (Table 1.1).

Table 1.1 Different types of vesicles in secretory and endocytic pathway (Lodish, 2000)

Vesicle	Coat and Adapter Protein	Small GTP Binding Protein	Transport Step
Clathrin	Clathrin heavy and light chains, AP2	ARF	Plasma membrane→ endosome (endocytosis)
	Clathrin heavy and light chains, AP1	ARF	Golgi→ endosome
	Clathrin heavy and light chains, AP3	ARF	Golgi→ lysosome, vacuole, melanosome or platelet vesicles
COPI	COP α, β, β', γ, δ, ε, ζ	ARF	Golgi→ ER
COPII	Sec23/Sec24 complex; Sec13/Sec31 complex, Sec 16	Sar1	ER→ Golgi

Three types of transport vesicle have been functionally characterized at molecular level and can be defined by both their membrane origin and their coat proteins: i. Clathrin coated vesicles, ii. COPI, iii. COPII. Clathrin-coated vesicles are formed from both the plasma membrane and the trans-Golgi network and mediate vesicular trafficking within the endosomal membrane system. Both COPI- and COPII-coated vesicles are transport intermediates of the secretory pathway (Memon, 2004).

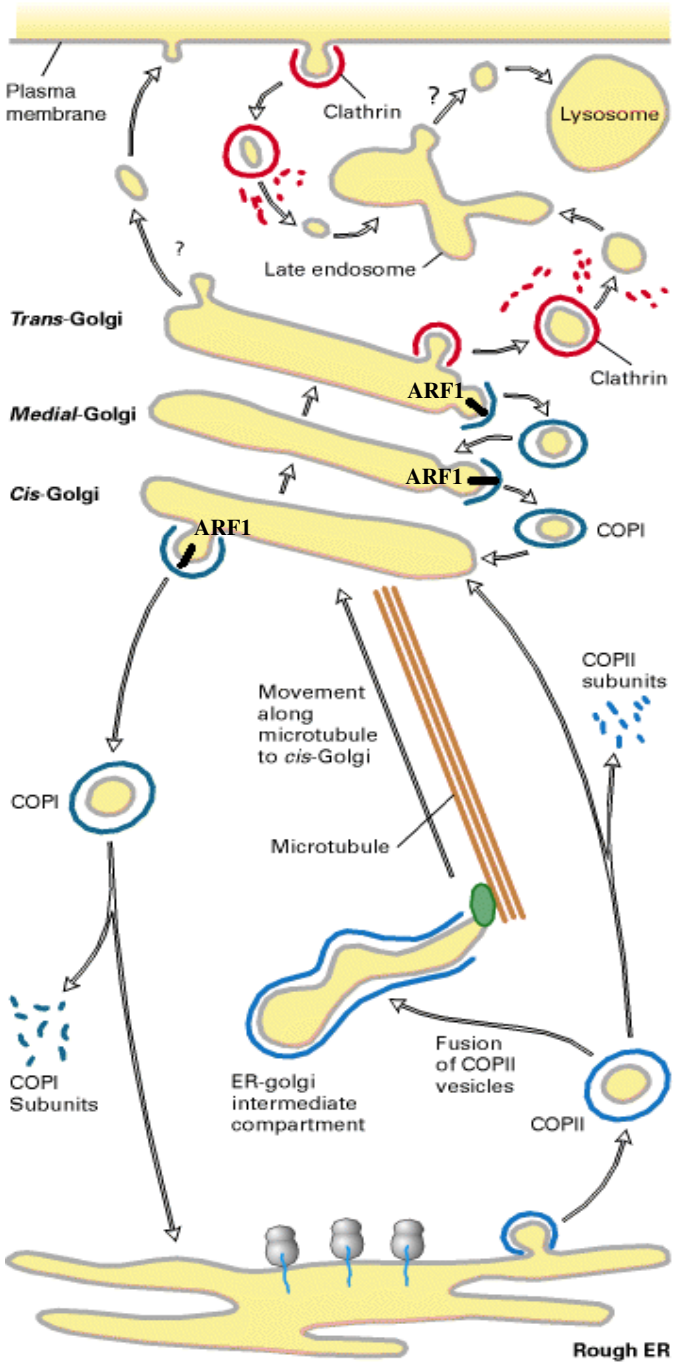


Figure 1.2 Involvement of the three known types of coat proteins — COP I, COP II, and clathrin — in vesicular traffic in the secretory and endocytic pathways (Modified from Lodish, 2000).

1.4.1 Clathrin Coated Vesicles

Clathrin Vesicles Mediate Several Types of Intracellular Transport. They mediate protein transport from the plasma membrane to endosomes (endocytosis) and trans-Golgi to endosomes. Cells that engage in extensive endocytosis have numerous clathrin-coated pits on the cytosolic face of their plasma membrane (Lodish, 2000). In animal cells and yeast, endocytosis occurs via clathrin coated vesicles (CCVs) that act in plasma membrane recovery and in cycling of vesicles in the endomembrane system. Clathrin-coated pits in the plasma membrane and CCVs have been described widely also in plant cells (Battey, 1999). The subunits that build the outer layer of these vesicles are three-legged structures, consisting of three clathrin heavy-chain and three clathrin light-chain polypeptides that are recruited as a hexameric complex, the triskelion, from the cytosol onto the donor membranes (Holstein, 2002; Lodish, 2000; Battey, 1999). Plant clathrin heavy chains have a number of well-conserved regions in common with animal and yeast cells (Battey, 1999). The other prominent protein complex of the clathrin coat at the mammalian PM is the heterotetrameric adaptor (AP-2)-complex. A similar complex, the AP-1 complex is involved in clathrin-coated vesicle (CCV) budding from the trans-Golgi-network (TGN) (Holstein, 2002; Lodish, 2000; Battey, 1999). In mammalian cells *ARF*6 functions exclusively in the endosomal- plasma membrane system where it is involved in recycling to the plasma membrane, regulated secretion, and in coordinating actin cytoskeleton changes at the plasma membrane (Holstein, 2002; Krauss *et al.*, 2003). Although the plant *ARF* GEF has been described to function in PM membrane protein recycling, the role of an *ARF*-type GTPase in plant endocytosis has not been described (Holstein, 2002).

1.4.2 COP Coated Vesicles

Traffic within secretory pathway follows directional routes, and each step involves a unique type of vesicle, which originates on one compartment and is targeted to another (Donohoe *et al.* 2007). Pathway starts with the export of newly synthesized and properly folded proteins from ER to the trans Golgi network. This is named as *anterograde transport*. The transport of proteins from Golgi to ER is named as *retrograde transport* (Lodish, 2000).

There is general agreement that coat protein II (COPII) vesicles are the carriers involved in anterograde ER-to-Golgi transport. Coat protein I (COPI) vesicles arise from Golgi cisternae and mediate the recycling of proteins from the Golgi back to the endoplasmic reticulum (ER) in retrograde direction and the transport of Golgi resident proteins between cisternae. There is still much confusion surrounding the trafficking patterns of COPI vesicles. There is strong evidence to support the notion that COPI vesicles originating from *cis*-Golgi cisternae recycle membrane molecules back to the ER. The targets of the COPI vesicles that bud from medial- and trans-Golgi cisternae are less clear, in part because of conflicting data, and in part because of conflicting hypotheses of Golgi trafficking. The vesicle shuttle model postulates that COPI vesicles are involved in both anterograde and retrograde transport between cisternae, whereas the cisterna progression/maturation model proposes that COPI vesicles are used in retrograde transport only (Donohoe *et al.*, 2007). Donohoe *et al.* (2007) showed that there are two distinctive types of COPI vesicles; COPIa and COPIb. COPIa vesicles bud exclusively from cis cisternae and occupy the space between cis cisternae and ER export sites, whereas the COPIb vesicles bud exclusively from medial- and trans-Golgi cisternae and are confined to the space around these latter cisternae; indicating that COPIa vesicle-mediated recycling to the ER occurs only from cis cisternae, that retrograde transport of Golgi resident proteins by COPIb vesicles is limited to medial and trans cisternae.

It was shown that, low molecular weight GTP binding proteins play important roles in the formation of these vesicles and binding of vesicles to the target membrane in endomembrane system (Clark *et al.*, 1993). ARF1 is the key component in the formation of COPI vesicles and SAR1 is the main GTPase in the formation of COPII vesicles (Memon, 2004).

In order to form a fully functional vesicle, three major prerequisites should be satisfied:

1. The formation of different vesicles requires different specific coating protein. So, correct cytosolic proteins should attach to the source membrane.
2. Some specific membrane proteins should be involved in the structure of the vesicle in order to attach to the target membrane properly
3. The vesicle should take its cargo properly as it leaves the source compartment.

The studies conducted with yeast and higher eukaryotic cells show that these three events occur in a single mechanistic step which is the formation of a "priming complex" of a small GTPase (*ARF* or SAR1), a membrane protein and a coat subunit (Memon, 2004).

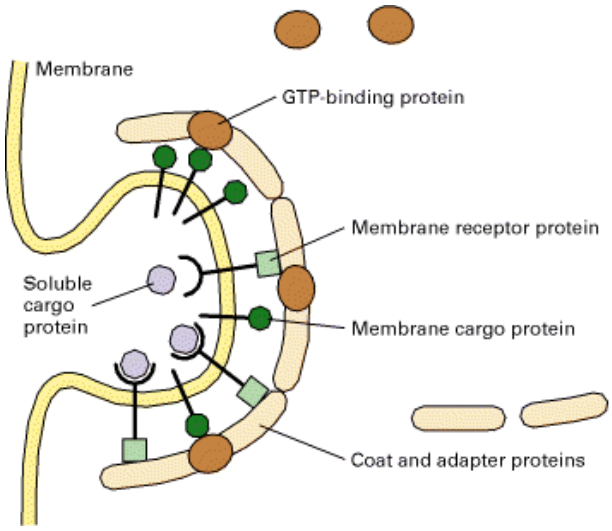


Figure 1.3 Components that participate in budding of coated vesicles (Lodish, 2000).

1.5 ARF/SAR Family of Small GTP Binding Proteins

ARF/*SAR* family low molecular weight GTP binding proteins are controlling factors for the protection of organellar structures (Bischoff, 1999). ARF (ADP Ribosylation Factor) proteins are highly conserved, 21 kDa GTP binding proteins which are involved in the maintenance of organelle structure, formation of two types of coated vesicles in the secretory and endocytic pathway and other cellular processes (Memon, 2004).

Based on phylogenetic analysis, deduced amino acid sequences, protein size and gene structure, *ARF* can be divided into three main classes: Class I (ARF1, *ARF*2, *ARF*3), Class 2 (*ARF*4, *ARF*5) and Class 3 (*ARF*6). Although all classes of *ARF*s are structurally similar and have shown to possess similar activities, partially in *in vitro* assays, the cellular roles of each *ARF* seem to be diverse. Class 1 *ARF*s are currently the best understood and have shown to regulate the assembly of several types of vesicle coat complexes including COPI on the Golgi apparatus, clathrin-AP1 on the trans Golgi network (TGN), clathrin-AP3 on endosomes and the recruitment of AP4 to the Trans Golgi Network (TGN). (Memon, 2004) There are 6 ARFs identified in mammalian systems, 3 in yeast and 21 in *Arabidopsis thaliana*. All 6 *ARF* proteins identified in mammalian systems have been cloned (Memon, 2004; Vernaud *et al.*, 2003).

Studies with yeast and mammalian cells show that ARF1 plays an important role in vesicular transport in retrograde (from Golgi to ER), anterograde (from ER-Golgi Intermediate compartment to Golgi) and cis-medial and medial-trans Golgi cisternae. It is thought that ADP Ribosylation Factor 1 (ARF1) is a universal GTPase which has an important role in vesicular trafficking between ER and Golgi (Memon, 2004). *ARF*3 appears to be functionally interchangeable with ARF1. Class II ARFs, *ARF*4 and *ARF*5, are likely to have similar roles as ARF1 and *ARF*3 in the Golgi. In contrast, *ARF*6 regulates a variety of processes including some forms of regulated secretion, endosomal recycling, desensitization of some

G-protein coupled receptors and actin assembly at the plasma membrane (Casanova, 2003). Through its effects on endosomal membrane trafficking and actin organization, ARF6 modulates several cell-surface-associated activities. In fusion-competent cell types, ARF6 can regulate plasma-membrane fusion, whereas in epithelial cells ARF6 controls intercellular adhesion by regulating the endocytic trafficking of E-cadherin. ARF6 might also regulate cell adhesion to the extracellular matrix (ECM) by regulating the distribution of β1 integrins. ARF6-regulated membrane recycling facilitates the delivery of essential cargo to the cell surface, which in turn enables various cellular processes at the plasma membrane such as phagocytosis, cell migration and invasion (Schorey and Chavrier, 2006). Yeast ARF1 and *ARF*2 are functionally analogous to mammalian ARF1, *ARF*2 and *ARF*3 and localize primarily to the Golgi complex. Although little is known about *ARF*4 and *ARF*5, they probably act primarily at the Golgi too (Casanova, 2003).

The first *ARF* protein in plants was identified in pea (Memon *et al.*1993). In green algae *Chlamydomonas reinhardtii*; a cDNA clone which has 90% similarity with human ARF1 was isolated later (Memon *et al.*, 1995). Six of the ARF genes identified in Arabidopsis had been demonstrated to code amino acid sequences which have 98-100% similarity to class I ARFs and three genes code proteins which has 60% similarity to human ARF1. It was also shown in Cauliflower that, ARF1 is accumulated in ER and Golgi membranes (Jürgens and Geldner, 2002). All these evidences suggest that ARF1 in plant systems has a homologous function to mammalian *ARF*s.

1.5.1 Regulation of ARFs

GTPase cycle of ARFs is regulated by two classes of accessory proteins, guanine nucleotide exchange factors (GEFs) which stimulate GTP loading, and GTPase-activating proteins (GAPs), which promote GTP hydrolysis. The number of

mammalian GEFs (fourteen) and GAPs (twelve) far exceeds the number of ARFs, indicating that individual ARFs must be regulated by more than one GEF or GAP. Most of the Golgi-associated GEFs are sensitive to the fungal toxin brefeldin A (BFA). Since ARF–GTP is required for carrier vesicle formation at multiple Golgi sites, secretion is effectively inhibited by BFA treatment (Casanova, 2003).

ARF is activated by GEFs that share a conserved 200 amino-acid catalytic Sec7 domain. Many ARF GEFs have been identified, including the large multidomain GBF1 (Golgi associated brefeldin A (BFA)-resistant) and the BIG1 and BIG2 (BFA-inhibited) GEFs, which localize to early Golgi and late Golgi/endosome subcompartments, respectively. These have been grouped according to their sequence similarity outside their *Sec7* domain. GBF1 mediates the recruitment of the COPI coat to cis-Golgi membranes, whereas BIG2 regulates the association of the components of clathrin coated vesicles, AP-1 and GGAs, to the trans-Golgi network (TGN). These findings indicate that site-specific targeting of GBF- and BIG-family GEFs to Golgi subcompartments might have a prominent role in the formation of coats at specific locations (Schorey and Chavrier, 2006).

GTP hydrolysis on ARF1 is required for the dissociation of COPI from transport vesicles. This process is mediated by a family of ARF GAPs that contain a conserved zinc-finger motif catalytic domain. In an earlier model, GTP hydrolysis is required for coat disassembly and ARF GAP functions primarily to induce vesicle uncoating. However, several recent studies indicate other roles for ARF1 GAPs. GTP hydrolysis on ARF1 is required for cargo packaging. Also, there is evidence that ARFGAP1 might be a component of the COPI coat and might couple cargo sorting with vesicle formation (Schorey and Chavrier, 2006).

GEFs and GAPs themselves are also subject to regulation. The activity of ARF-GAP1, which is incorporated into COP I coated vesicles during their formation, is inhibited by interaction with specific cargo molecules. As unregulated GAP activity would cause dissociation of the coat before vesicles could even form, this mechanism allows completion of coat assembly and budding only in the presence of the appropriate cargo (Casanova, 2003).

1.5.2 ADP Ribosylation Factor 1 (ARF1)

ARF1 is a basic component of COPI vesicles which has functions in both formation of coatomer complex and recruitment of cargo proteins (Lodish, 2000; Memon, 2004). ARF1 has been shown to interact directly with components of the COP I vesicle coat, and nucleates assembly of the clathrin–AP-1 and clathrin–AP-3 coats by recruiting the linker proteins to the trans-Golgi network. Additionally, at least some of the functions of ARF in cells are linked to their ability to modulate phospholipid metabolism. All ARFs are allosteric activators of PLD, which generates phosphatidic acid from phosphatidylcholine. ARFs can also stimulate the activity of phosphoinositide kinases, leading to enhanced local production of PI(4,5)P2 at the Golgi, plasma membrane and endosomes. These charged lipids may stimulate the recruitment of selected proteins (including coat proteins) to the membrane, alter membrane fluidity (affecting budding and fusion) and also facilitate remodeling of cortical actin in response to ARF activation (Casanova, 2003).

COPI vesicle formation requires at least ARF1 and coatomer *in vitro*. It has been shown that ARF1, nucleotides and coatomer are sufficient to create COPI coated vesicles from chemically defined liposomes but *in vivo*, the situation is different. In the structure of a newly budding vesicle, at least four components exist: SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) which are required for fusion with the target membrane, ARF1, ARF GAP and coatomer subunits (Spang, 2002). In the formation of COPI vesicles, ARF1-GDP binds to membrane phospholipids at low affinity. Upon binding to a p23 oligomer, this interaction is stabilized. Later on, a nucleotide exchange factor acts on ARF1-GDP and the resulting ARF1- GTP released from p23 receptor. Two binding sites; one membrane-bound ARF1-GTP and the other a p23 oligomer; have now been generated and this interaction induces a conformational change and polymerization of the complex, which shapes the membrane into a coated bud (Figure 1.3) (Memon, 2004).

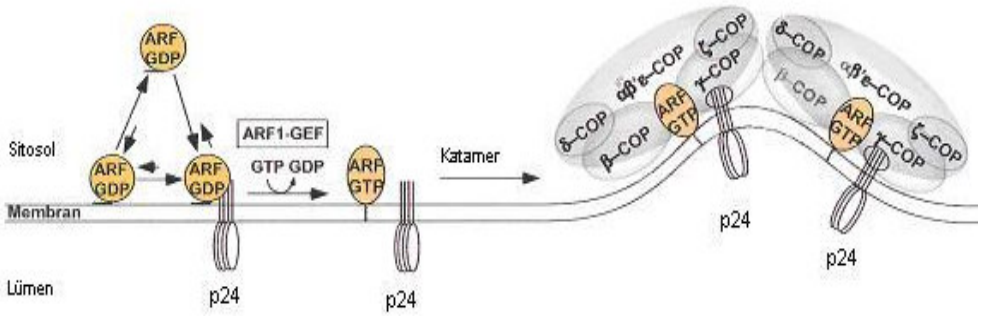


Figure 1.4 A model showing the mechanism of COPI coatomer polymerization in Golgi membranes (Gommel *et al.*, 2001).

The association of cytosolic ADP-ribosylation factor-1 (ARF1)–GDP to the Golgi membranes is facilitated by interactions with the p23 and p24 transmembrane Golgi–cargo receptors and the endoplasmic reticulum–Golgi SNARE protein, membrin, as well as through hydrophobic interaction of the myristoyl group (zig-zag line) with Golgi lipids. The Sec7-domain-containing protein GBF1 (Golgi-associated brefeldin A-resistant protein), which functions as an ARF1 GEF in cis-Golgi compartments, associates with Golgi membranes through interaction(s) with as yet unknown receptor(s) to stimulate nucleotide exchange. Stimulation of GDP–GTP exchange and GTP loading on ARF1 by GBF1 promotes the release of the myristoylated N-terminal amphipatic helix so the affinity of ARF1–GTP for membranes increases dramatically. ARF1–GTP then recruits the pre-assembled heptameric coatomer complex from cytosol to form a coated bud (Figure 1.4-a). ARFGAP1 bound to ligand-coupled KDEL receptors (KDEL-R) and packaged into budding vesicles is activated by coatomer and by membrane curvature. This results in maximal GTP hydrolysis on ARF1 at the distal end of the budding vesicle. Localized cycles of binding and release of ARF1, coatomer and ARFGAP1 might have a role in cargo selection and concentration (Figure 1.4-b) (Schorey and Chavrier, 2006).

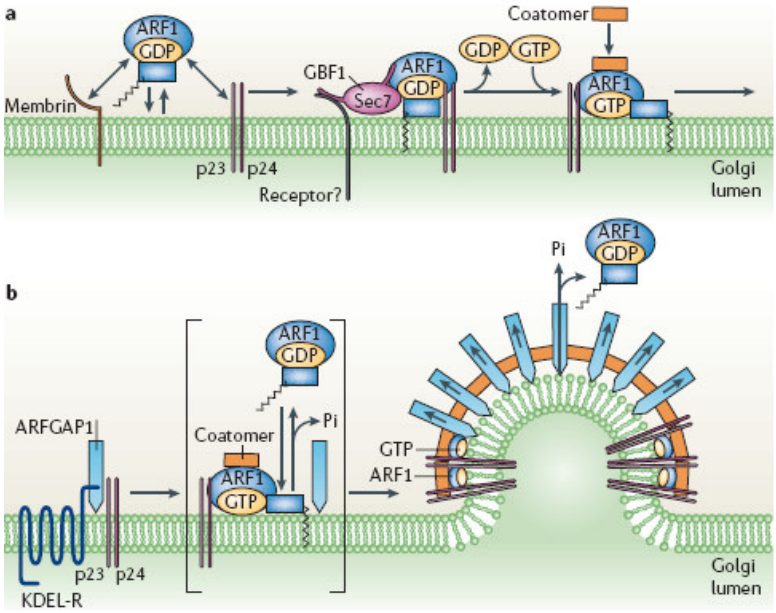


Figure 1.5 Regulation of COPI-coat assembly and vesicle budding by ARF1. a. The association of cytosolic ADP-ribosylation factor-1 (ARF1)–GDP to the Golgi Membranes b. Release of ARF1 from newly formed vesicles (Schorey and Chavrier, 2006).

1.6 Plant Hormones and Their Effects on Small GTPases

In higher plants, regulation and coordination of metabolism, growth and morphogenesis often depend on chemical signals from one part of the plant to another. These signals are plant hormones, i.e. phytohormones which are produced throughout the plant which are simple molecules of diverse chemical composition and function. Until recently plant development was thought to be regulated by five types of hormones: auxins, Gibberellins, cytokinins, ethylene and Abscisic acid which are sometimes referred as “classical five”. However, now, there are also steroid hormones identified in plants; Brassinosteroids, and Jasmonic acid (Taiz and Zeiger, 2002; Srivastava, 2002).

In the context of this thesis, four of the “classical five”; Auxin, Giberellin, cytokinin and Abscisic acid were used to examine their effect on the expression of

ARF1. Here, the major cellular functions of these hormones will be discussed in correlation with their effects on the expression of plant GTPases where available.

1.6.1 Auxins

Auxin, which is an indole compound, is the first growth hormone to be discovered in plants (Appendix 1, Figure A1.1). The principle Auxin in higher plants is Indole-3-Acetic Acid (IAA) (Taiz and Zeiger, 2002; Srivastava, 2002) and is used in this study. Although virtually all plant tissues appear to be capable of producing low levels of IAA, shoot apical meristems, young leaves and developing fruits and seeds are the primary sites of IAA synthesis. It is transported polarly to the root through parenchyma cells and non-polarly in the phloem.

IAA is involved in many aspects of plant growth and development, from embryo to adult reproductive plant. The processes regulated include pattern formation in embryo development, induction of cell division, stem and coleoptile elongation, apical dominance, induction of rooting, vascular tissue differentiation, fruit development and tropic movements such as bending of shoots toward light or roots toward gravity (Srivastava, 2002, Taiz and Zeiger, 2002).

1.6.1.1 Auxins and Small GTPases

It has been shown that, a BFA sensitive ARF-GEF, GNOM, control auxin transport in plants. When BFA-resistant version of the GNOM protein was expressed in plants, PIN1 (a protein functioning in auxin transport) localization and auxin transport lost sensitivity towards BFA treatment (Steinmann *et al.*, 1999; Muday et. al, 2003; Geldner *et al.*, 2004). A similar response is observed with PIN7 protein (Benjamins *et al.*, 2005). Another study indicates the importance of

Rice ARF-GAP (OsAGAP) in IAA transport. The constitutive expression of ARF-GAP phenocopied the wild type rice with exogenous IAA treatment and the analysis of the whole OsAGAP transgenic *Arabidopsis* seedlings showed a sharp increase of free IAA (Zhuang *et al.*, 2005). All these evidences suggest the active involvement of COPI vesicles and ARF1 in the auxin transport in plants.

1.6.2 Gibberellins

Gibberellins are a family of compounds defined by their structure rather than their biological activity. They are all cyclic diterpenes with an *ent*-giberellane ring structure (Appendix 1, Figure A1.2). Two main types of GAs are recognized; those with the full complement of 20 C atoms, the C_{20}-GAs, and the C_{19}-GAs, which have lost one carbon atom and possess a lactone. The biologically active form of GAs in higher plants is C_{19} compounds. Although the number of naturally occurring GAs is high, the number of GAs that are biologically active is quite few. Only certain GAs, notably GA_1, GA_3, GA_4, GA_7 and a few others are responsible for the effects in plants. The others are precursors or metabolites (Taiz and Zeiger, 2002; Srivastava, 2002). Results from numerous bioassays indicate that in pea epicotyl elongation GA_1 and GA_3 show greater activity than GA_4 or GA_7 (Croizer *et al.* 1970). In the present study, GA_3 was applied to the plants in order to observe its effect on ARF1 expression.

Gibberellins stimulate stem growth by promoting both cell elongation and cell division. The activity of some wall enzymes has been correlated with gibberellin-induced growth and cell wall loosening. Other physiological effects of gibberellin include changes in juvenility and flower sexuality, and the promotion of fruit set, fruit growth and seed germination (Taiz and Zeiger, 2002). In cereal grains, GAs induce the *de novo* synthesis of and/or activation of several different enzymes for hydrolysis of storage products. Other responses include cambial reactivation in trees in spring, phloem tissue differentiation, germination of certain seeds, floral

development and in low concentration, stimulation of root growth (Srivastava, 2002).

In pea seedlings, the gibberellin biosynthetic enzymes and GA_3 are specifically localized in young, actively growing buds, leaves, and upper internodes which appear to be the principle sites of GA synthesis (Elliott *et al.,* 2001). Gibberellins that are synthesized in the shoot can be transported to the rest of the plant via phloem (Taiz and Zeiger, 2002).

The GA biosynthetic pathway can be divided into three stages, each residing in a different cellular compartment: the plastid, the endoplasmic reticulum, and the cytosol (MacMillan, 1996). *Stage 1* comprises the production of terpenoid precursors in plastids. Terpenoids are compounds made up of 5-carbon isoprenoid building blocks, joined head to tail. The GAs are diterpenoids that are formed from *four* such isoprenoid units. The basic biological isoprenoid unit is isopentenyl diphosphate (IPP). IPP synthesis pathway in the green parts of plants and in algae uses glyceraldehyde 3-phosphate and pyruvate, and the pathway is named for an important intermediate, methyl erythritol phosphate (MEP). Once synthesized, the 5-carbon units condense to produce intermediates of 10 carbons (geranyl diphosphate, GPP), 15 carbons (farnesyl diphosphate, FPP), and 20 carbons (geranylgeranyl diphosphate, GGPP). GGPP is a precursor of many diterpenoid compounds, including the phytol side chain of chlorophyll, and tetraterpenoids (40 carbons), including carotenoids. There are two cyclization reactions that convert linear GGPP to ent-kaurene (Appendix 1, Figure A1.5) (MacMillan, 1996; Plant physiology online). In the *second stage* of GA biosynthesis, kaurene is oxidized in three steps to give *ent*-kaurenoic acid (KA). Kaurenoic acid is then oxidized in two steps to give GA12-aldehyde. GA12-aldehyde is then oxidized to GA12, which is the first-formed GA, and thus the precursor, of all the other GAs (MacMillan, 1996). *Stage three* is the production of other Gibberellins from GA12 in cytosol.

During active growth, the plant maintains gibberellin homeostasis by metabolizing most gibberellins by rapid hydroxylation to inactive conjugates. Plant metabolizes most gibberellins quickly with the exception of GA3. GA3 is degraded much

slower. Inactive conjugates might be stored or translocated via the phloem and xylem before their release (activation) at the proper time and in the proper tissue. The irreversible deactivation of GAs is achieved by 2β-hydroxylation of the active form. (www.arabidopsis.org:1555/ARA/).

1.6.2.1 Gibberellins and Small GTPases

It has been shown by Chen and An (2006) with microarray differential expression analysis that four GTP-binding protein genes were regulated by GA. Two putative Rho GTPase genes were up-regulated 17-to 27-fold , one putative Rac-like GTPase was up-regulated by 40 fold whereas one Ras-related protein was down-regulated by 3 fold by GA (Table 2) (Chen and An, 2006). There is no recorded evidence for the regulation of ARF GTPases by GA.

Table 1.2 GA regulated G-protein genes in barley (Chen and An, 2006).

Function annotation	Fold-change (GA)
Rho GTPase	27.77
Rho GTPase	17.04
Rac-like GTP-binding protein	40.15
Ras-related protein	−3.37

1.6.3 Cytokinins

Cytokinins are N^6-substituted aminopurines that initiate cell proliferation in many plant cells (Appendix 1, Figure A1.3). The first identified cytokinin is kinetin, which was discovered as a breakdown product of herring sperm DNA. The principle cytokinin of the higher plants is Zeatin (Taiz and Zeiger, 2002; Srivastava, 2002). Kinetin is a stable compound, because, unlike zeatin, its side

chains are immune to attack by cytokinin oxidase. In this study, Kinetin is used as a Cytokinin to examine its effect on the expression of ARF1.

Cytokinins serve very important functions in plant development and morphogenesis. They participate in the regulation of many plant processes including cell division, morphogenesis of shoots and roots, chloroplast maturation, cell enlargement and senescence. They retard senescence of leaves and promote the light independent deetiolation response, including greening of dark grown seedlings.

Cytokinins are synthesized in roots, in developing embryos, young leaves, fruits and crown gall tissues. They are transported passively into the shoot from the root through xylem, along with water and minerals (Taiz and Zeiger, 2002). In pea, the shoot regulates the flow of cytokinin from the root (Beveridge *et al.*, 1997). Cytokinins can occur free in the cytoplasm or as components of tRNAs (Srivastava, 2002).

1.6.3.1 Cytokinins and Small GTPases

There are several microarray studies examining the effect of cytokinin on the expression profile of plants, specifically *Arabidopsis thaliana* (Kiba *et al.*, 2005; Rashotte *et al.*, 2005; Rashotte *et al.*, 2003). There are no precisely defined GTPases which are up- or down-regulated by cytokinin but one putative GTP binding protein and one unidentified GTP binding family protein are reported to be up-regulated by the external application of cytokinin (Kiba *et al.*, 2005).

1.6.4 Abscisic Acid

ABA is a 15 carbon terpenoid compound derived from the terminal portion of caretonoids (Appendix 1, Figure A1.4). It is synthesized in mature leaves, developing seeds and fruits, roots and in most parts of the plant. So, it is synthesized in almost all cells that contain plastids and is transported via both the xylem and phloem (Taiz and Zeiger, 2002; Srivastava, 2002).

ABA has important roles in seed development and maturation, in the synthesis of proteins and compatible osmolytes which enable proteins to tolerate stresses due to environmental or biotic factors, and as a general inhibitor of growth and metabolic activity. ABA is required for the development of desiccation tolerance in the developing embryo, the synthesis of storage proteins and the acquisition of dormancy. Seed dormancy and germination are controlled by the ratio of ABA to GA and ABA deficient embryos may exhibit precocious germination and vivipary. Water stress brings about an increase in ABA synthesis and ABA stimulates the closure of stomata under water stress (Taiz and Zeiger, 2002; Srivastava, 2002).

ABA belongs to a class of metabolites known as isoprenoids, also called terpenoids. They derive from a common five-carbon (C5) precursor, isopentenyl (IDP). As indicated in GA biosynthesis, plastidic isoprenoids, including carotenoids, originate from IDP synthesized from 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway (Appendix 1, Figure A1.5) (Milborrow , 2001). The biosynthetic pathways for ABA and GA coincide in MEP pathway until GGPP formation. Although ABA contains 15 carbon atoms, in plants it is not derived directly from the C15 sesquiterpene precursor, farnesyl diphosphate (FDP), but is rather formed by cleavage of C40 carotenoids originating from the MEP pathway (Nambara and Marion-Poll, 2005).

ABA catabolism is largely categorized into two types of reactions, hydroxylation and conjugation. PA and DPA are the most widespread and abundant ABA catabolites. In addition to hydroxylation pathways, ABA and its hydroxylated

catabolites [8'-hydroxy ABA, PA, DPA, and epi-DPA] are conjugated to glucose. A minor inactive form, 2- trans-ABA, was also identified (Nambara and Marion-Poll, 2005).

1.6.4.1 ABA and Small GTPases

The first small GTP binding protein that was shown to be up-regulated by ABA is Ypt/Rab protein in *Fagus sylvatica* (Nicolas, 1998). It has been shown by Chen and An with microarray differential expression analysis that four GTP-binding protein genes were regulated by ABA treatment: Two putative Rho GTPase genes were down-regulated 2-to 3-fold , one putative Rac-like GTPase was down-regulated by 1 fold whereas one Ras-related protein was up-regulated by 1 fold by ABA (Table 3) (Chen and An, 2006).

Table 1.3 ABA regulated G-protein genes in barley (Chen and An, 2006).

Function annotation	Fold-change (ABA)
Rho GTPase	–2.49
Rho GTPase	–3.24
Rac-like GTP-binding protein	–1.08
Ras-related protein	1.20

1.7 Aim of the study

The aim of this study is to elucidate the importance of plant hormones indole acetic acid, kinetin, gibberellin and abscisic acid in the expression and regulation of ADP Ribosylation Factor 1 (ARF1) in pea seedlings.

To achieve this aim following methods were used:

i. Pea plants were grown for 3 weeks under normal conditions. 50 µM concentrations of indole acetic acid, kinetin, gibberellin and abscisic acid were applied separately 3 times on alternate days on the fourth week. Plants were harvested after 4 weeks of growth.

ii. Protein extraction from roots and shoots of the plants was achieved as described by Memon *et al.* (1993).

iii. Different cell fractions (13.000 x g supernatant and 100.000 x g fractions) were obtained by differential centrifugation.

iv. The protein fractions were run on SDS polyacrylamide gel electrophoresis (PAGE). Western blot and immunoblot analysis with *At*ARF1 polyclonal antibody were conducted.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The chemicals were obtained from Bio-Rad Company, Carlo Erba Chemical Company, Fermentas Chemical Company, Fluka Chemical Company, J. T. Baker Chemical Company, Merck Chemical Company, Riedel Chemical Company, Roth Chemical Company and Sigma Chemical Company.

2.1.2 Plant material

In this study Araka variety of pea (*Pisum sativum* cv. Araka) was used. The seeds were obtained from Istanbul Tohumculuk.

2.2 Methods

2.2.1 Growth of Plants

2.2.1.1 Developmental Stages

Newly imbibed embryo, 2 days and 6 days old radicles were obtained to examine the expression of ARF1.

2.2.1.1.1 Embryo

Pisum sativum cv. Araka seeds were imbibed in deionized water for 3 hours. The embryos were excised with a razor blade, weighed, frozen in liquid nitrogen and kept at -80 °C until used. Tissues were used for protein analysis.

2.2.1.1.2 2 Days Old Pea Radicles

Pisum sativum cv. Araka seeds were imbibed in deionized water for 3 hours and transferred to water damped coarse filter papers and germination and subsequent early development was achieved in dark growth chamber at 20 °C. 2 days after first radicle protrusion, radicles were excised using a razor blade. Samples were weighed, frozen in liquid nitrogen and kept at -80 °C until used. Tissues were used for protein analysis.

2.2.1.1.3 6 Days Old Pea Radicles

Pisum sativum var. Araka seeds were imbibed in deionized water for 3 hours and transferred to 14 mm perlite damped with ¼ strength Hogland's solution. Seedlings were grown in dark growth chamber at 20 °C until 6 days after first radicle protrusion. 6 days old radicles were excised using a razor blade. Samples were weighed, frozen in liquid nitrogen and kept at -80 °C until used. Tissues were used for protein analysis.

2.2.1.2 Effect of Hormones

14 mm diameter perlite was wetted with ¼ strength Hogland's solution. Three seeds were planted to plastic pots and pots were covered with aluminum foil. After germination, seedlings were grown for 3 weeks at 20 °C under greenhouse conditions. 50 mL of ¼ diluted Hogland's solution were supplied to the seedlings 3 times per week.

2.2.2 External Application of Hormones

After 3 weeks of growth, following hormone treatments were applied to the seedlings: i. 50 µM IAA, ii. 50 µM GA, iii. 50 µM Kinetin, iv. 50 µM ABA

Stock hormone solutions were diluted to 50 µM in ¼ strength Hogland's solution and 50 mL of each hormone solution were supplied to the seedlings on alternate days for 3 times. 50 mL of ¼ diluted Hogland's solution were given to the control seedlings

After 4 weeks of growth, seedlings were harvested. Roots and shoots were separated. Each tissue sample was frozen in liquid nitrogen immediately after harvesting and kept at -80 °C until used.

2.3 Protein Analysis

2.3.1 Protein Extraction

Protein extraction was performed as explained by Memon *et al.*, 1993.

Harvested samples (2-8 grams) were homogenized with a mortar and pestle in liquid nitrogen. The homogenized samples were transferred to extraction buffer (1 M Sucrose, 10 mM HEPES pH:7, 5 mM MgCl, 1 mM EDTA, 10 mM DTT, 0,1 mM PMSF, 5 mM Benzamidine). 1 mL of extraction buffer was used for 1 g of tissue. The homogenates were passed from 3 layers of cheesecloth to remove insoluble cell wall fragments**.**

2.3.2 Fragmentation of Plant Extracts by Differential Centrifugation

Different cellular fragments from plant extracts were separated using differential centrifugation. Centrifugation steps were outlined below:

- The total homogenates were centrifuged at 3000 x g (Sorvall RC5C, rotor code HS4) for 10 minutes to pellet cell debris.

- The supernatants were recentrifuged at 13.000 x g (Sorvall RC5C, rotor code SS34) for 20 minutes. Pellet contains large organelles and the supernatant consists of cytoplasm and microsomes. Supernatant

contains cytosol and microsomal fraction which contains total pool of ARF1 in both inactive, GDP bound cytosolic form and active, GTP bound, membrane attached form.

- Finally the supernatants were ultracentrifuged at 100.000 x g (Beckman Optima Max, rotor code MLA 130) for 1 hour. Pellet and supernatant fractions were used as microsomal fraction and cytosolic fraction respectively. Microsomal fraction contains active, GTP bound, membrane attached form of ARF1. Cytosolic fraction contains inactive, GDP bound form of ARF1. With this step of the cellular fractionation, we examine the regulation of ARF1 between active and inactive forms.

2.3.3 Protein Determination

Protein concentrations in different subcellular fragments of pea roots and shoots were determined according to Bradford method (Bradford, 1976).

2.3.3.1 Optimization of Bradford Protein Assay for Microtiter Plates

Bradford protein determination assay was optimized for 96 well micro titer plates. To determine the optimum sample : Bradford reagent ratios for protein determination with small volumes in micro titer plates, three different ratios were used. BSA : Bradford reagent ratios used were 1:20 (final volume 210 µL), 1:10 (final volume 165 µL) and 1:3 (final volume 200 µL). The mixtures were incubated for 10 minutes and absorbance was red at 595 nm in elisa micro plate reader (Bio-Rad Mode 3550 micro plate reader). In the optimization studies, 50 µg/mL, 100 µg/mL, 150 µg/mL and 200 µg/mL BSA standards were used and the standard curves were compared in terms of R^2 values.

2.3.3.2 Determination of Pea Extract Protein Concentrations

1:10 and 1:20 sample : Bradford reagent ratios were determined to be the optimum conditions for protein determination in small volumes (see Appendix III). In the rest of the study, 1:10 ratio was used as it is for larger volumes (Bradford, 1976).

After the determination of optimum working conditions, protein determination of pea protein samples was carried out as follows:

1/5 and 1/10 dilutions of shoot samples and 1/20 and 1/40 dilutions of the root samples were used for protein determination. 15 µL of standard / diluted sample was mixed with 150 µL of Bradford reagent in micro titer plates. The mixture was incubated for 10 minutes and absorbance was red at 595 nm in elisa micro plate reader (Bio-Rad Mode 3550 micro plate reader).

The protein concentrations of the samples were calculated with the following formula:

$$\mathbf{C = A_{595} \times m \times DF}$$

Where A_{595} is absorbance of the mixture at 595 nm, m is the slope of the standard curve and DF is the dilution factor of the sample.

2.3.4 SDS Polyacryamide Gel Electrophoresis (SDS-PAGE)

SDS PAGE was performed as described by Laemmli, 1970.

2.3.4.1 Preparation of Electrophoresis Unit

Glass plates and plastic combs were cleaned with 70% (v/v) ethanol and assembled in a gel caster. In this study, 5% stacking and 12 % separating gels were used. Preparation of the gels is given in Appendix 2. SDS-PAGE electrophoresis unit (Bio-Rad mini vertical slab gel apparatus) was assembled according to manufacturer's instructions.

2.3.4.2 Sample Preparation for SDS PAGE

10 µg of protein samples for silver staining and 50 µg of protein samples for western blotting were mixed with 5x sample buffer so that sample buffer was diluted to 1x. The samples were heated at 95°C for 5 min. Prepared samples were loaded to wells using a micropipette. 0.5 µL of molecular weight marker (Fermentas cat.# SM 0671) was used for silver staining and 1.5 µL was used for western blotting. The empty wells, if any, were loaded with 1x sample buffer.

Protein samples were separated at 80 V in the stacking gel and 120 V in the separating gel. After electrophoresis, the gels were removed from the glass plates and either used for western blotting or stained for protein visualization by silver staining.

2.3.5 Silver Staining of SDS PAGE Gels

Each step of silver staining protocol is performed on a slowly rotating orbital shaker (50-60 rpm) at room temperature. The steps indicated below were performed consecutively.

- Fixation with freshly prepared fixer solution (40% Methanol, 10% acetic acid) for 30 minutes
- Washing with ultrapure water 3 times for 10 minutes
- Incubation with 320 µM DTT solution for 30 minutes
- Gentle wash with ultrapure water
- Incubation with 0.2 g/L $AgNO_3$ solution for 30 minutes
- Gentle wash with ultrapure water
- The gel was soaked in freshly prepared developer solution (Appendix 2). The solution was replaced when a smoky brown precipitate appeared. The final development was watched to avoid over staining the gel. Development was stopped by using 1% Acetic acid when the background intensity increased at about the same rate as the band intensity.
- Destaining with Farmer's reducer solution (Appendix 2), if necessary.

2.3.6 Western Blotting

Western blot analysis was performed as described by Gommel *et al.*, 2001.

SDS-PAGE was run with pea root or shoot protein samples. Prestained molecular weight marker was used (Fermentas cat.# SM 0671). Nitrocellulose membrane and 3mm whatman papers were cut to gel size and equilibrated in Transfer Buffer for 5 min. Nitrocellulose membrane was marked using a pencil. The gel was removed from glass plates and gel sandwich was prepared as shown in Figure 2.1

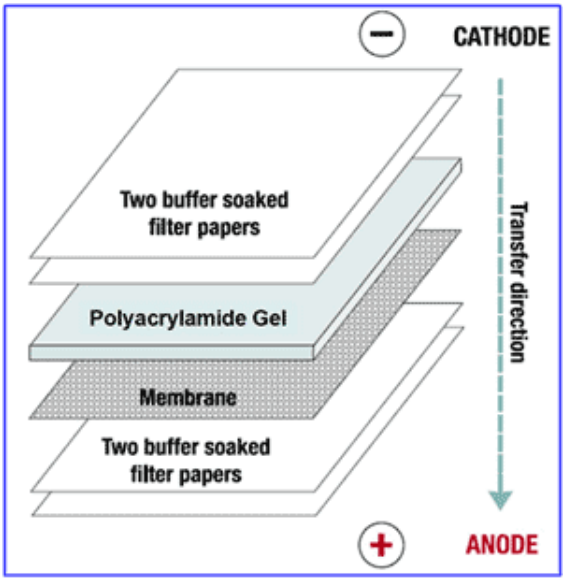


Figure 2.1 Preparation of sandwich system for western blotting

Air bubbles were rolled out between layers, using glass pipette and gel sandwich was inserted to transfer cassette according to manufacturer's instructions (Bio-Rad midi trans blot cell). Ice cooling unit was inserted into the tank to prevent over heating of the system. Transfer was carried out at 350 mA for 1 hour with Bio-Rad Power Pac 200 power supply.

After the transfer, nitrocellulose membrane was removed from the cassette and stained by Panceu S solution in order to detect protein transfer and Immunoblot analysis was carried out.

2.3.7 Panceu Staining of Nitrocellulose Membranes

Membranes removed from the cassette were immersed into Panceu S solution (Appendix II) for 1 min. Excess dye was removed from the membranes by gently shaking in distilled water.

2.3.8 Immunoblot Analysis

All steps of immunoblot analysis were performed on a slowly rotating orbital shaker (50-60 rpm). The steps indicated below were performed consecutively

- Blocking in TBS + 5% skimmed milk for 1 hr at room temperature.
- Washing in TBST twice for 2 min
- Incubation with anti-*At*ARF1 (1:2500 diluted in 1%BSA + TBST) overnight at +4°C
- Washing in TBST three times for 20 min
- Incubation with HRP tagged secondary antibody (1:10.000 diluted in 1%BSA + TBST) (Goat anti-rabbit IgG(H+L) – HRP conjugate, Bio-Rad cat. # 170-6515) for 1 hour at room temperature,
- Washing in TBST three times for 20 min

Immunoblotted membranes were developed by using Lumigen PS-3 acridan substrate (Pierce ECL Western Blotting Substrate Cat.# 32209) which gives Acridinium ester intermediate as the florescent product of the reaction with Horse Radish Peroxidase (HRP). Visualization is performed using blue X-Ray films (Fuji Super RX safelight glass no: 8u) in dark room.

2.3.9 Interpretation of Data

The specific bands in the raw immunoblot data were converted to numerical values using Photoshop CS3 program. The calculations were made using two parameters: Area and Integrated Density. The films were transferred to computer using HP 1100 scanner and the images were opened with Photoshop CS3. The images were inverted so that brighter areas show darker bands. The desired band was selected using magnetic lasso tool and the data for each selection was recorded using analysis option. In the data records, *area* represents the area of selection in square

pixels. *Integrated density* was the sum of the values of the pixels in the selection. This was equivalent to the product of Area (in pixels) and Mean Gray Value where *gray value* was a measurement of brightness. The recorded data was converted to graphs using Microsoft Excel.

CHAPTER 3

RESULTS AND DISCUSSION

In the first part of this study, the expression of ADP Ribosylation Factor 1 (ARF1), which is a critical component of COPI vesicles, at different early developmental stages of pea was examined. In the second part, the effects of different phytohormones; which are differentially expressed at different developmental stages; were examined on the protein expression of ADP Ribosylation Factor 1 (ARF1). ARF1 protein expression was examined both in roots and shoots.

To examine the expression differences at early developmental stages, pea seeds were imbibed to excise embryos. 2 days and 6 days old radicles after germination were obtained. For hormonal difference work, pea plants were grown on perlite for 4 weeks. In the first three weeks plantlets were grown under normal conditions with mineral supply from Hogland's reagent. In the last one week, plants were supplied with indicated concentrations of exogenous hormones. Effects of different plant hormones on physiological parameters (lengths and wet weights of the pea roots and shoots) and protein profiles of different cellular fractions were examined. ARF1 expression differences were detected by immunoblotting.

3.1 ARF1 Expression at Different Early Developmental Stages

ARF1 expression in embryo, 2 days and 6 days old radicles were examined with immunoblotting to see developmental stage dependent regulation of ARF1.

In cytosolic fraction (i.e. GDP bound inactive form of ARF1) ARF1 expression was highest at 6 days old plants and lowest at 2 days old plantlets (~2.5 fold less than 6 days old plants). The expression was lower than 6 days but higher than 2 days at embryos (~1.5 fold less than 6 days old plants) (Figure 3.1, 3.2).

Figure 3.1 Immunoblot carried out with *At*ARF1 antibody, 100.000 x g supernatant protein samples of the radicles at early developmental stages
E: embryo, R2: 2 days old radicle, R6: 6 days old radicle

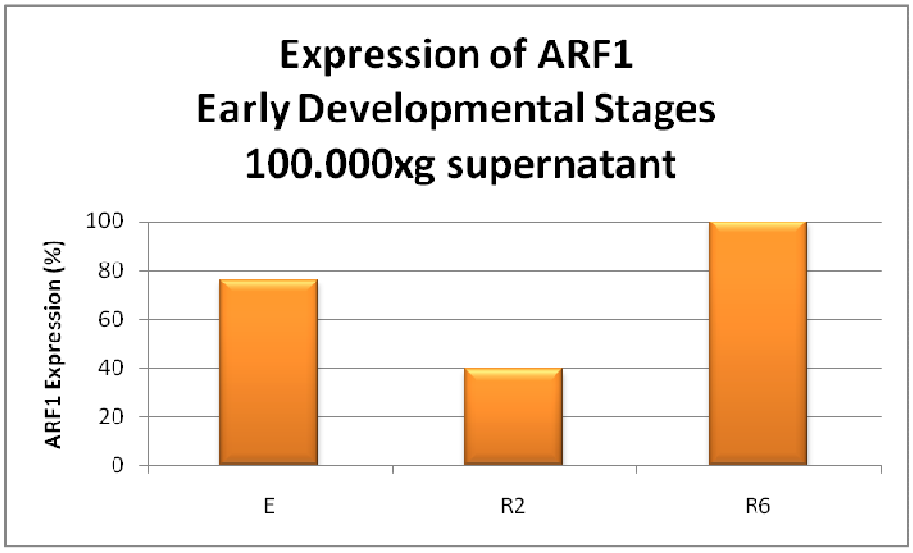


Figure 3.2 Graphical demonstration of the amount of ARF1 protein in 100.000 x g supernatant protein samples of the radicles at early developmental stages
E: embryo, R2: 2 days old radicle, R6: 6 days old radicle

In microsomal fraction (i.e. GTP bound active form of ARF1); the expression of ARF1 at embryo and 2 days old radicle was ~2.5 fold lower than 6 days old radicle (Figure 3.3, 3.4).

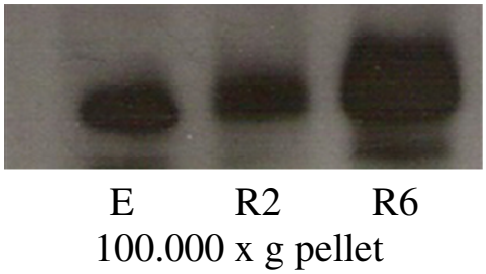


Figure 3.3 Immunoblot carried out with *At*ARF1 antibody, 100.000 x g pellet protein samples of the radicles at early developmental stages
E: embryo, R2: 2 days old radicle, R6: 6 days old radicle

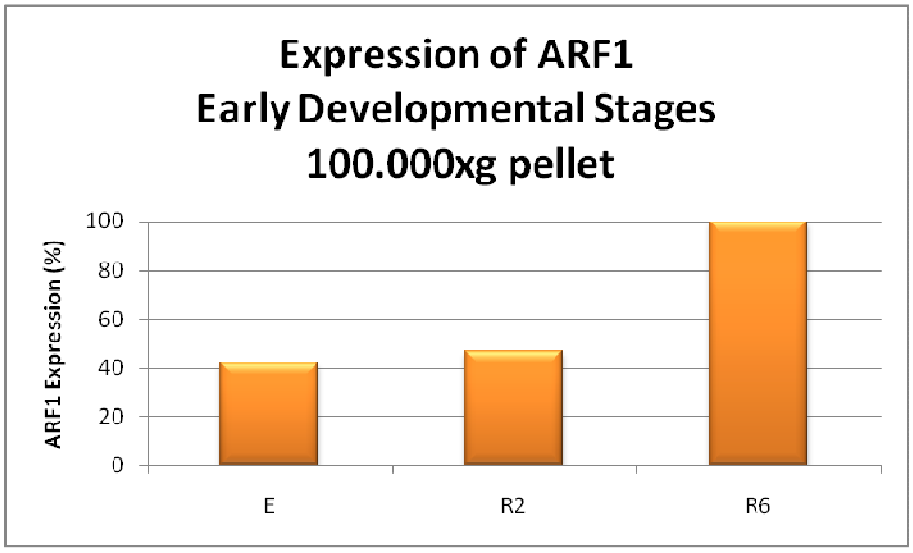


Figure 3.4 Graphical demonstration of the amount of ARF1 protein in 100.000 x g pellet protein samples of the radicles at early developmental stages
E: embryo, R2: 2 days old radicle, R6: 6 days old radicle

In both cytosolic fraction and microsomal fraction, the highest ARF1 expression was observed at 6 days old radicle. At 2 days old radicle, ARF1 in cytosolic fraction was less when compared to embryo but, in microsomes, there was an equal amount of active ARF1 at both E and 2 days old radicle (Figure 3.1 - 3.4). These results indicated that, there was a larger active pool of ARF1 at 2 days old radicle, and thus, more extensive membrane trafficking at 2 days old radicle compared to embryos. At 6 days old radicle, on the other hand, the total pool of active and inactive ARF1 was greater than the other developmental stages, which implied both more extensive membrane trafficking and higher ARF1 protein expression. It is natural to observe such a regulatory pattern because of plant growth and cell division. The largest active pool of ARF1 was observed at 6 days old radicles which are growing rapidly and smallest ARF1 expression is observed at embryo, where cell division and growth is slow.

These data show the regulation of ARF1 at different early developmental stages but, interestingly, the figures 3.2 and 3.4 indicate that the balance between ARF-GDP and ARF-GTP is maintained in both 2 days and 6 days old radicles. In both

microsomal and cytosolic fractions, the ratio of ARF1 at 6 days old radicles to ARF1 at 2 days old radicles is ~2.5 fold meaning the balance between GDP and GTP bound form of ARF was maintained in the cell.

To understand the reasons of the difference observed in ARF1 expression at the indicated developmental stages, we should first understand the physiological changes at these stages. Early developmental stages of a plant are the most dynamic stages when we consider the hormonal status of the plant.

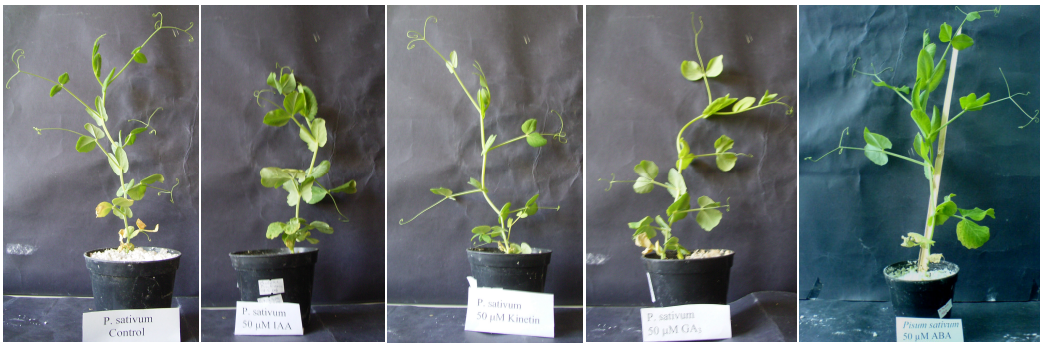
In many plant species endogenous ABA is involved in the induction and maintenance of the dormancy of the seeds. ABA inhibits embryo growth potential and endosperm cap weakening during seed germination but a transient rise in ABA content in the embryo is evident early during imbibition (Kucera *et al.,* 2005). So, the samples taken from the newly imbibed seeds have a high content of endogenous ABA. GAs play a key role in dormancy release and in the promotion of germination. Bioactive GAs accumulate just prior to radicle protrusion (Kucera *et al.,* 2005). 2 days old pea plantlets, used in this study, with emerged radicle and newly emerging plumule has a ready content of accumulated active GAs endogenously (Kucera *et al.,* 2005).

Taken together, in embryo, there is a high content of ABA and in two days old seedlings, the predominant hormone is GA. So, in the second part of this study, we applied different plant hormones to developing pea seedlings exogenously to see their effects on ARF1 expression.

3.2 Hormone Application

3.2.1 Physiological Parameters

Despite the hormones were applied to mature plants with no defects in hormone biosynthesis, the physiological effects of the hormones were observed after one week of hormone application (Figure 3.5, 3.6). The physiological results indicated below are given according to three independent replications.



Control **50µM IAA** **50µM Kinetin** **50µM GA** **50µM ABA**

Figure 3.5 Pea shoots after 3 weeks of normal growth and 1 week of hormone treatment (4 weeks old plantlets). From left to right, Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

Control **50µM IAA** **50µM Kinetin** **50µM GA** **50µM ABA**

Figure 3.6 Pea roots after 3 weeks of normal growth and 1 week of hormone treatment (4 weeks old plantlets). From left to right, Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants

3.2.1.1 Root and Shoot Lengths and Organization of Axillary Buds

Upon exogenous application of indole acetic acid (IAA), the plant showed retarded development in axillary buds and rather showed apical growth, which may be attributed to the effect of IAA on apical dominance (Pessarakli, 2002). A contrasting effect of Kinetin was observed with well developed axillary buds since kinetin has an effect on the release of axillary buds from apical dominance (Pessarakli, 2002) (Figure 3.5).

Gibberellin (GA) treated plants were slightly longer than control plant, in correlation with the promotion of stem elongation by GA (Pessarakli, 2002). ABA treated plants were shown to be shorter than control (Figure 3.7). No significant root development difference was observed upon hormone application with the exception of kinetin (Figure 3.7). In kinetin applied roots, root development was retarded, probably because of low auxin / kinetin ratio in the roots. Higher rooting is observed in cultured plant tissues where high auxin / kinetin ratio is applied (Pessarakli, 2002).

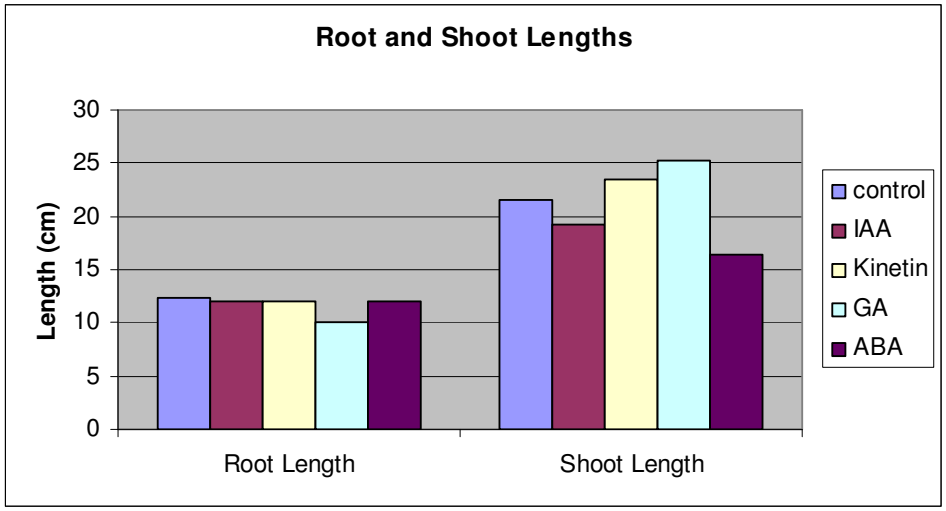


Figure 3.7 Root and shoot lengths of control and 50 µM hormone treated 4 weeks old pea seedlings. The results are given as the average of three independent replications.

3.2.1.2 Wet Weight of Roots and Shoots

Wet weight measurements of the pea shoots and roots were recorded just after harvesting. The highest wet weight of roots was observed in ABA treated plants. There was approximately 2 times increase in the wet weight of the roots of ABA treated plants. However, there was no significant change in the shoot wet weights of the plants (Figure 3.8). It was shown that, ABA, the stress hormone, inhibits shoot growth but a contradictory effect was observed in roots. Exogenous ABA has been observed to inhibit shoot growth and maintain root growth in maize seedlings. This root response may be attributed to the hydrotropic movement which induces the organ to reach deeper in the soil to find more available water.

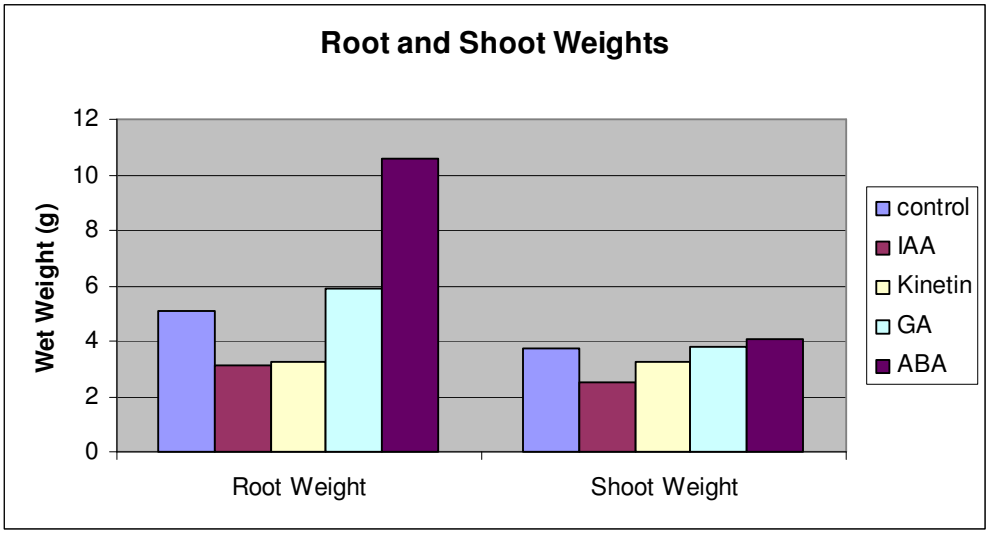


Figure 3.8 Root and shoot wet weights of control and 50 µM hormone treated 4 weeks old pea seedlings. The results are given as the average of three independent replications.

3.2.2 Protein Analysis

3.2.2.1 SDS PAGE Profiles of Protein Extracts

Protein profiles of the pea extracts after 13000 x g supernatant (consists of cytoplasm and microsomes), 100.000 x g supernatant (cytoplasmic fraction) and 100.000 x g pellet (microsomal fraction) samples of shoots and roots were observed with SDS-PAGE. Dominant Light Harvesting Complex (LHC) bands at 55 KDa were visible in the shoot proteins. The differential protein bands in different subcellular fractions can be seen in the SDS PAGE (Figure 3.9, 3.10).

ARF1 protein band was not visible in SDS PAGE gels since ARF is not a dominant protein in the proteome of pea and since purified proteins were not used (Figure 3.9, 3.10).

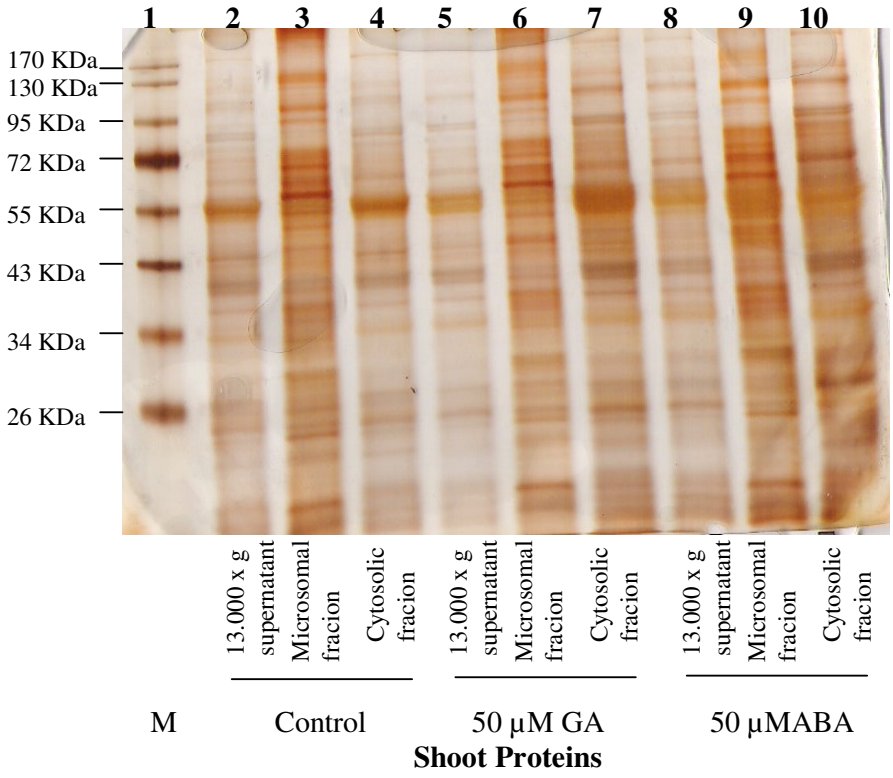


Figure 3.9 Silver stained SDS PAGE gel (12%) of shoot extracts. 25µg of proteins were loaded to each well. 1. Molecular weight marker 2. 13.000 x g supernatant fraction of control pea shoots 3. 100.000 x g pellet fraction of control pea shoots 4. 100.000 x g supernatant fraction of control pea shoots 5. 13.000 x g supernatant fraction of 50 µM GA treated pea shoots 6. 100.000 x g pellet fraction of 50 µM GA treated pea shoots 7. 100.000 x g supernatant fraction of 50 µM GA treated pea shoots 8. 13.000 x g supernatant fraction of 50 µM ABA treated pea shoots 9. 100.000 x g pellet fraction of 50 µM ABA treated pea shoots 10. 100.000 x g supernatant fraction of 50 µM ABA treated pea shoots

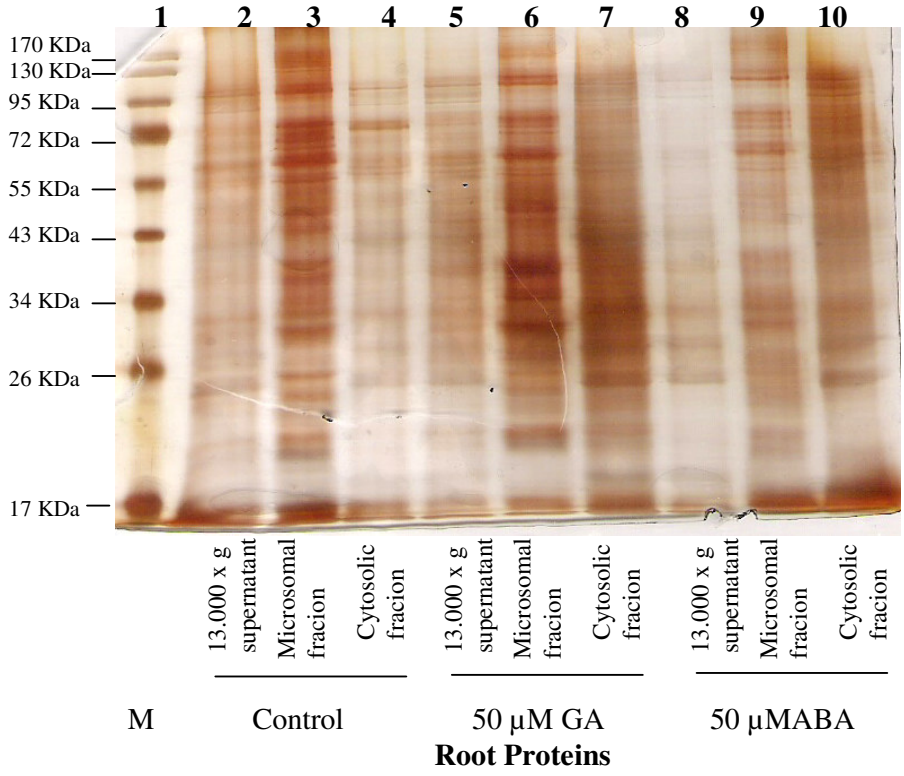


Figure 3.10 Silver stained SDS PAGE gel (12%) of root extracts. 25µg of proteins were loaded to each well. 1. Molecular weight marker 2. 13.000 x g supernatant fraction of control pea roots 3. 100.000 x g pellet fraction of control pea roots 4. 100.000 x g supernatant fraction of control pea roots 5. 13.000 x g supernatant fraction of 50 µM GA treated pea roots 6. 100.000 x g pellet fraction of 50 µM GA treated pea roots 7. 100.000 x g supernatant fraction of 50 µM GA treated pea roots 8. 13.000 x g supernatant fraction of 50 µM ABA treated pea roots 9. 100.000 x g pellet fraction of 50 µM ABA treated pea roots 10. 100.000 x g supernatant fraction of 50 µM ABA treated pea roots

3.2.2.2 Effect of Hormones on ARF1 Expression

Western blot and subsequent immunoblot analysis were performed and interpreted as described in materials and methods. Equal amount of protein was loaded to each well according to the Bradford protein assay results (Appendix 3, Table A3.1). Equal protein loading to the gels was controlled by panceu staining and immunoblot analysis was performed with the membranes which has equal amount of protein in all wells. The results of panceu staining are given in Appendix IV. Data obtained in the films were scanned and "*Integrated density"* of the bands, which is the product of the area and intensity of the bands in pixels, were calculated. The integrated density of the bands of different hormone treatments was compared using bar graphs. The results represented with graphs were totally consistent with the expression profile observed in the western films.

ARF1 protein expression was higher than control at all fractions of the control samples compared to the experiment plants of all hormone treatments (Figures 3.11 - 3.22). This might be a result of the inhibitory effect of the hormones applied exogenously. The plants that were used in this study can synthesize all hormones normally. As the hormones were supplied, ARF1 expression decreased when compared to control plants. But plant growth goes on in all the plants healthily and in a healthy growing plant, vesicular trafficking should go on normally. So, there is a small, but very efficiently recycling ARF1 pool in the cell. So we can conclude that, there is a significant regulation of ARF1 turnover with the effect of the hormones.

3.2.2.2.1 ARF1 expression in Shoot Tissue

In the shoot samples, when we examine the total pool of active and inactive ARF1 (13.000 supernatant fraction) we observe a decrease in ARF1 expression with the

application of all the hormones; IAA, Kinetin, gibberellin and Abscisic acid where the decrease is ~2 fold with IAA, Kinetin and ABA and ~4 fold with GA application (Figure 3.11, 3.12). The more significant decrease in the expression of ARF1 with the application of GA is strongly consistent with the results obtained in developmental stage work, where ARF1 expression was lower at 2 days old plantlets – the developmental stage where active GAs accumulated.

Figure 3.11 Results of the immunoblot carried out with AtARF1 antibody, 13.000 x g supernatant protein samples of the shoots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

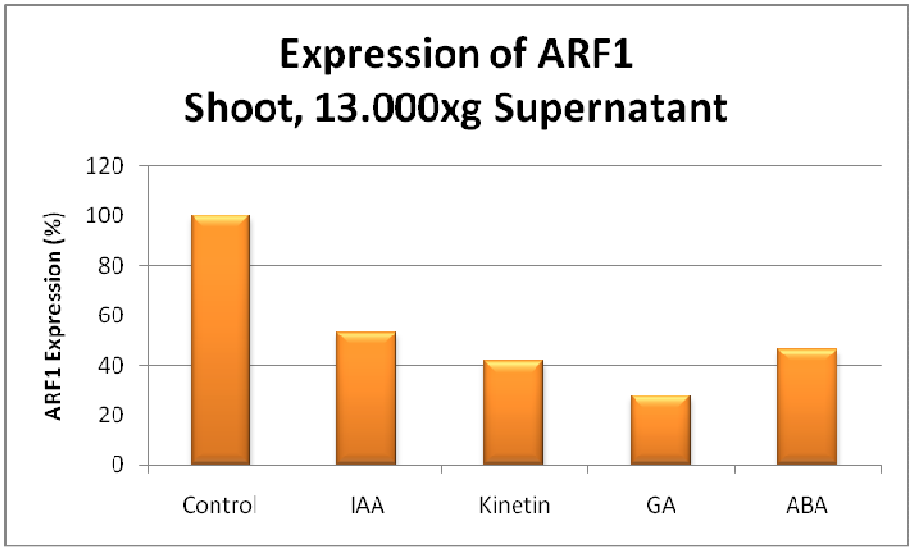


Figure 3.12 Graphical demonstration of the amount of ARF1 protein in 13.000 x g supernatant protein samples of the shoots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

In cytosolic fraction of the shoot samples, where the inactive, soluble form of ARF1 is present, we observe an increase in the amount of ARF1-GDP in all hormone treated samples, where the most significant increase is observed with GA and ABA where ~2 times more ARF1 protein was observed compared to control (Figure 3.13, 3.14).

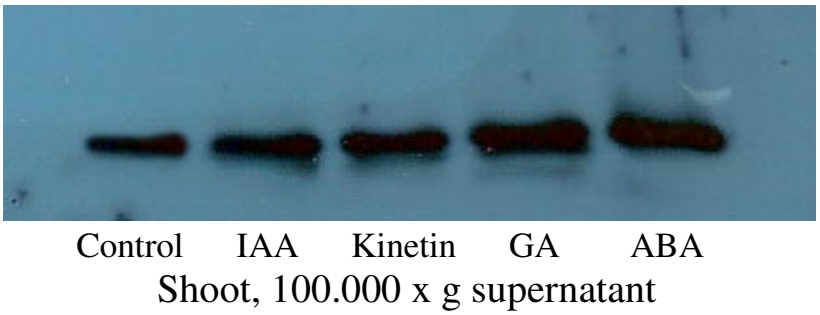


Figure 3.13 Results of the immunoblot carried out with AtARF1 antibody, 100.000 x g supernatant protein samples of the shoots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

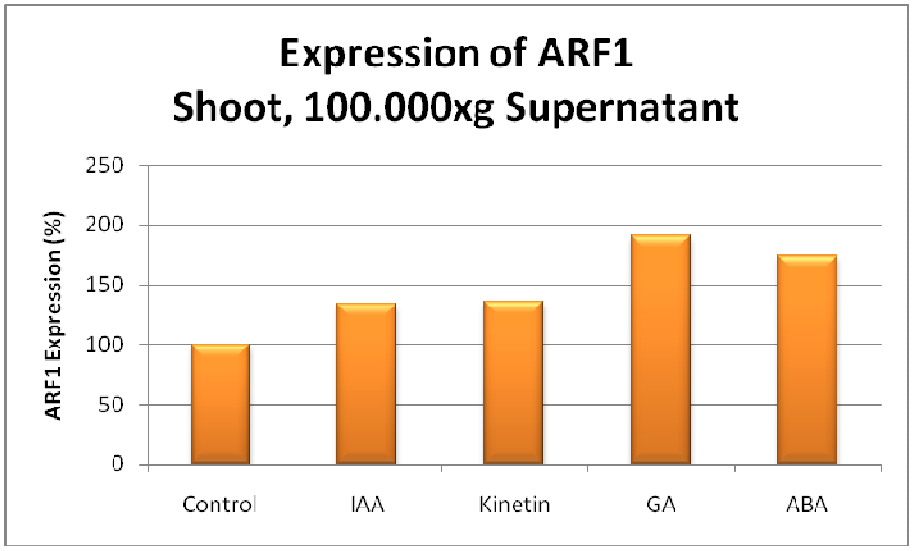


Figure 3.14 Graphical demonstration of the amount of ARF1 protein in 100.000 x g supernatant protein samples of the shoots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

In contrast to cytosolic fraction, ARF1 protein was shown to decrease in the microsomal fraction (where membrane bound, active form of ARF1 exists) significantly upon application of exogenous ABA (~7 fold) and GA (~3 fold) (Figure 3.15, 3.16).

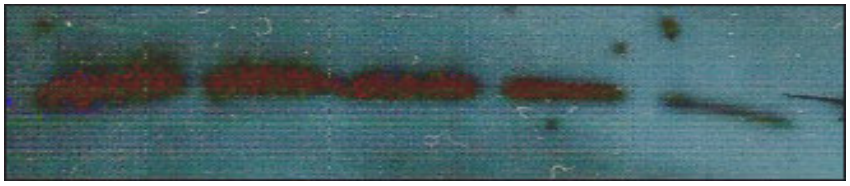


Figure 3.15 Results of the immunoblot carried out with AtARF1 antibody, 100.000 x g pellet protein samples of the shoots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

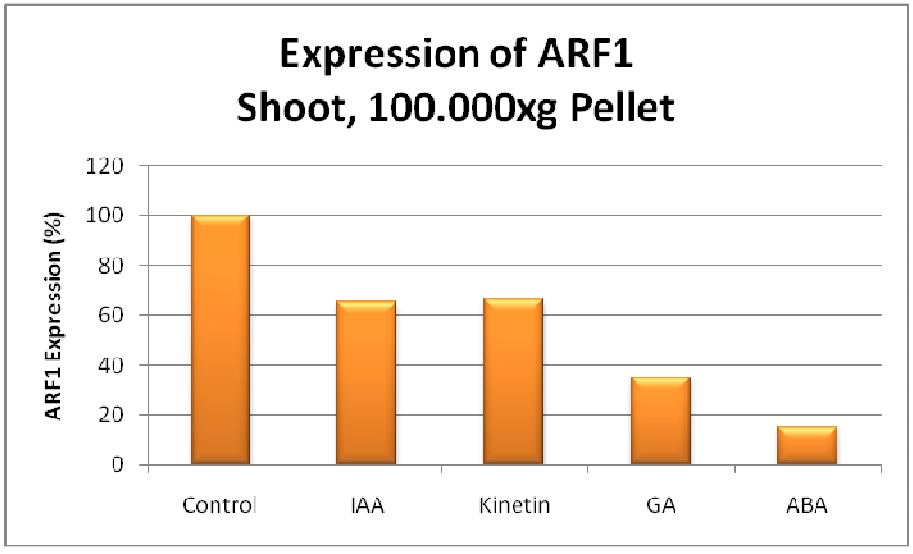


Figure 3.16 Graphical demonstration of the amount of ARF1 protein in 100.000 x g pellet protein samples of the shoots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

The decrease observed in microsomal fraction upon ABA application is natural, since ABA retarded the growth of shoot of the plant (Figure 3.7) and in a slowly growing plant, there should be less extensive membrane trafficking. When we consider GA, growth continues actively, even faster than the control plant (Figure 3.7) but ARF1-GTP seems to be 3 fold less than control. This implies a small but very actively recycling ARF1 pool.

Overall, in the shoot samples, we both observed the regulation of expression of ARF1 in the total pool, and also regulation of the activity of ARF1 – there was an increase in the inactive form of ARF1. So, in this case, there was an effect of the hormones ABA and GA on the activity of ARF-GAP (GTPase activating protein), which promotes the hydrolysis of active ARF-GTP to inactive ARF-GDP, and/or ARF-GEF (Gunanine nucleotide exchange factor) which converts inactive, cytosolic form of ARF to active membrane bound form. ABA and GA; the hormones which had the greatest effect on the regulation of ARF1; either activated ARF-GAP or decreased the activity of ARF-GEF.

3.2.2.2.2 ARF1 expression in Root Tissue

The modulation of ARF1 expression in roots in response to hormone treatment was observed especially with GA and ABA where less significant expression difference was observed with IAA and Kinetin. Gibberellin and ABA down regulates ARF1 expression where the effect observed with gibberellin is greater than ABA. Both Gibberellin and ABA shows down regulatory effect in all subcellular fractions (Figures 3.17 – 3.22). In roots, growth continued normally in GA treated plants but growth rate increased significantly in case of ABA application (Figure 3.8). But yet, there was a significant decrease in ARF1 expression at all fractions in the root samples, including total ARF1, ARF1-GDP and AFR1-GTP. This was the indication of high rate of ARF1 turnover with the application of ABA and GA.

In 13.000 x g supernatant fraction; total ARF1; the expression of ARF1 was more than ~11 fold less in both 50 µM GA and ABA treated plants (Figures 3.17, 3.18).

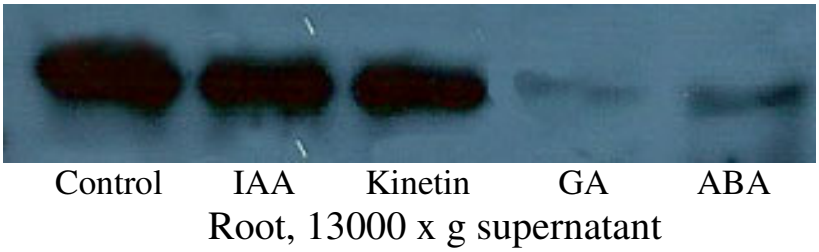


Figure 3.17 Results of the immunoblot carried out with AtARF1 antibody, 13.000 x g supernatant protein samples of the roots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

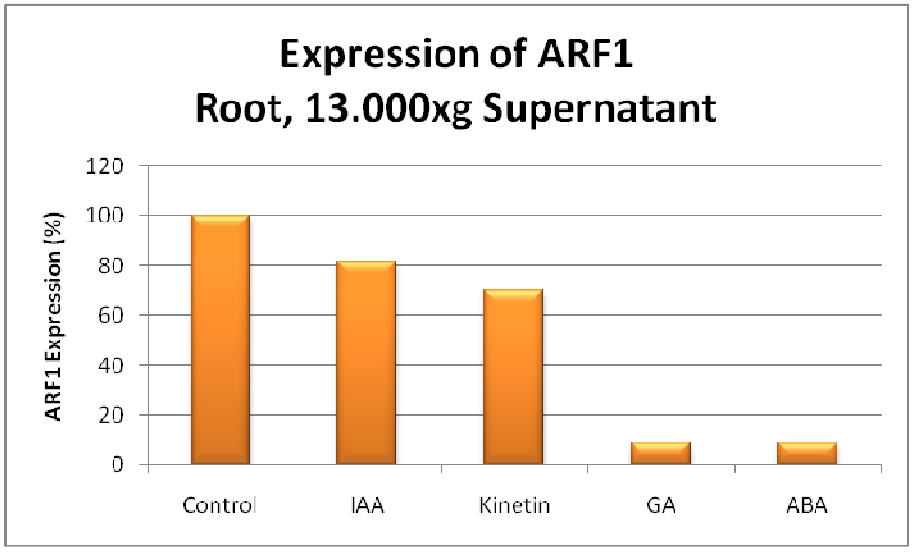


Figure 3.18 Graphical demonstration of the amount of ARF1 protein in 13.000 x g supernatant protein samples of the roots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

In cytosolic fraction, ARF1 was 19 fold down-regulated by GA treatment and 7 fold decrease was observed upon ABA treatment (Figures 3.19, 3.20).

Figure 3.19 Results of the immunoblot carried out with AtARF1 antibody, 13.000 x g supernatant protein samples of the roots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

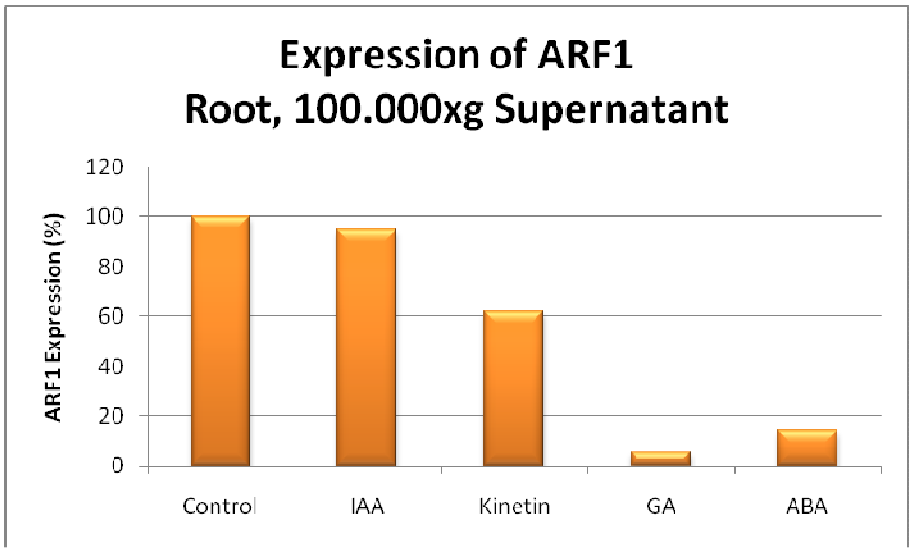


Figure 3.20 Graphical demonstration of the amount of ARF1 protein in 100.000 x g supernatant protein samples of the roots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

In microsomal fraction of the root samples, all hormones decreased membrane bound active form of ARF1. The expression differences are ~4 fold for IAA and Kinetin, ~11 fold for GA and ~3 fold for ABA. The most significant down-regulation was observed in response to 50 µM GA treatment with ~11 fold (Figures 3.21, 3.22).

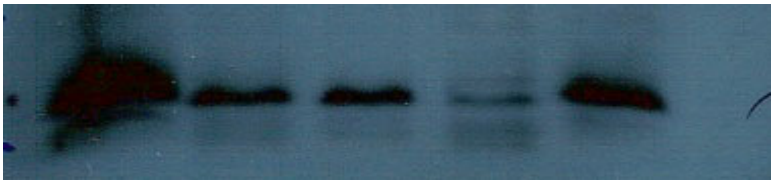


Figure 3.21 Results of the immunoblot carried out with AtARF1 antibody, 100.000 x g pellet protein samples of the roots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

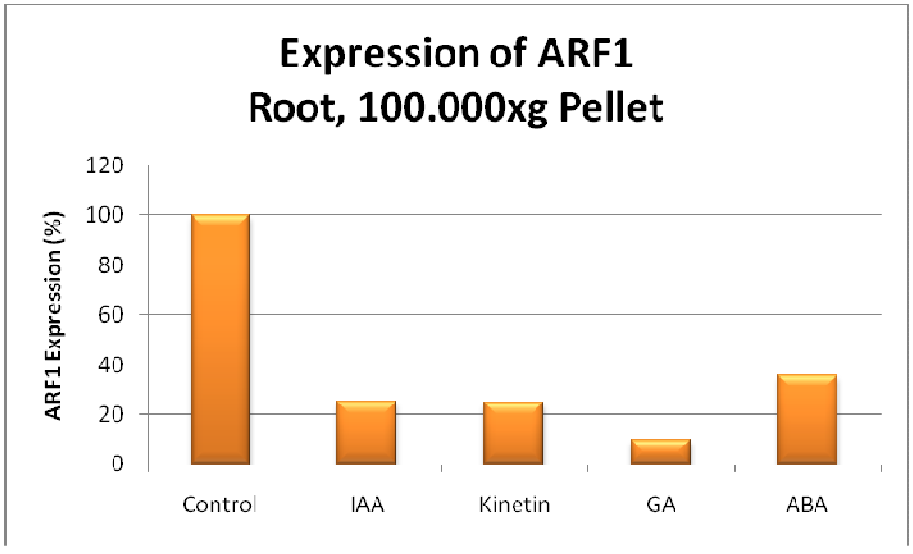


Figure 3.22 Graphical demonstration of the amount of ARF1 protein in 100.000 x g pellet protein samples of the roots. From left to right; Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

As a summary of the effect of GA on the expression of ARF1; gibberellin down-regulated ARF1 in all subcellular fractions. In 13.000 x g supernatant fraction, the expression of ARF1 was 11 fold less in GA treated plants (Figures 3.17, 3.18). In cytosolic fraction ARF1 was 19 fold down-regulated (Figures 3.19, 3.20) and, in microsomal fraction, 11 fold down-regulation was observed (Figures 3.21, 3.22). These results bring a strong evidence that GA down-regulated ARF1 protein expression significantly in both active GTP bound and inactive GDP bound forms. The second hormone which shows a significant down-regulatory effect is ABA with 11 fold decrease in 13.000 x g supernatant fraction (total pool of active and inactive forms of ARF1) and 7 fold decrease in cytosolic fraction (inactive, GDP bound form of ARF1). The down-regulatory effect of GA was more prominent than ABA.

When we consider root and shoot tissues together, in both cases, ABA and GA decreases active form of ARF1 (Figure 15, 16, 21, 22) but in all cases, there is a high rate of ARF1 turnover in the cell. In case of shoots, inactive pool of ARF1 (ARF1-GDP) is larger than the active pool (Figure 13, 14), unlike the case in roots where inactive pool also decreases (Figure 19, 20).

These results were strongly consistent with the data obtained from the study of regulation of ARF1 expression in early developmental stages. In the study; ARF1 expression was highest in 6 days old seedlings and lower in embryo and 2 days old seedlings in both microsomal and cytosolic fractions (Figure 3.1 – 3.4). When we consider 2 days old embryo, there was a smaller pool of ARF1-GTP but the stage was known to be a stage where extensive growth takes place. When the hormonal status of these developmental stages was considered, the consistency of the data with the hormonal regulation work was clear. In 2 days old seedlings, where GA content is thought to be high, there was a high rate of growth and hence vesicular trafficking, but a small pool of rapidly recycling ARF1 is sufficient for the activity of the cell. In case of embryo, where growth and cell division is slower when compared to 2 and 6 days old plants, ABA content is high, which decreases rate of

division and so, metabolic events. So, it is natural to observe lower ARF1 expression in that case.

The regulatory effect of the hormones was clear with the results indicated above, but the fact is that, ABA and GA both decreases the amount of total ARF1 (Figure 3.11, 3.12, 3.17, 3.18). The question is how do two hormones which are known as antagonistic cause the same effect on the expression of ARF1. In the light of the data obtained from the study of ARF expression at early developmental stages, one possible explanation for differential expression of ARF under different hormonal conditions is the regulation of expression with the precursors of the hormones. ARF expression is down-regulated by ABA and GA application. And as indicated in the introduction part, ABA and GA biosynthetic pathways overlap until the production of carotenoid precursor GGPP. It is possible that one intermediate of the MEP pathway down-regulates ARF1 expression. But this hypothesis cannot explain the modulation of ARF expression with the external application of these hormones since hormone biosynthesis is not operative in this case. The solution should lie in the metabolic events after hormone biosynthesis. At this point, two factors may be considered.

The first factor is the genes induced by ABA and GA. There are several studies using microarray platforms, hybridization, and data normalization, that investigated ABA and GA regulated genes (Yazaki and Kikuchi, 2005; Chen and An, 2006). Most of the genes which are responsive to both hormones under the same experimental conditions were regulated antagonistically. But in *Arabidopsis* (Yazaki and Kikuchi, 2005) and Barley (Chen and An, 2006) There are plenty of genes showing coordinated response to ABA and GA. In barley, interestingly, 27% of genes differentially regulated by both GA and ABA showed a coordinated response to GA and ABA. In pea, however, there is no expression profiling data conducted with ABA and GA up to date but the findings indicated above shows the coordinated effect of the two hormones. The difference on the protein expression of ARF1 which occurs synergistically for ABA and GA may be a result of the

activity of the products of the coordinated-response genes, or a direct effect of the hormones on ARF gene.

The second factor to be considered to explain the results presented in this thesis is the catabolism of the effective hormones; ABA and GA. The degradation or inactivation products of these hormones may alter the expression of ARF1. There are no common catabolites of ABA and GA as seen in the introduction part. But the major degradation product of ABA, phaseic acid (PA) and inactive GA conjugates may alter the expression of ARF1.

CHAPTER 4

CONCLUSION

*ARF*1 is an important regulator of vesicular transport between Golgi and ER which control the formation of Coatomer Protein I (COPI) coated vesicles. So, it is a critical GTPase which affects the regulation of protein synthesis and in turn, cell viability and productivity.

In this study, the importance of plant hormones indole acetic acid (IAA), kinetin, gibberellin (GA) and abscisic acid (ABA) in the regulation of ADP Ribosylation Factor 1 (ARF1) expression, in pea was elucidated. As an overall picture of the results, it was shown that, GA and ABA down regulated ARF1 expression in pea roots. It was shown that the effect of Gibberellin was more potent than the effect of ABA.

With this study, hormonal regulation of ARF1 was revealed for the first time in literature. The next step to be revealed in the light of these results is the RNA expression of ARF1 under different hormone treatments. It is necessary to show the expression differences in the mRNA level in order to confirm these results. So, future studies may include the northern blot analysis to see the mRNA transcription levels. Furthermore, there is no published microarray studies conducted with pea under different hormone treatments. Future studies may also include the microarray analysis conducted with pea, to show the expression differences of the genes regulated with plant hormones.

It will also be beneficial to see the combinatorial effects of the hormones on the expression of ARF1. Future prospects of this study also includes the application of different combinations of the plant hormones to pea plants and see the expression profiles of ARF1 in both protein and RNA level.

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APPENDIX I

CHEMICAL STRUCTURES OF THE HORMONES

Figure A1.1 Chemical Structure of Auxins

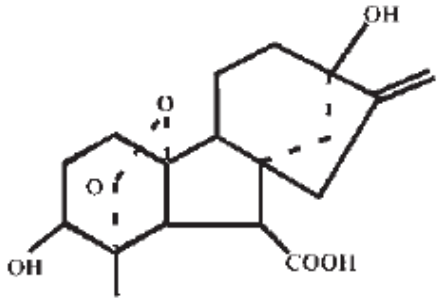

Figure A1.2 Chemical Structure of Gibberellins

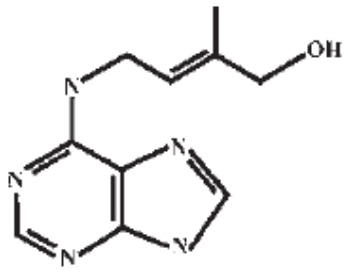

Figure A1.3 Chemical Structure of Cytokinins

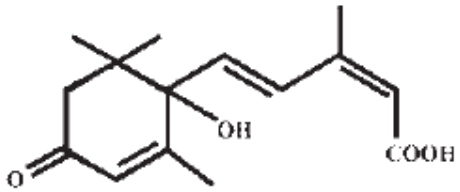

Figure A1.4 Chemical Structure of Abscisic acid

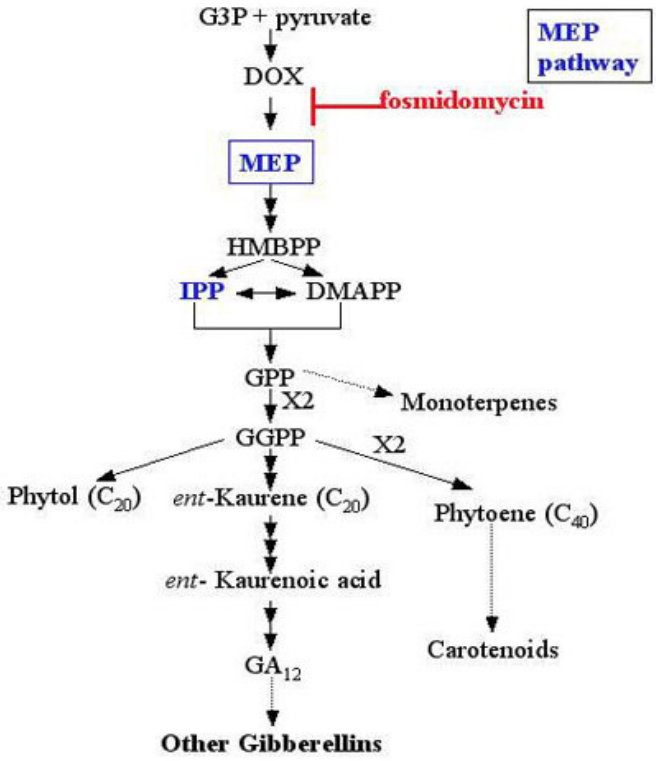

Figure A1.5 Overview of Gibberellin synthesis (Plant Physiology online).

APPENDIX II

SOLUTIONS

A2.1 Solutions Used in Plant Growth

A2.1.1 Hogland Solution:

Hogland solution was prepared as described in Gatz, 1995.

Hogland Stock Solutions:

1 M $NH_4H_2PO_4$: 11.5 g $NH_4H_2PO_4$ (Sigma-MW=115.0) was dissolved in water to final volume of 100 mL.

1 M KNO_3: 10.11 g KNO_3 (Sigma-MW=101.1) was dissolved in water to final volume of 100 mL.

1 M $Ca(NO_3)_2.4H_2O$: 23.62 g $Ca(NO_3)_2.4H_2O$ (Sigma-MW=236.2) was dissolved in water to final volume of 100 mL.

1 M $MgSO_4.H_2O$: 16.9 g $MgSO_4.H_2O$ (Sigma-MW=169.0) was dissolved in water to final volume of 100 mL.

Solution III:

H_3BO_3 (CARLO ERBA-MW=61.843)	2.86 g/L
$MnCl_2.2H_2O$ (Fluka-Garantie-MW=197.91)	1.81 g/L
$ZnCl_2$ (Merck-MW=136.28)	0.11 g/L
$CuCl_2.2H_2O$ (Riedel-MW=170.48)	0.05 g/L
$Na_2MoO_4.2H_2O$ (Merck-MW=241.95)	0.025 g/L

Fe-EDTA:

$Na_2EDTA.2H_2O$ (J.T.BAKER-MW=372.24)	37.3 mg/l
$FeSO_4.7H_2O$ (BAKER-MW=278.02)	27.5 mg/l

5X Hogland's solution (Gatz, 1995):

Stock solution	**Amount per Liter**
1 M $NH_4H_2PO_4$	1 ml
1 M KNO_3	6 ml
1 M $Ca(NO_3)2.4H2O$	4 ml
1 M $MgSO_4$	2 ml
Solution III	1 ml
Fe-EDTA	0.2 ml

Indicated amounts of each stock solution was added to dH_2O, pH was adjusted to 5,5

A2.1.2 Hormone Solutions

Hormone Stock Solutions:

100 mM GA3 0.346 g Gibberellin *(*Sigma, $C_{19}H_{22}O_6$, MW: 346.37) was dissolved in 3 mL of Ethanol and completed to 10 mL with dH_2O. Solution is sterilized through 0.22µm pore size filters and kept at -20°C until use.

100 mM IAA 0.175 g IAA *(*Merck, $C_{10}H_9NO_2$, MW: 175.18) was dissolved in 10 mL of 1 N NaOH. Solution is sterilized through 0.22µm pore size filters and kept at -20°C until use.

100 mM Kinetin 0.215 g Gibberellin *(*Sigma, $C_{10}H_9N_5O$, MW: 215.21) was dissolved 10 mL of 1 N NaOH.. Solution is sterilized through 0.22µm pore size filters and kept at -20°C until use.

100 mM ABA 0.264 g ABA *(*Sigma, $C_{15}H_{20}O_4$, MW: 264.32) was dissolved in 3 mL of Ethanol and completed to 10 mL with dH_2O. Solution is sterilized through 0.22 µm pore size filters and kept at -20°C until use.

Hormone Solutions in Use

50 µM GA3 25 µL of 100 mM GA3 stock solution is added to 50 mL of ¼ diluted Hogland's solution

50 µM IAA 25 µL of 100 mM IAA stock solution is added to 50 mL of ¼ diluted Hogland's solution

50 µM Kinetin 25 µL of 100 mM Kinetin stock solution is added to 50 mL of ¼ diluted Hogland's solution

50 µM ABA 25 µL of 100 mM ABA stock solution is added to 50 mL of ¼ diluted Hogland's solution

A2.2 Solutions Used in Protein Analysis

A2.2.1 Protein Extraction

Protein Extraction Buffer Stock Solutions

2 M Sucrose: 342.3 g sucrose (Fluka - $C_{12}H_{22}O_{11}$, MW= 342.3g), was dissolved in water to final volume of 1 L.

0.1 M HEPES: 11.915 g HEPES (Sigma- N-[2-hidroksietil]piperazin- N'-[2-etansulphonic acid], $C_8H_{18}N_2O_4S$, MW= 238.3) was dissolved in water to final volume of 500 mL. pH was adjusted to 7.2

1 M $MgCl_2$: 20.331 g $MgCl_2.6H_2O$ (Fluka- MW= 203.31g) was dissolved in water to final volume of 100 mL.

0.1 M Benzamidine: 0.1566 g benzamidine (Sigma-$C_7H_8N_2$.HCl-MW= 156.6 g) was dissolved in water to final volume of 10 mL. Stock solution was preserved at -20 °C.

1 M DTT: 3.084 g DTT (Fermentas- Dithiotreitol- $C_4H_{10}O_2S_2$, MW= 154.2 g) was dissolved in water to final volume of 20 mL. Stock solution was preserved at -20 °C.

0.1 M PMSF: 0.1742 g PMSF (Sigma-Phenylmethylsulfonylfloride-$C_6H_5CH_2SO_2F$ MW=174.19 g) was dissolved in ethanol to final volume of 10 mL. Stock solution was preserved at -20 °C.

Protein Extraction Buffer

1 M sucrose, 10 mM HEPES pH:7.5 mM MgCl, 1 mM EDTA, 10 mM DTT, 0.1 mM PMSF, 5 mM Benzamidine

Stock solution	**Amount per Liter**
2 M Sucrose	500 ml
0.1 M HEPES	100 ml
1 M $MgCl_2$	5 ml
0.1 M EDTA	10 ml
0.1 M PMSF	1 ml
0.1 M Benzamidine	5 ml
1 M DTT	10 ml

Indicated amounts of each stock solution was mixed to a final volume of 1 L. DTT, PMSF and Benzamidine are added freshly before use.

A2.2.2 Bradford Protein Determination

1 X Bradford Reagent 100 mg Coomassie Brilliant Blue G-250 (Roth-$C_{47}H_{48}N_3NaO_7S_2$, MW: 854.02) was dissolved in 50 ml 95% ethanol, 100 ml 85% (w/v) phosphoric acid was added. Solution is diluted to 1 L. When the dye has completely dissolved, reagent was filtered through coarse filter paper.

A2.2.3 SDS-PAGE

30% Acrylamide-Bisacrylamide mix: 291.7 g acrylamide (J. T. BAKER-CH_2:$CHCONH_2$, MW= 71.08 g), 8.3 g bisacrylamide (Sigma- N,N'-Methylene-bis-Acrylamide, MW=154.2 g) was dissolved in water to final volume of 400 mL.

10% SDS : 1 g of SDS (Sigma- Sodium Dodecyl Sulfate - $C_{12}H_{25}O_4SNa$, MW= 288.4 g) was dissolved in water to final volume of 10 mL.

Separating Gel Buffer (1.5 M Tris-HCl pH 8.8): 18.16 g Tris (Sigma-$NH_2C(CH_2OH)_3$ - MW=121.1 g) was dissolved in water to final volume of 100 mL. pH was adjusted to 8.8.

Stacking Gel Buffer (1 M Tris-HCl pH 6.8): 11.12 g Tris was dissolved in water to final volume of 100 mL. pH was adjusted to 6.8.

10% APS: 0.1 g APS (Aldrich- Ammoniumpersulfate-MW=228.2 g) was dissolved in water to final volume of 1 mL. Solution was prepared freshly before use.

Table A2.1 SDS PAGE gel solutions

	12% separating gel	**5% stacking gel**
H_2O	1.6 mL	1.4 mL
1 M Tris-HCl, pH 6.8	-	0.25 mL
1.5 M Tris-HCl, pH 8.8	1.3 mL	-
10% (w/v) SDS	0.05 mL	0.02 mL
Acrylamide/Bis-acrylamide (30%/0.8% (w/v))	2.0 mL	0.33 mL
10% (w/v) APS	0.05 mL	0.02 mL
TEMED	0.002 mL	0.002 mL

Acrylamide monomer, dd.H_2O, gel buffer and SDS were mixed with fresh ammonium persulfate and TEMED according to the quantities given in Table A2.1, Appendix II. The mixture was poured between plates up to ~1.5 cm below the top. The gel was then overlaid with water saturated butanol to exclude oxygen and allowed to polymerize.

After polymerization, the butanol was removed by washing the gel with dH_2O. The stacking gel was prepared as described for the separating gel according to the quantities given in Table A2.1, Appendix II. Plastic comb was inserted into the stacking gel and polymerization was allowed to occur. The combs were then removed and wells were washed thoroughly with water to remove acrylamide and excess radicals.

5x Sample loading Buffer: 10% w/v SDS , 10 mM Dithiothreitol, 20% v/v Glycerol, 0.2 M Tris-HCl, pH 6.8, 0.05% w/v Bromophenol blue

5X SDS Running Buffer: 15.1 g Tris, 72 g Glycine, 5 g SDS was dissolved in water to final volume of 1 L. pH was adjusted to 8.3.

A2.2.4 Silver Staining of SDS-PAGE Gels

All solutions used were prepared using ultrapure water

Fixer solution: 40 mL Methanol and 10 mL acetic acid was mixed and completed to final volume of 100 mL with water. Solution is prepared freshly before use.

320 µM DTT solution: 80 µL 1M DTT solution was mixed with 250 mL of water. Solution was prepared freshly before use.

2% Silver Nitrate solution: 0.02 g Silver Nitrate (Sigma, $AgNO_3$, M.W.: 169.9) was dissolved in 100 mL water.

Developer: 2.968 g Na_2CO_3 (Merck-MW: 105.99) and 500 µL formaldehyde was dissolved in water to final volume of 100 mL. Solution was prepared freshly before use.

Farmer's Reducer: 0.19 g K-ferricyanide (Sigma-$K_3Fe(CN)_6$-M_A=329.2), 2.4 g Na-thiosulfate (Merck-$Na_2S_2O_3.5H_2O$-M_A=248.18) was dissolved in water to final volume of 100 mL. Solution was prepared freshly before use.

A2.2.5 Western Blotting and Immunoblotting

0.1% Panceu S : 0.1 g Panceu S (Sigma- 3-hydroxy-4-[2-sulfo-4-(4-sulfo-phenylazo)phenylazo]-2,7-naphtelenedisulfonic acid- $C_{22}H_{12}N_4O_{13}S_4Na_4$-MW=760.6) was dissolved in 5% acetic acid solution to final volume of 1 L.

Transfer Buffer: 12.08 g Tris base (Sigma), 56.25 g glycin (Merck-H_2NCH_2COOH-MW=75.07) and 1 L Methanol was dissolved in water to final volume of 5 L.

10X TBS: 24.2 g Tris base (Sigma) and 80 g of NaCl were dissolved in water to final volume of 1 L. pH was adjusted to 7.5

TBST: 500µl Tween 20 (Merck-MW=1227.72) is added to 1 L of TBS solution

Blocking solution: 5 g skimmed milk (Pınar) was dissolved in 100 mL TBS.

Primary antibody: Polyclonal AtARF1 antibody provided from the laboratory of Prof. Dr. David Robison was prepared in 1% BSA TBST with 1:2500 dilution

Secondary antibody: HRP labeled secondary Antibody (Bio-Rad) was prepared in 1% BSA TBST with 1:10.000 dilution.

APPENDIX III

BRADFORD PROTEIN ASSAY

The proteins were extracted from 4 weeks old pea seedling grown under normal conditions and hormone treatments. The amounts of proteins were determined by modified Bradford protein assay (Bradford 1976). The assay was modified to find optimum working conditions for 96 well elisa micro titer plates. Protein concentrations were determined according to the optimized method.

A3.1 Optimization of Bradford Protein Assay for Small Volumes

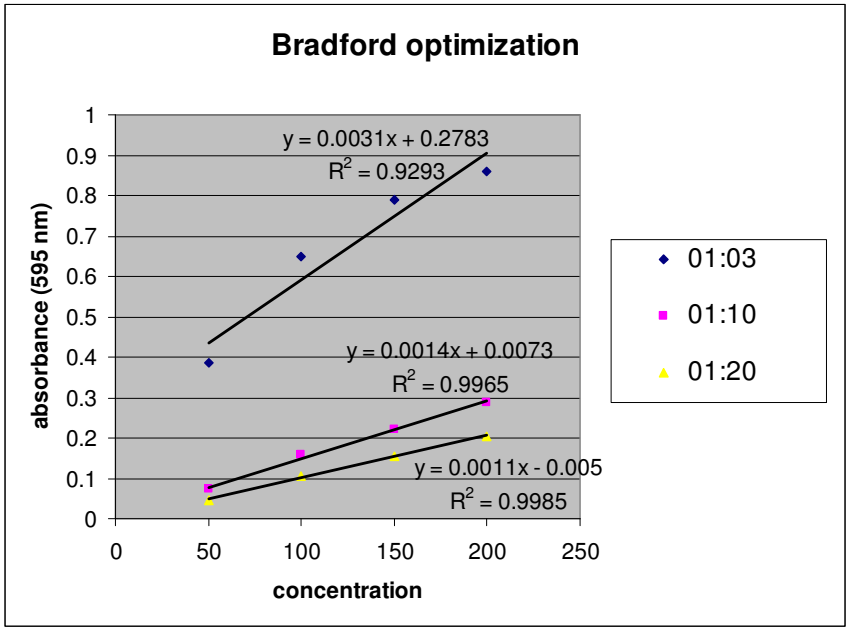

Figure A3.1 Optimization of Bradford protein analysis for micro plates

Optimization studies were conducted as described in materials and methods. According to the results, best fit standard curves were obtained from 1:10 and 1:20 sample: Bradford reagent ratios with R^2 values 0.9965 and 0.9985, respectively. (Graph 1) In the rest of the study, 1:10 ratio was chosen for further analysis.

A3.2 Protein Concentrations of different Cellular Fractions

The protein concentrations of different cellular fractions of pea roots and shoots are given, in Table A3.1. In the table, the plants were named according to the hormones applied. The amounts of proteins to be loaded to SDS PAGE gels were determined according to these concentrations. Experiments were conducted as three independent replications.

Table A3.1 Protein concentrations of the samples determined by Bradford protein assay in different fragments of root and shoot tissues.

Sample		Concentration(mg/mL)
Control (13.000 x g supernatant)	Root	8.33
	Shoot	7.38
50µM IAA (13.000 x g supernatant)	Root	7.20
	Shoot	10.94
50µM Kinetin (13.000 x g supernatant)	Root	1.87
	Shoot	7.73
50µM GA (13.000 x g supernatant)	Root	4.07
	Shoot	13.04
50µM ABA (13.000 x g supernatant)	Root	1.13
	Shoot	20.64
Control (100.000 x g supernatant)	Root	1.30
	Shoot	1.91
50µM IAA (100.000 x g supernatant)	Root	0.285
	Shoot	0.85
50µM Kinetin (100.000 x g supernatant)	Root	0.292
	Shoot	0.592
50µM GA (100.000 x g supernatant)	Root	0.192
	Shoot	0.754
50µM ABA (100.000 x g supernatant)	Root	0.08
	Shoot	0.808
Control (100.000 x g pellet)	Root	4.116
	Shoot	1.229
50µM IAA (100.000 x g pellet)	Root	1.342
	Shoot	1.119
50µM Kinetin (100.000 x g pellet)	Root	1.662
	Shoot	1
50µM GA (100.000 x g pellet)	Root	1.954
	Shoot	0.938
50µM ABA (100.000 x g pellet)	Root	0.954
	Shoot	2.33

APPENDIX IV

PANCEU STAINED MEMBRANES

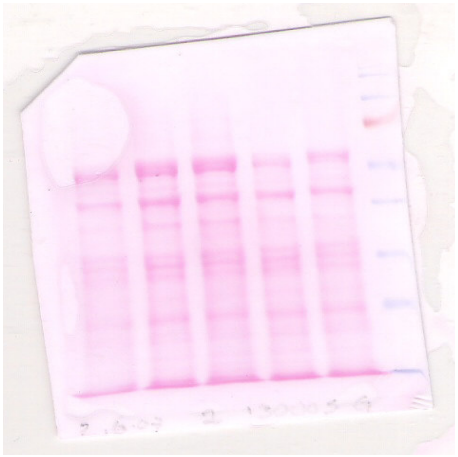

Figure A4.1 Panceu stained membrane. From left to right; 13.000 x g supernatant protein samples of the shoot tissues of Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants, Molecular weight marker.

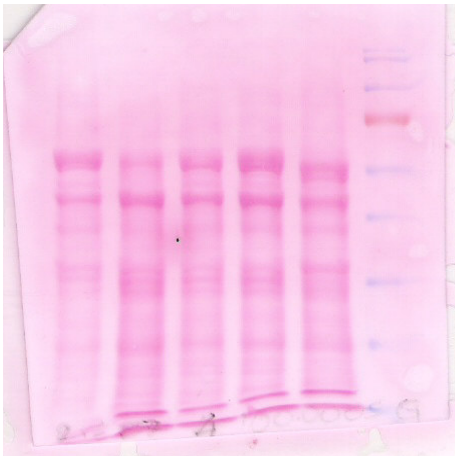

Figure A4.2 Panceu stained membrane. From left to right; 100.000 x g supernatant protein samples of the shoot tissues of Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants, Molecular weight marker.

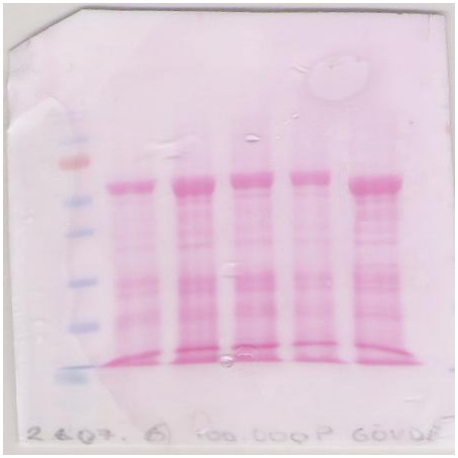

Figure A4.3 Panceu stained membrane. From left to right; Molecular weight marker, 100.000 x g pellet protein samples of the shoot tissues of Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

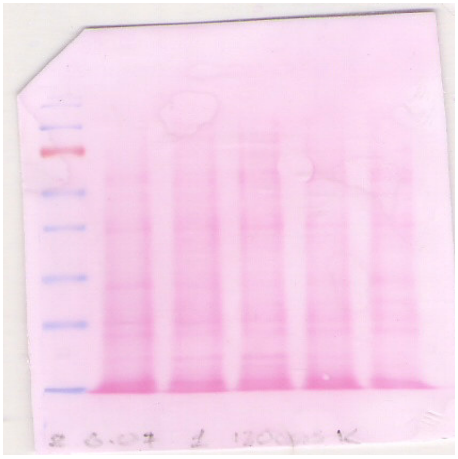

Figure A4.4 Panceu stained membrane. From left to right; Molecular weight marker, 13.000 x g supernatant protein samples of the root tissues of Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

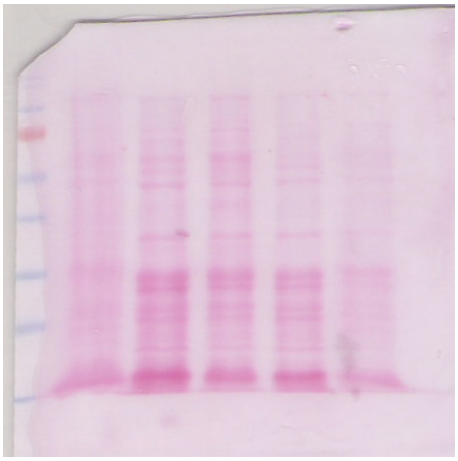

Figure A4.5 Panceu stained membrane. From left to right; Molecular weight marker, 100.000 x g supernatant protein samples of the root tissues of Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.

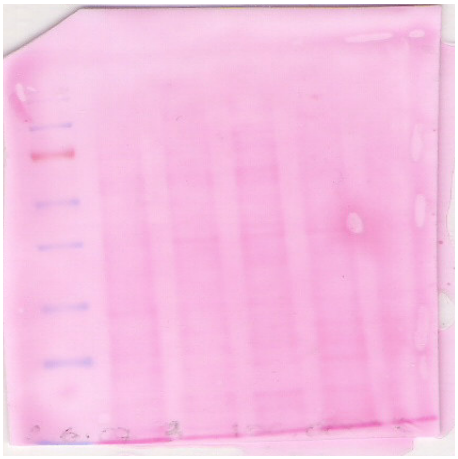

Figure A4.6 Panceu stained membrane. From left to right; Molecular weight marker, 100.000 x g pellet protein samples of the root tissues of Control, 50µM IAA, 50µM Kinetin, 50µM GA, 50µM ABA applied plants.